COMPARATIVE ANALYSIS OF LIVE, HEAT-INACTIVATED, AND ELECTRON BEAM INACTIVATED *SALMONELLA* TYPHIMURIUM INFECTION IN HUMAN HOST CELLS

A Thesis

by

CAROLINA CORKILL

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Chair of Committee,	Suresh D. Pillai
Co-Chair of Committee,	Huaijun Zhou
Committee Member,	James A. Byrd
Head of Department,	Jimmy T. Keeton

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ABSTRACT

Salmonella Typhimurium continues to be a leading cause of human gastroenteritis worldwide. This organism is a facultative intracellular pathogen, meaning that it is able grow and reproduce within the host cell it inhabits. *S*. Typhimurium has the ability to invade and replicate within human intestinal epithelial cells, which in turn causes induced cell death or apoptosis.

The human intestinal epithelial cells, HCT-8, were challenged with live, heat inactivated, and electron beam inactivated *S*. Typhimurium for various time points. Infected cell monolayers were collected for RNA extractions, and Real-time PCR was performed on the samples to analyze differential gene expression. Genes of the host cell that were expected to be differentially expressed were shortlisted and Real-Time PCR analysis was performed.

Internalized *Salmonella* within the host cell was unable to be successfully visualized using fluorescent light microscopy. However, differential gene expression for a common transcriptional regulator and inflammatory chemokine were observed to be expressed significantly higher in response to e-beam inactivated *Salmonella* infection. Genes coding for extracellular and intracellular pattern-recognition receptors of the host cells were shown to be up-regulated in response to e-beam inactivated *Salmonella* infection at 4 and 24 hours, but were not statistically significant. Additional studies must be conducted to definitively confirm e-beam irradiated *Salmonella* has the ability to invade human host cells.

Understanding the mechanisms of invasion and host cell response to live and electron-beam inactivated *S*. Typhimurium can possibly provide insight on what treatments work best to inhibit bacterial infection. These studies will also provide additional information on how electron beam irradiated Salmonella can be used as a novel therapeutic in the vaccine industry.

DEDICATION

I would like to dedicate this work to my mother, my family, and loved ones.

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NOMENCLATURE

EBST	Electron-beam irradiated *Salmonella* Typhimurium
LST	Live *Salmonella* Typhimurium
HKST	Heat-inactivated *Salmonella* Typhimurium
E-beam	Electron Beam

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimates roughly 48 million foodborne illness cases occur, and approximately 3,000 people die of foodborne disease in the United States each year (1). There are thirty-one known foodborne pathogens that make up roughly 21% of total foodborne related illnesses. One of the top five pathogens that cause domestically acquired foodborne illnesses is nontyphoidal *Salmonella*. It is also the number one pathogen that causes domestically acquired foodborne illnesses resulting in death (1). According to *FoodNet* data released in 2011, there has been a lack of progress in reducing infections caused by *Salmonella spp.* There was a 3% increase of incidence of laboratory-confirmed *Salmonella* infections in the United States in 2010 compared to the years of 1996 to 1998 (2). It is obvious that there is an ongoing need for novel preventative measures of *Salmonella* infections caused by contaminated foods.

Efforts to formulate a *Salmonella* vaccine have been studied extensively in the past and continue to be a widely researched topic. Different types of avirulent but immunogenic *S.* Typhimurium vaccines exist, but more research is required to determine which vaccines elicit the best immune response. Many of these vaccines have shown to be effective oral vaccines in mice, sheep, cattle, and chickens, however there lacks sufficient evidence that proves these vaccines to be effective in humans (3).

For the purpose of this study, it was hypothesized that e-beam inactivated *Salmonella* Typhimurium would not have the capability of invading the human host cells. Because the *Salmonella* had been irradiated at a lethal dose, it was believed that the host cell machinery would not be able to function in such a way where it retains its virulence factors, including invasion.

This study utilized *in vitro* infection techniques to monitor the response of human host cells to e-beam inactivated *S.* Typhimurium. With the use of e-beam irradiation technology there may be strong potential in constructing an effective *S.* Typhimurium vaccine that could, in the near future, be administered to humans if it proves to elicit an appropriate immune response.

This study aimed to determine if e-beam inactivated *Salmonella* could be used as a potential vaccine as a preventative measure of *Salmonella* caused foodborne illnesses. Human intestinal epithelial host cells were infected with e-beam inactivated *Salmonella* to get initial knowledge of what type of response would be elicited by the host cells. The specific objectives and underlying hypotheses of this study were:

- **Objective #1:** To determine if e-beam irradiated *Salmonella* Typhimurium had the ability to invade human host cells.
- **Hypothesis #1:** It was hypothesized that *S.* Typhimurium did not have the ability to invade human host cells.
- **Objective #2:** To determine if differential gene expression of the human host cells would occur in response to e-beam inactivated *S.* Typhimurium infection.

- **Hypothesis #2:** It was hypothesized that there would be a detectable level of differential gene expression of the host cells in response to e-beam inactivated *S.* Typhimurium infection.
- **Objective #3:** To investigate what genes were differentially expressed in response to e-beam inactivated *S.* Typhimurium infection.
- **Hypothesis #3:** It was hypothesized that genes which coded for pattern-recognition receptor proteins and inflammatory mediators would be differentially expressed in response to e-beam inactivated *S.* Typhimurium infection.

The negative controls used in all performed experiments were healthy, non-infected human host cells, and the positive controls were live *S.* Typhimurium infected human host cells. Heat-inactivated *S.* Typhimurium infection was a treatment used as a parallel comparison for e-beam inactivated *S.* Typhimurium infection. The ultimate goal of this study was to determine the effects of e-beam inactivated *S.* Typhimurium infection in human host cells.

CHAPTER II

LITERATURE REVIEW

Foodborne Illnesses

The Centers for Disease Control and Prevention (CDC) estimates that approximately 1 in 6 Americans, or 48 million people, become ill with a foodborne illness annually. It has also been estimated that about 3,000 people die each year due to a foodborne illness (1). These statistics are representative of the number of cases that have been reported each year from health departments. As we might know, many cases typically go unreported, and thus the source of a foodborne illness is left unknown. Based off of the cases that have been reported and confirmed through laboratory testing, 5.5 million foodborne illnesses were caused by viruses, and 3.6 million by bacteria. Norovirus is the leading cause of foodborne illnesses; followed by nontyphoidal *Salmonella* spp. *Salmonella* is also the leading cause in both hospitalizations and deaths as a result of foodborne illnesses (1). It is clear that nontyphoidal *Salmonella* spp. play a pivotal role in the cause of foodborne illnesses, and leads us to believe that it is of the top bacteria responsible for food contamination.

The top five pathogens that contribute to domestically acquired foodborne illnesses include Norovirus, nontyphoidal *Salmonella*, *Clostridium perfringens*, *Campylobacter* spp., and *Staphylococcus aureus* (4). Noroviruses remain the number one cause of foodborne illnesses, more than any other bacterial, viral or protozoa pathogen (5). Norovirus (NoV) is responsible for 58% of acquired foodborne illnesses

and 26% of acquired foodborne illnesses resulting in hospitalization (4). However, the statistics of this virus are thought to be highly underestimated due to lacks of reporting, culturing, and detection methods (6). NoV is highly infectious, easily transmissible, resistant to environmental stress, and ubiquitous (7). Because fresh produce undergoes minimal processing it is considered a significant vehicle in transmission of NoV (7).

Nontyphoidal *Salmonella* spp. are responsible for 11% of total acquired foodborne illnesses, 35% of foodborne illnesses resulting in hospitalization, and 28% of foodborne illnesses resulting in death (4). *Salmonella* can colonize a wide range of hosts including all major livestock species (poultry, cattle, and pigs) eventually producing contaminated meat and other food products (8). *Salmonella* is often associated with contamination of foods of animal origin; however, fruits and vegetables can also be contaminated with *Salmonella* spp. by coming into contact with animals or animal manure (9).

Clostridium perfringens is responsible for 10% of total acquired foodborne illnesses in the United States (4). This microorganism is an anaerobic, Gram-positive, spore-forming bacillus (10). *C. perfringens* produces a total of 12 toxins divided into 5 groups – A through E – where only types A and C produce disease in humans (11). However, most identifiable instances of foodborne disease due to *C. perfringens*, in the United States, appear to be due to type A strains (10). The vehicle for this pathogen is typically a high protein product, such as meat, that is allowed to cool slowly after cooking. Because the organism is a spore, its physical structure allows for its survival during the cooking process. When the meat is allowed to cool for long periods of time,

the spores germinate, multiply, and produce illness if ingested unless the food is reheated to adequate temperatures (10).

Campylobacter spp. is also a leading cause of gastrointestinal illness worldwide. It responsible for 9% of total domestically acquired foodborne illnesses and 15% of acquired foodborne illnesses resulting in hospitalization (4). Within the genus *Campylobacter*, the species *C. jejuni* makes the greatest contribution to human disease, accounting for approximately 90% of cases (12). The ecology of *C. jejuni* is often found in birds – more specifically poultry (13). The intestines of poultry are easily colonized, and most chickens in commercial operations are colonized by 4 weeks (14).

Salmonellosis continues to be a significant problem in developed and developing countries. Because *Salmonella* is the leading cause of hospitalizations and deaths as a result of foodborne illnesses, it is crucial that novel preventative measures be researched and implemented to reduce the number of infections within the United States and worldwide.

***Salmonella* Typhimurium**

The Enterobacteriaceae family of bacteria is a large group of Gram-negative, facultative anaerobes, of which *Salmonella enterica* are members. There are over 2,500 serovars of *S. enterica,* and only very few are commonly associated with disease in humans (2). According to the CDC, *Salmonella* serovars Enteritidis, Typhimurium, and Newport account for about half of culture-confirmed *Salmonella* isolates reported by public health laboratories to the National Salmonella Surveillance System (15). The diseases typically acquired due to *Salmonella* infection are severe typhoid fever, or self-

limiting gastroenteritis. *S. enterica* serovar Typhimurium (*S.* Typhimurium) is specifically responsible for causing gastroenteritis in humans. It is one of the most frequent causes of foodborne illnesses, which could be due to its ability to infect a wide phylogenetic range of hosts, including birds, meat-producing animals, and mammals (2).

S. Typhimurium is a facultative, intracellular anaerobe that can be found within a variety of phagocytic and non-phagocytic cells *in vivo* (16). *S.* Typhimurium is a highly invasive pathogen that has evolved an array of mechanisms to breach the integrity of the intestinal epithelial barrier. After intestinal colonization has been established, *S.* Typhimurium enters enterocytes, M cells, and dendritic cells in the small intestine of the host (17). The ability for *S.* Typhimurium to survive in a variety of host cells is a key component in its success as a pathogen (16).

The first significant cellular contact enteric pathogens have with its host will occur at the intestinal epithelium (3). Internalization of *Salmonella* into host cells can occur via two distinct processes (18). Phagocytes, such as macrophages, efficiently recognize bacterial pathogens and will utilize phagocytic uptake of the bacteria (18). Phagocytosis is a complex system that involves pattern-recognition receptors that recognize pathogen-associated molecular patterns, including lipopolysaccharide (LPS) in Gram-negative bacteria (19). The pattern-recognition receptors can activate downstream signaling pathways within the phagocyte, which will ultimately result in the engulfing of the bacterial pathogen (20). This internalization can be categorized as host cell-mediated internalization (21). However, bacterial pathogens, such as *Salmonella,* have evolved complex mechanisms to breach the integrity of the intestinal epithelial barrier (22). The

type III secretion system, which includes the required set of proteins T3SS1 and T3SS2, is a mechanism that *Salmonella* utilizes to actively invade both phagocytic and non-phagocytic cells (22). T3SS-mediated internalization, or pathogen-mediated invasion, is a highly specific process that depends on tightly regulated expression of a number of bacterial factors (23). T3SS1 effectors are translocated across the plasma membrane and act cooperatively to induce actin rearrangements and membrane ruffling of the host cell, resulting in the internalization if *Salmonella* (24). Both *in vitro* (25) and *in vivo* (26) evidence strongly suggests that upon internalization into non-phagocytic cells via the T3SS1 effectors, *Salmonella* becomes enclosed within an intracellular phagosomal compartment called the *Salmonella*-containing vacuole (SCV) (27). The T3SS2 effectors are translocated across the SCV membrane and promote intracellular survival (27).

The maturing SCV translocates towards the Golgi apparatus, undergoing interactions with the host endocytic pathway. Once positioned within the perinuclear area, the SCV-enclosed bacteria replicate (24). *S.* Typhimurium infections will remain localized in the small intestine where stimulation of inflammatory responses, and decreased epithelial ion absorption will contribute to diarrhea (27).

Because *Salmonella* is the leading cause of both hospitalizations and deaths due to foodborne bacterial illnesses, and has the ability to infect a wide range of hosts, vaccine development remains to be a high priority.

Salmonella Vaccines

The importance of vaccination in the control of infectious disease continues to grow. Past and recent developments have suggested the use of vaccines to manage

disease causing bacterial infections. Vaccination is a powerful tool for the control of salmonellosis and the development of safer and more effective *Salmonella* vaccines is needed. Vaccines can be generally sorted into three main categories: live attenuated, subunit vaccines, and inactivated whole-cells (28, 29).

Live attenuated vaccines are the most utilized because of their efficiency to produce an immune response (29).

Live attenuated vaccines have multiple advantages over nonviable vaccines because of their ease of administration, ability to carry antigens, and capacity to induce cellular and humoral immune responses (28). Attenuated strains of intracellular bacteria are ideal candidates for the elicitation of T-cell mediated immunity due to their capacity to mimic the lifestyle of intracellular pathogens and replicate (30). The goal of attenuation is to diminish the virulence of the pathogen, while retaining its immunogenicity. The use of genomics allows for the selective knockouts of virulence genes while maintaining the viability of the organism (28). The advantage of this type of vaccine strategy is that important antigenic determinants can be retained by the attenuated strains. Some disadvantages of this type of vaccine are that they could cause severe complications in immunocompromised patients in that secondary mutations can cause a reversion to virulence (31).

The World Health Organization (WHO) currently recommends the use and administration of a live attenuated vaccine for humans - *S.* Typhi Ty21a, which protects against typhoid (32). Ty21a has been evaluated as a typhoid vaccine in several efficacy trials and shown to be safe and effective (33, 34). Unfortunately, Ty21a is only modestly

immunogenic and requires 3 to 4 initial doses (35).This particular vaccine is administered orally, which allows for not only cellular and humoral immunity, but mucosal immunity as well (36). It is usually administered by 3 to 4 capsules containing bacteria on alternate days, and requires boosters every 5 years (37). In the *S.* Typhi Ty21a strain lipopolysaccharide (LPS) biosynthesis is restricted in its ability to produce complete LPS, however a sufficient amount of complete LPS is made in order to induce a protective immune response. Other live attenuated *Salmonella* vaccines include *S.* Typhimurium strains carrying defined mutations in the *aroA* gene. This mutation renders the microbe avirulent by making it dependent for growth on specific aromatic compounds that are not found in mammalian tissues (38). The *aroA* vaccine is not commercially available for human use. Most published studies and research that use this vaccine typically use mice as host subjects (38); however, one study was conducted using human volunteers where a *S.* Typhi strain, containing an *aroA* mutation, was administered (39). All of the volunteers in the study developed serologic responses against *S.* Typhi (39).

Because a significant proportion of human salmonellosis is caused by the consumption of contaminated poultry products there have been extensive research conducted in the field of *Salmonella* vaccines for the poultry industry. One study showed that a commercially available live attenuated vaccine, Megan®Vac 1 vaccine (Megan Health Inc., St. Louis, MO), was effective in enhancing the cell-mediated immunity (CMI) of 18- and 32-week-old chickens (29). A follow-up study proved that same live attenuated vaccine administered to chickens was not only effective in increasing the

CMI, but also showed the live vaccine was more effective than killed vaccines in clearing *Salmonella* infection in chickens (28).

Poor performance of killed vaccines forced researchers in the 1980s to develop other types of Salmonella vaccines that employ sub-cellular components of *Salmonella* (40). As a result, several subunit vaccines came into being. Common cellular components of Salmonella used for development of vaccines are: outer membrane proteins, porins, toxins, and ribosomal fractions (40). A subunit vaccine presents an antigen to the immune system without introducing whole bacterial cells (41). Instead of the entire microbe, subunit vaccines include only the antigens that best stimulate the immune system (42). Because the subunit vaccines contain only the essential antigens that will elicit an immune response, the chances of an adverse reaction are much lower in comparison to vaccines that use whole microbes (42). Subunit vaccines are safe, immunogenic, and are currently licensed for human use. *S. typhi* and some *S. dublin* strains present the Vi surface antigen, which is polysaccharide that is capable of producing an immune response in hosts (43). The Vi antigen is thought to prevent antibodies from binding to the O antigen, allowing *S. enterica* var. Typhi to survive in the blood, and is also associated with inhibition of complement activation and resistance to complement-mediated phagocytosis (43). Vi-based vaccines, such as Typhim Vi® (Sanofi Pasteur SA) are currently used in humans in the United States. Studies and trials conducted using these types of vaccines have proven to confer 55 to 75% protection against typhoid fever (41). Other subunit vaccines that utilize *Salmonella* components include detoxified LPS and O-polysaccharides. These vaccines have been shown to be

sufficiently immunogenic only when repeatedly administered or coupled with protein carriers (41). This evidence is a clear indication that the sole use of subunit vaccines is not sufficient.

Other widely used *Salmonella* vaccines include killed whole-cells. Inactivated whole- cell vaccines given parentally have been used to provide protection against typhoid fever; however, due to high incidence of associated adverse systemic and local reactions, they are generally considered to be unsuitable for use as a public health vaccine (43, 44). In humans, killed whole-cell vaccines have shown to provide significant protection after parenteral, but not oral administration (45) Killed whole-cell *Salmonella* vaccines have been widely used in the mouse model and are typically orally administered. This vaccine type elicits sufficient humoral immune response but lacks in providing cell-mediated immune responses (41). Killed whole-cell *Salmonella* vaccines have been proven to confer only partial protection against intestinal colonization in chickens (46). These vaccines have also proven to show some protection against salmonellosis in calves, but are less protective than live attenuated vaccines (47). Nobilis® Salenvac (MERK Animal Health, Summit, NJ) is a commercially available, inactivated *Salmonella* vaccine that is used to fight against *S.* Enteritidis and *S.* Typhimurium infection in chickens. This vaccine is rendered inactive by growing it in conditions of iron restriction (48). Killed vaccines, while not very effective, are still the best options and preferred choice for eradication of an endemic strain from a herd or when dealing with an outbreak of salmonellosis.

There is a justifiable need for novel vaccine construction to control infectious diseases. Many of the existing vaccines have one or more factors that render them ineffective. Current research is exploring the use of new technology to inactivate microbes, while maintaining their immunogenicity.

Electron Beam Irradiation Technology

Because most inactivation processes include formalin killing or heat killing, the vaccine loses its potential efficacy by rendering its immunogenicity (49). Many times surface structures of the microbe lose their native configuration, and an effective induction of the host immune response cannot be produced. The use of ionizing radiation is a popular technology that is currently being used in food processes to decrease the population of, or prevent the growth of, undesirable biological organisms in food (50). Some studies have implemented different forms of ionizing radiation on bacterial cells in an attempt to produce killed whole-cell vaccines (49).

Ionizing radiation can be generally described as the use of energetically charged particles such as electrons and alpha particles, or energetic photons such as gamma rays and x-rays, to inactivate undesirable biological organisms (50). This technology has been used for food applications since the 1950s. Ionizing radiation has sufficient energy to remove electrons from atoms or molecules leading the formation of ions. The main sources of ionizing radiation include: naturally radioactive isotopes like Uranium 238, artificially created isotopes using nuclear reactors such as Cobalt-60 (Co^{60}), and linear accelerators. Isotope based radiation involves the use of Co^{60} and Cesium-137 (Cs^{137}), which emit penetrating gamma rays. While gamma rays from radioisotopes such as Co^{60}

are effective for many food irradiation applications, facilities are beginning to gravitate towards the use of accelerator sources of ionizing radiation. This is partly due to the rising prices of Co^{60} and because of public concern related to the nuclear industry (50). The use of accelerator sources of ionizing radiation includes electron beams and penetrating x-rays. For the purpose of this review and its relevance to the remainder of this work, techniques associated with irradiation using electron beams will be the main focus of this section.

The accelerator system is a very sophisticated and complex set of machinery. A microwave accelerator produces ionizing radiation, in the form of an electron beam. The electrons are linearly accelerated to form a beam that emerges from the accelerator, through a thin titanium exit window at the end of the scan horn (50). Thus, the product is directly treated with electrons at a specified, desired dose. These electrons have enough energy to eject electrons from atoms and molecules within the product (50).

The basic effects of ionizing radiation can be divided into two categories: the primary effects and the secondary effects. The direct result of strong, ionizing collisions is the breaking of chemical bonds and the formation of cations and energetic secondary electrons. The products can also be referred to as free radicals, because they have an unpaired electron. The primary effects are non-specific, which means the electrons will strike any molecule that is in the track of the ionizing radiation. There is no preference to a particular atom or group of atoms (50). The secondary effects are the various reactions of the primary species that result in the ultimate molecular products. The chemically active free radicals produced during the primary events can combine with themselves or

with other atoms and molecules to produce secondary effects – usually scavengers and/or sensitizers (50).

For the purpose of this review, the biological organisms of primary interest when discussing electron beam (e-beam) irradiation include disease-causing bacteria. It is now known that the biological effects caused by ionizing radiation are primarily the disruption of deoxyribonucleic acid (DNA) molecules in the nuclei of the bacterial cells (51). Disruption or damage caused by primary ionizing events or through secondary free radical attack, can prevent successful replication and could potentially cause death (50). The DNA has comparatively high sensitivity to the effects of ionizing radiation, because it is much larger than the other molecular structures within a cell. E-beam irradiation of a bacterial cell will produce single and double-stranded lesions within the DNA molecule. If the dose applied, measured in kilograys (kGy), is large enough it could produce enough single and double-stranded breaks in the DNA, which would render the bacteria unable to replicate (50).

Irradiated Vaccines

Ionizing radiation technology has been used to inactivate bacterial cells while being able to maintain their immunogenicity. Studies have suggested that the use of ionizing radiation to inactivate bacteria can induce protective humoral and cellular immune responses when administered as a vaccine to certain hosts.

One study evaluated the use of gamma-irradiated *Listeria monocytogenes* as a killed bacterial vaccine to determine if irradiation preserved the antigenic and adjuvant structures destroyed by traditional chemical or heat inactivation (49). *L. monocytogenes*

is a Gram-positive, facultative intracellular pathogen that can cause systemic disease in immunocompromised hosts. The results from this study established that gamma irradiated killed *L. monocytogenes* induced protective T-cell responses, which were previously thought to require live infection. They also stated that gamma irradiation could potentially be applied to other bacterial candidates to use as vaccines (49).

Another study utilized gamma irradiation to inactivate *Brucella abortus*. *B. abortus* is an intracellular, Gram-negative bacterial pathogen that can cause abortions in pregnant cattle, as well as fever in humans (52). Their findings suggested *B. abortus,* irradiated at lethal doses, are unable to replicate but maintains some level of metabolic activity. They also found that gamma irradiated *B. abortus* elicits a better immune response than heat-killed *B. abortus* when administered to mice; the authors discovered that irradiated *B. abortus* confers a greater cell mediated immune response when compared to heat-killed (52).

While many studies have been published using gamma irradiation for the inactivation of pathogens in hopes to develop vaccines, there have been no publications on using electron beam irradiation to develop vaccines. Extensive searches in multiple databases including: Agricola (EBSCO), PubMed, Proquest Central, and the Library of Congress, yielded no relevant results when using key phrases such as: electron beam, irradiation, vaccine and *Salmonella*. Therefore, there were no studies published using e-beam irradiation of *S.* Typhimurium as a vaccine, and a review specifically dedicated to this subject could not be conducted.

Molecular Methods

Infections with nontyphoidal *Salmonella* are the second most common cause of foodborne illness in the United States and one of the most common causes of foodborne illness worldwide (4, 53). This type of infection can be life threatening in susceptible individuals such as the very young, the elderly, and those who are immunocompromised. The spread of salmonellosis is crucial to monitor because of its ability to infect a wide range of animal species used for food (53). *Salmonella* Typhimurium infection begins with the ingestion of organisms in contaminated foods or water. Once in the gastrointestinal tract, the bacterium must traverse the intestinal mucus layer before adhering to the intestinal epithelial cells. Shortly after adhesion, the *Salmonella* has the ability to penetrate the intestinal epithelium (54). The ability of this organism to invade and penetrate the intestinal epithelial cells is required for its pathogenicity. This processes is not a passive consequence of bacterial contact with the epithelial cells, but instead requires the active participation of bacterium (53).

The pathogenesis triggered by *S.* Typhimurium has been extensively studied over the last few years. To study the molecular details of the entry process of *Salmonella*, *in vitro* assays have been developed to assess its invasion capabilities in cultured mammalian cells (55). In the *in vitro* system, the bacteria interact with the apical surface of the cells, where the ability of the *Salmonella* to enter the mammalian cells appears to correlate with their ability to invade the ileal mucosa *in vivo* (55). Many studies have been conducted where the *in vitro* invasion assay is utilized to assess the invasion capabilities of live, heat-inactivated, and mutated *Salmonella* and other bacteria;

however, there have been no publications utilizing the *in vitro* invasion assay to assess the invasion capabilities of irradiated bacteria. For this reason, this body of work used the *in vitro* invasion method to analyze the invasion capabilities of e-beam irradiated *S.* Typhimurium.

The *in vitro* invasion assay begins with establishing a host cell monolayer in tissue culture treated flasks or well plates (56). The human intestinal adenocarcinoma cell line, HCT-8, has been shown to be of use as a model of enteric pathogen activity (56-58), which is why it was used for this research. Once monolayers have been established, bacterial suspensions are added to each flask or well containing the cell monolayers at a specified concentration and for a specified time (57). After the host cells and bacterial cells have co-incubated for a specific time point, the bacterial suspensions are aspirated, and the host cells go through a series of buffer washes (59). The host cells are then subjected to an antibiotic treatment to kill the extracellular bacteria (60). The host cells are then lysed using varying reagents depending on what downstream assays follow.

Host pathogen interaction is a widely studied area of research, where a numerous amount of publications have studied pathogen and/or host responses. While *Salmonella* induces its own uptake into intestinal epithelial cells, the host uptake processes involve different host receptors and cell signaling pathways (61). While the intestinal epithelial layer serves as a critical barrier to luminal bacteria, it is also an active participant of the intestinal innate immune response (62). Intestinal epithelial cells respond to signals in

both the apical and basal compartments, and the invasion of pathogenic bacteria will cause injury and elicit pro-inflammatory gene expression (63).

Common molecular methods used to study the bacterial induced gene expression of the host cells include: nucleic acid isolation, cDNA synthesis, and Real-time PCR gene expression analysis (64). There are many commercially available kits used to isolate DNA and RNA. Many publications report the use of TRIzol® for DNA and RNA extraction from cell cultures (64). The understanding of the molecular events taking place in cells under physiological or pathological stress is the major goal of molecular biology (64). The amount of an expressed gene in a cell can be measured by the number of copies of a messenger RNA (mRNA) transcript of that gene in a sample (65). Measurement of gene expression (mRNA) has been used extensively in monitoring biological responses to various stimuli (65). Once the RNA has been extracted from the cell culture sample of interest, the RNA serves as a template to synthesize equal amounts of complimentary DNA (cDNA). The enzymes reverse transcriptase and DNA polymerase catalyze the cDNA synthesis process, commonly referred to RT-PCR. (65)

Real-time PCR is the method in which data is collected throughout the PCR process as it occurs by combining amplification and detection into a single step (66). This is conducted by utilizing fluorescent reagents that correlate PCR product concentration to fluorescence intensity (67). Reactions are characterized by the point-in-time, or PCR cycle, where the target amplification is first detected. This is referred to as the cycle threshold (C_t), where the fluorescence intensity is greater than that of the background fluorescence (65). So, the greater quantity of target DNA in the starting

material the faster a significant increase in fluorescent signal will appear, directly corresponding to gene expression (66). Real-time PCR assays can reliably detect gene expression differences as small at 23% (66), which is why it was used for the experiments in this research.

Table 1. A complete list of target host genes and corresponding functions for real-time PCR gene expression analysis.

Gene	Function
B-actin	House-keeping gene
Nf-kappab	Protein complex that controls the transcription of DNA; involved in cellular responses to stimuli such as stress and bacterial or viral antigens (68)
Lbp	Binds to bacterial lipopolysaccharide (LPS) to elicit immune responses by presenting the LPS to cell surface pattern recognition receptors (69)
Lyz	Human lysozyme is an anti-microbial agent whose substrate is bacterial cell wall peptidoglycan (70)
Rela	Part of the Nf-kappab complex (most abundant form) (71)
Cd14	Co-receptor for the detection of bacterial LPS (72)
Tlr4	Detects LPS from Gram-negative bacteria and couples with CD14 to mediate signal transduction pathways (73)
Il6	Inflammatory cytokine (74)
Nod2	Intracellular pattern recognition receptor that recognizes LPS and peptidoglycan (75)
Il8	Mediator of the inflammatory response and functions as a chemotractant (76)
Slpi	Secreted inhibitor that protects epithelial cells from serine proteases and provides antibiotic activity (77)

CHAPTER III

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The *Salmonella* enterica serovar Typhimurium strain American Type Culture Collection (ATCC, Manassas, VA) 14028 was used as the positive control in this study. This strain was also used in the preparation of the heat-inactivated and electron beam (e-beam) inactivated *S.* Typhimurium used in this study. The *S.* Typhimurium strain 14028 was grown in two 15 ml conical tubes each with 3 ml of tryptic soy broth (TSB), for approximately 16 hours. To prepare the heat-inactivated *S.* Typhimurium, a 500 µl aliquot from one of the 15 ml conical tubes containing 3 ml of the bacterial culture was transferred to a 1.5 ml microfuge tube. The 500 µl aliquot was centrifuged at 12,000 rpm in a Microfuge®18 Centrifuge (Beckman Coulter, Fullerton, CA) at room temperature, for 1 minute. The sample was then resuspended in 1 ml of phosphate buffered saline (PBS). The resuspension process was repeated 3 times to ensure that residual traces of TSB were washed out of the pellet. After the final resuspension with PBS, the culture was adjusted to an optical density (OD_{600}) of approximately 1.0 with PBS. The sample was then incubated in a 70°C water bath for 30 minutes. The heat-inactivated *S.* Typhimurium was stored at 4°C until further use was required.

The second of the 3 ml samples of *S.* Typhimurium was centrifuged using a Sorvall® RT7 Benchtop Centrifuge at 4,000 rpm (Sorvall, Newtown, CT), at room temperature for 5 minutes. The supernatant was decanted, and the bacterial pellet was

resuspended in 3 ml of PBS and washed as mentioned earlier. The resuspension process was repeated 3 times to adequately remove any remaining traces of TSB within the pellet. After the final PBS wash, the resuspension was adjusted to an OD_{600} of approximately 1.0, also using PBS. Finally, the sample was double-bagged into sterile Whirl-Pak® (Nasco, Salida, CA) plastic bags, heat sealed, and placed into a 95 kPa biohazard specimen transport bag (Therapak®, Duarte, CA). The sample was transported to the e-beam facility of the National Center for Electron Beam Research on the Texas A&M University campus. The sample was exposed to a target e-beam dose of 7 kGy. The e-beam irradiated *S.* Typhimurium was stored at 4°C until further use was needed.

The live *S.* Typhimurium samples were prepared similarly, by transferring 500 µl of the overnight bacterial culture from the 15 ml conical tube into a 1.5 ml microfuge tube. The culture was centrifuged at 14,000 rpm, at room temperature, for 1 minute then resuspended in 1 ml of PBS. The resuspension process was repeated 3 times to remove any remaining TSB. The final resuspension was adjusted to an OD_{600} of approximately 1.0 and stored at 4°C until it was needed for further downstream assays.

Human Epithelial Cell Culture

The human cell line, American Type Culture Collection (ATCC, Manassas, VA) HCT-8, is an ileocecal colorectal adenocarcinoma epithelial cell (57, 78). The cell line demonstrates considerable intestinal-like differentiation and is capable of forming epithelial monolayers (79-81). The HCT-8 cells are adherent cells and were grown using complete media composed of RPMI-1640 with 2.05 mM of L-glutamine media (HyClone, Thermo Scientific, Rockford, IL), supplemented with 10% fetal bovine serum

(Invitrogen, Grand Island, NY), 1 mM of sodium pyruvate (Thermo Scientific), and 1,000 U of penicillin/10 mg streptomycin (Sigma, St. Louis, MO) per 1 ml (57, 82-84). This cell line was incubated at 37°C in an air-jacketed incubator (NuAire, Plymouth, NM) with 5% CO_2 and constant humidity until an appropriate confluent monolayer was established. The HCT-8 cells were adherent cells, therefore the cell cultures were passaged by using trypsin treatments with TrypLE™ Express (1X) (Gibco®, Carlsbad, CA). The HCT-8 cells were passaged every 2 days, at 90% confluence to keep the cells alive and growing under cultured conditions for extended periods of time. The confluence was confirmed by visualizing the cell monolayer using a Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc., Melville, NY).

Invasion Assay

Studies to determine the infectivity of live, heat-inactivated, and e-beam inactivated *Salmonella* on the HCT-8 cell line were conducted by using the gentamicin protection assay (85, 86). Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while the intracellular bacteria remain viable (86). Because the gentamicin protection assay relies on the poor ability of gentamicin to permeate eukaryotic cell membranes, the quantification of intracellular bacteria is possible (85). This particular study was not aimed at quantifying intracellular bacteria but at determining if e-beam irradiated *Salmonella* could invade host cells; therefore, a modified gentamicin protection assay was implemented.

Invasion assays on the cell line were preformed using at least three independent experiments, using triplicate sample wells at each trial. HCT-8 cells were enumerated by using the Countess™ automated cell counter (Invitrogen, Eugene, OR) by briefly mixing 5 µl of Trypan Blue stain (Invitrogen, Eugene, OR) and 5 µl of HCT-8 cell suspension. The host cell dye mixture was pipetted into a cell counting chamber slide where the cells were visualized and enumerated. This process was required to make a 2 x 10^5 HCT-8 cells per 1 ml concentration mixture. There were 2 x 10^5 HCT-8 cells seeded per well in 12-well plates along with 1 ml of complete media. The host cell monolayers were established for 24 hours. At 24 hours of incubation the growth media for the host cells were aspirated and replaced with 1 ml of HCT-8 complete media containing no penicillin or streptomycin. The negative control for this assay was HCT-8 cells with complete media containing no penicillin or streptomycin. This negative control was selected to compare healthy HCT-8 cells to *Salmonella* challenged HCT-8 cells. The positive control was HCT-8 cells infected with complete media, without penicillin and streptomycin, containing live *S.* Typhimurium. Live *S.* Typhimurium was used as a positive control to mimic traditional *Salmonella* infection in host cells. A total of three 12-well plates were used for 1-hour, 4-hour, and 24-hour. All 12 of the wells for the 1-hour time point plates were seeded, while the 4 and 24-hour time point plates had only 9 wells seeded. Only the 1-hour time point plates were seeded with live *Salmonella*; the 4-hour and 24-hour plates were not challenged with live *Salmonella*, because preliminary studies indicated that the host cells would lose adherence properties after 3 hours of co-incubation with live *Salmonella*. Also, 1 hour of co-incubation with live *Salmonella* and

HCT-8 cells was sufficient time for the invasion process to occur, thus providing a suitable positive control (86, 87). HCT-8 cells were challenged with live, heat-inactivated, and e-beam irradiated *Salmonella* at a multiplicity of infection (MOI) of 10, or $2x10^6$ *Salmonella* cells per 1 ml, by replacing the media with cell culture media containing live, heat-inactivated, or e-beam *Salmonella.* Please refer to the Appendix for a sample calculation on how the *Salmonella* treatments were prepared. As indicated previously, the 4 and 24-hour time point plates contained only the negative control, heat-inactivated *Salmonella* treatment and e-beam inactivated *Salmonella* treatment. Immediately upon challenge, plates were centrifuged at 500 x *g* for 5 minutes at room temperature to induce an even distribution of bacteria to the cell monolayers. The samples were placed in a CO_2 incubator for either 1, 4, or 24 hours of co-incubation at 37°C (84, 88, 89). There have been no published studies involving e-beam irradiated bacterial infection of cell cultures. These time points were selected based on published literature of heat-inactivated infection of host cells (90, 91); we took into consideration that dead, or non-replicating, bacteria are thought to be incapable of invading host cells.

After the required time of co-incubation, the media containing the *Salmonella* treatments was aspirated from each well. The host cells were then washed with 1 ml of PBS 3 times. After the buffer wash, 1 ml of complete media containing 100µg/ml of gentamicin was added to each well. The antibiotic treatment ensured that any remaining extracellular bacteria would be killed (87, 88, 92, 93). Preliminary gentamicin dose response studies defined the antibiotic concentration and time required to achieve bactericidal effects on live *Salmonella*. The antibiotic treatment was mainly required for

host cells challenged with live *Salmonella*; however, for consistency throughout the assay, all HCT-8 cells received the gentamicin treatment. Once gentamicin was added to each well, the plates were placed a CO_2 incubator for 2 hours (88). When the antibiotic incubation was completed, the media was aspirated from each well, and 100 µl of the antibiotic media from each well was plated on TSA plates. This was performed to ensure the gentamicin treatment was effective and to confirm that the negative control was not contaminated either. The cell monolayers received a final wash with 1 ml of ice-cold PBS. The HCT-8 cells were then lysed with 1ml of 1% Triton X-100 or 380 µl of TRIzol® reagent depending on the down stream assay.

Bacterial and Cell Lysate Staining

Bacterial suspensions of live and e-beam *Salmonella* preparations were stained with the commercially available bacterial viability stain (LIVE/DEAD *Bac*Light) Bacterial Viability Kit (Invitrogen, Grand Island, NY). The manufacturer's recommended protocol was followed. Briefly, equal volumes of Component A and Component B were mixed together in a sterile 1.5 ml microfuge tube – 4 µl of each. Three microliters of the Component A and Component B mixture were added to 1 ml of live and e-beam irradiated *Salmonella* preparations in separate microfuge tubes. The samples were incubated in the dark for 15 minutes at room temperature. A total of 5 µl of each stained bacterial suspension was placed between a pre-cleaned microscope slide and an 18 mm square coverslip. The coverslip edges were sealed with clear nail polish, were allowed to dry, and then visualized under a fluorescence microscope. Three slides for each sample were prepared.

After the cell monolayers received their final wash of PBS, 1 ml of 1% Triton X-100 was added to each well, and the plates were incubated at 37°C for 20 minutes with continuous agitation to allow complete lysing of the host cells. The cell lysates were pipetted up and down to mix thoroughly, stained with 3 µl of prepared *Bac*Light™ mixture, and incubated for 15 minutes in the dark at room temperature. A total of 5 µl of the stained cell lysate was placed between a pre-cleaned microscope slide and an 18 mm square coverslip. The coverslip edges were sealed with clear nail polish, were allowed to dry, and then visualized under a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Inc., Melville, NY). Three slides of each sample were prepared as well.

RNA Extraction

Total RNA was extracted using TRIzol® reagent (Ambion, Grand Island, NY). After the cell monolayers received the final 1 ml wash with ice-cold PBS, the PBS was then aspirated and 380 µl of TRIzol® Reagent was added to each well. TRIzol mixtures were gently pipetted up and down several times to thoroughly homogenize the samples, and transferred to a sterile 1.5 ml microfuge tube. RNA extraction was conducted as per manufacturers instructions. A total volume of 76 µl of chloroform was added to each tube containing homogenized sample. The tubes were securely capped, shaken vigorously for 15 seconds, and incubated at room temperature for 2-3 minutes. The samples were centrifuged at 12,000 x *g* for 15 minutes at 4°C to separate the samples into an aqueous phase, interphase, and organic layer. Because the total RNA remained exclusively in the aqueous phase, it was removed by gentle pipetting, avoiding the interphase and organic layer. The aqueous phase of each sample was transferred into a

pre-labeled, sterile 1.5 ml microfuge tube. Then, 190 µl of 100% isopropanol was added to the aqueous phase and incubated at room temperature for 10 minutes. The samples were centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was removed by gentle pipetting, leaving only the RNA pellet. The RNA pellets were washed with 380 µl of 75% ethanol, vortexed briefly, and centrifuged at 7500 x *g* for 5 minutes at 4°C. The ethanol wash was discarded, and the RNA pellets were allowed to air dry for 10 minutes. Extra precaution was taken to make sure the pellets did not completely dry out, otherwise the RNA pellet would not resuspend easily. The RNA pellets were resuspended in 40 µl of RNase-free water by passing the solution up and down several times through the pipette tip.

The extracted RNA samples for each treatment were pooled over the three replicates within the plates. Therefore, there were a total of 4 tubes of RNA for each 1-hour plate, and 3 tubes of RNA for each 4 and 24-hour plate. The RNA was quantified using a Nanodrop (ND-1000) spectrophotometer (Nanodrop® Technologies, Palo Alto, CA) and dilutions of stock concentrations were made as needed.

cDNA Synthesis

Total extracted and pooled RNA was used for cDNA synthesis. cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, Grand Island, NY). The reaction contained 1 µg of total RNA from each sample in a 20-µl reaction. The reaction mixture contained the following: 1 µg of total RNA, 1µl of oligo$(dT)_{20}$ (50 µM),1 µl of 10mM dNTP Mix, and RNase free water to bring the mixture to 13 µl. The mixture was heated to 65°C for 5 minutes using the Gene Amp® PCR System 2700

(Applied Biosystems®, Foster City, CA) thermocycler and incubated on ice for at least 1 minute. After the 5-minute incubation in the thermocycler, the PCR tubes were centrifuged briefly to collect any condensation that may have formed while in the thermocycler. Then, the remaining reagents were added to each tube. The reaction mixture contained the following: 13 µl of RNA mixture, 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 M DTT, 1µl of RNaseOUT, and 1 µl of SuperScript™ III RT. The reaction mixtures were pipetted up and down to mix the contents thoroughly. First strand cDNA synthesis was synthesized using single Reverse Transcriptase polymerase chain reaction (RT-PCR). The RT-PCR reaction conditions were 25°C for 5 minutes, 50°C for 60 minutes, 70°C for 15 minutes, and held at 4°C until samples were removed from the thermocycler. The quantity of cDNA synthesized was measured using the Nanodrop (ND-1000) spectrophotometer and stored at -20°C for experimental studies.

Real-time PCR Amplification

Real-time PCR was carried out using a 96-well reaction plate (Applied Biosystems®, Foster City, CA). Each well contained 1 µl of cDNA template and 19 µl of master mix consisting of 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Foster City, CA), the respective primers (0.6 µl of each forward and reverse primers at 10 µM), and adjusted with 7.8 µl of DEPC treated deionized water. Refer to Table 1 for the gene targets and the primer pair sequences. The plate was sealed with an optical adhesive cover (Applied Biosystems, Foster City, CA) and placed in a 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems, Foster City, CA). The thermocycler conditions were programmed as follows: 50°C for 2 minutes, 95°C for 10

minutes, and 40 cycles of 90°C for 15 seconds and 60°C for 1 minute. A dissociation curve was added after the final cycle to assess the quality and specificity of each product. After real-time PCR processing, the raw data was edited using SDS v2.4 Software (Applied Biosystems, Foster City, CA), and cycle threshold (Ct) values were recorded. In a real-time PCR assay a positive reaction is detected by the accumulation of a fluorescent signal. The C_t is defined as the number of cycles required for the fluorescent signal to cross the threshold, or exceed the background level (86). The C_t levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the C_t value, the greater the amount of target nucleic acid there is in the sample. The real-time data in this study was conducted using the comparative C_t method (94). The C_t values of both the control and the treatment of interest are normalized to an appropriate endogenous housekeeping gene (94). The delta C_t samples are the C_t values for any sample normalized to the endogenous housekeeping gene (94, 95). The delta C_t values were uploaded to GraphPad Prism v5 software package where unpaired t-tests were conducted for all the target genes under each treatment and time point. Data was considered significant at a *p*-value less than 0.05 after statistical analysis was conducted. Column bar graphs were generated to compare the delta C_t values for all the target genes, under each treatment and time point. After real-time PCR was conducted the delta C_t values were uploaded to the GraphPad Prism v5 to conduct unpaired t-tests between target genes, under each treatment and time point. The delta C_t values entered into the GraphPad Prism v5 software were statistically analyzed by conducting unpaired t-tests for all target host genes, under each treatment for 1, 4, and 24-hour time points. The *p*-

values for each gene were also obtained to determine if treatment comparisons were significant. Treatment comparison values that contain an asterisk were considered statistically different. A column graph of the average delta C_t values for the target host genes and their corresponding standard deviation bars for each treatment were generated to analyze the gene expression levels.

Table 2. List of gene targets and respective primer sequences.

Gene Name			bp
B-actin		5'-3'	
	Forward	CATGTACGTTGCTATCCAGGC	21
	Reverse	CTCCTTAATGTCACGCACGAT	21
GAPDH			
	Forward	GGAGCGAGATCCCTCCAAAAT	21
	Reverse	GGCTGTTGTCATACTTCTCATGG	23
NF kappaB			
	Forward	AACAGAGAGGATTTCGTTTCCG	22
	Reverse	TTTGACCTGAGGGTAAGACTTCT	23
LBP			
	Forward	CTACAGGGCTCCTTTGATGTCA	22
	Reverse	CACGTCAGCGATGTCACTG	19
LYZ			
	Forward	CTTGTCCTCCTTTCTGTTACGG	22
	Reverse	CCCCTGTAGCCATCCATTCC	20
RELA			
	Forward	ATGTGGAGATCATTGAGCAGC	21
	Reverse	CCTGGTCCTGTGTAGCCATT	20
CD14			
	Forward	GACCTAAAGATAACCGGCACC	21
	Reverse	GCAATGCTCAGTACCTTGAGG	21

Table 2 Continued

Gene Name			bp
TLR4			
	Forward	TTTGGACAGTTTCCCACATTGA	22
	Reverse	AAGCATTCCCACCTTTGTTGG	21
NOD2			
	Forward	CACCGTCTGGAATAAGGGTACT	22
	Reverse	TTCATACTGGCTGACGAAACC	21
IL8			
	Forward	TTTTGCCAAGGAGTGCTAAAGA	22
	Reverse	AACCCTCTGCACCCAGTTTTC	21
IL6			
	Forward	ACTCACCTCTTCAGAACGAATTG	23
	Reverse	CCATCTTTGGAAGGTTCAGGTTG	23
SLPI			
	Forward	GAGATGTTGTCCTGACACTTGTG	23
	Reverse	AGGCTTCCTCCTTGTTGGGT	20

CHAPTER IV

RESULTS

Confirmation of Membrane Integrity of E-beam Irradiated *Salmonella*

Live *Salmonella* and e-beam irradiated *Salmonella* liquid cultures were stained with the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. These samples were visualized using a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Inc., Melville, NY) with a 535 emission filter to determine if e-beam irradiated *Salmonella* maintained its membrane integrity after being irradiated at a target dose of 7 kGy. The best images from each treatment were chosen for presentation in this thesis. When visualizing live and e-beam irradiated *Salmonella* under the 535 nanometers (nm) emission filter, the bacteria emitted a green fluorescence (Figure 1 and Figure 2). When visualizing heat-inactivated *Salmonella* under the 663 nm emission filter, the bacteria emitted a red fluorescence (Figure 3). The images were captured under the 100x objective. Figure 1 is an image of live *S.* Typhimurium that has been stained with *Bac*Light. This image is a confirmation that live *S.* Typhimurium maintains its membrane integrity. Figure 2 is an image if *S.* Typhimurium that has been e-beam irradiated at a dose of 7 kGy and stained with *Bac*Light at well. It was fluorescing green, similarly to that of live *S.* Typhimurium, indicating it maintained its membrane integrity after e-beam irradiation.

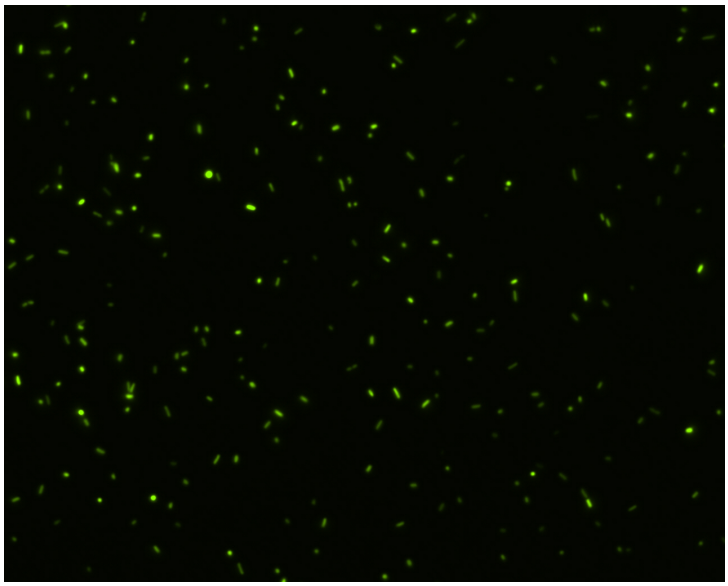

Figure 1. Live *Salmonella* stained with LIVE/DEAD *Bac*Light and captured under the 535 nm emission filter at the 100x objective at a total magnification of 1,000x.

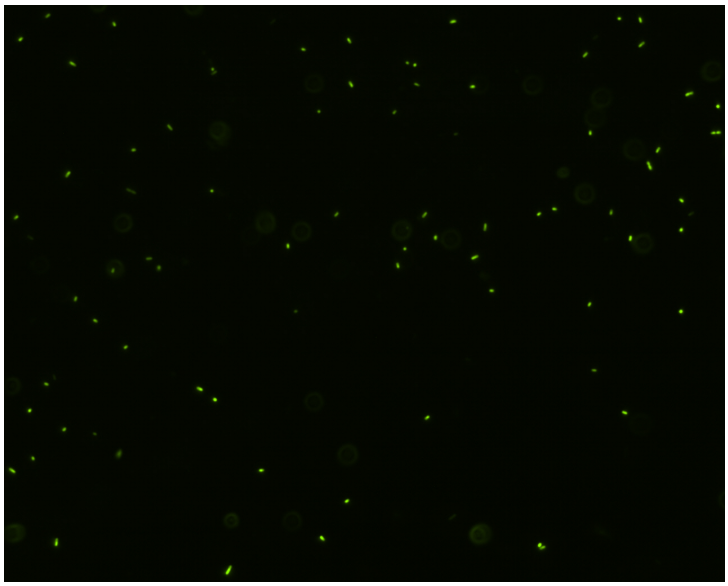

Figure 2. E-beam irradiated *Salmonella* stained with LIVE/DEAD *Bac*Light and captured under the 535 nm emission filter at the 100x objective at a total magnification of 1,000x.

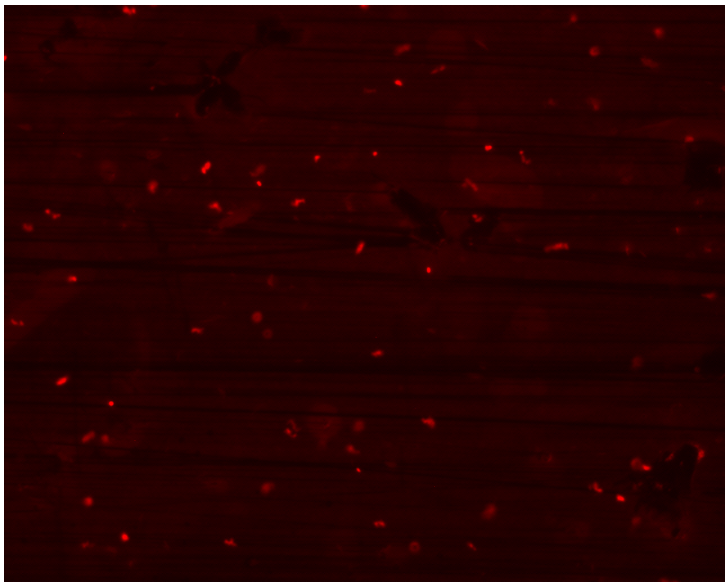

Figure 3. Heat-inactivated *Salmonella* stained with LIVE/DEAD *Bac*Light and captured under the 663 nm emission filter at the 100x objective at a total magnification of 1,000x.

Visualization of Internalized Bacteria in Host Cell Lysates

Live and e-beam irradiated *Salmonella* infected HCT-8 cells were lysed using the detergent 1% Triton X-100, collected into microfuge tubes, stained with the LIVE/DEAD *Bac*Light Bacterial Viability Kit as per manufacturer's instructions, and visualized using a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Inc., Melville, NY) with a 535 nm emission filter. The images captured (Figure 3 and Figure 4) are those of live and e-beam irradiated *Salmonella* infected HCT-8 cell lysates respectively taken after the 1-hour time point. These images were captured under the 535 emission filter and the 40x objective. Figure 3 and Figure 4 both show that host cell debris potentially containing internalized *Salmonella* from the cell lysate solutions

fluoresced green after the *Bac*Light staining procedures. In this situation, host cells debris could not be differentiated from internalized *Salmonella.*

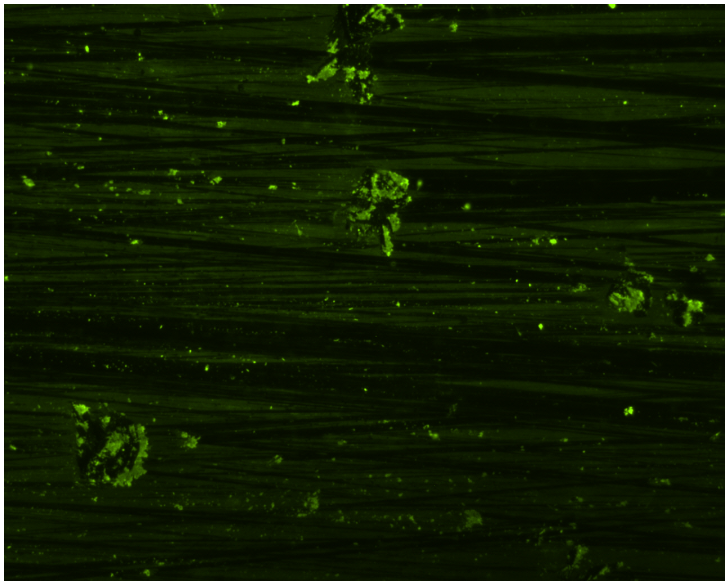

Figure 4. Live *Salmonella* infected HCT-8 cell lysate at the 1-hour time point captured under the 535 emission filter at the 40x objective at a total magnification of 400x.

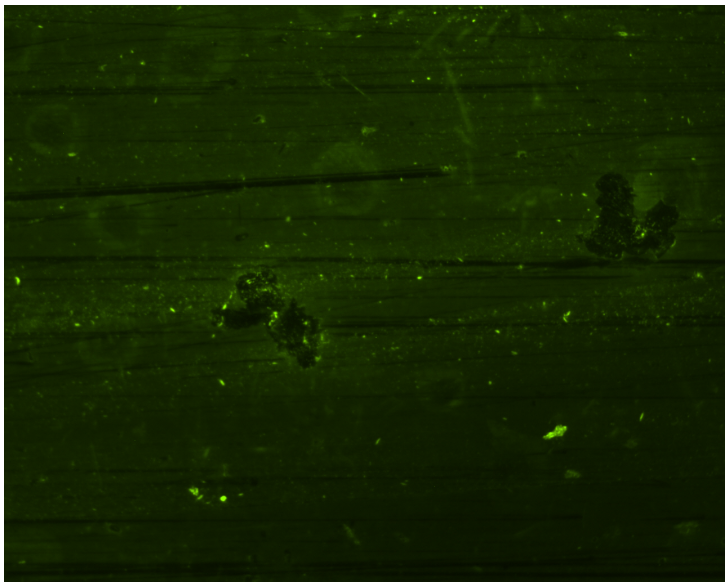

Figure 5. E-beam irradiated *Salmonella* infected HCT-8 cell lysate at the 1-hour time point captured under the 535 emission filter at the 40x objective at a total magnification of 400x.

Real-time Quantitative Reverse Transcription PCR Analysis of Host Gene Expression

A total of 11 host genes were shortlisted to study and analyze the gene expression of the host cells in response to live, heat-inactivated, and e-beam inactivated *Salmonella* infection (LST, HKST, EBST respectively) over a series of time points. A list of the genes analyzed is provided in Table 3.

Table 3. A shortlist of host target genes used to study and analyze gene expression of the HCT-8 human host cells.

Gene	Function
B-actin	House-keeping gene that codes for nonmuscle cytoskeletal actins involved in cell structure and integrity.
Nf-kb	Protein complex that controls the transcription of DNA; involved in cellular responses to stimuli such as stress and bacterial or viral antigens (68)
Lbp	Binds to bacterial lipopolysaccharide (LPS) to elicit immune responses by presenting the LPS to cell surface pattern recognition receptors (69)
Lyz	Human lysozyme is an anti-microbial agent whose substrate is bacterial cell wall peptidoglycan (70)
Rela	Part of the Nf-kb complex (most abundant form) (71)
Cd14	Co-receptor for the detection of bacterial LPS (72)
Tlr4	Detects LPS from Gram-negative bacteria and couples with CD14 to mediate signal transduction pathways (73)
Nod2	Intracellular pattern recognition receptor that recognizes LPS and peptidoglycan (75)
Il8	Mediator of the inflammatory response and functions as a chemoattractant (76)
Slpi	Secreted inhibitor that protects epithelial cells from serine proteases and provides antibiotic activity (77)

Comparison of Average Delta C_t Values of HCT-8 Cells When Exposed to Live, Heat-inactivated, and E-beam Inactivated *Salmonella* After 1 Hour of Infection

The delta C_t values for each gene across each experimental treatment are provided in Table 4. The delta C_t values indicated the level of gene expression observed in response to the different *Salmonella* infection treatments. The *p*-values for each gene were also obtained to determine if treatment comparisons were significant (Table 5). Treatment comparison values that contain an asterisk are considered statistically different. A column graph of the average delta C_t values for the target host genes and their corresponding standard deviation bars for each treatment was generated (Figure 6). It was observed that the only significant difference between gene expression levels detected at the 1-hour time point was when comparing HKST and EBST infected cells. HKST infected cells expressed *Nf-kb* at higher levels than EBST.

Table 4. Average delta C_t values of the target host genes for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 1 hour of infection. The first column lists the host genes, and the remaining columns represent the different treatments.

Gene	NC	HKST	EBST	LST
Nf-kb	8.142	7.329	8.217	8.045
Lbp	15.05	14.05	14.19	14.72
Lyz	13.74	12.03	12.77	12.39
Rela	8.238	8.7	8.776	9.129
Cd14	10.21	10.62	11.1	11.2
Tlr4	14.84	13.89	15.2	14.43
Nod2	15.38	15.15	16.09	15.35
Il8	9.38	7.327	8.438	8.69
Slpi	5.252	5.147	5.324	5.335

Table 5. *P*-values for each gene for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 1 hour of infection.

Gene	LSTvs NC	EBvs NC	HKvs NC	EBvs LST	HKvs LST	EBvs HK
Nf-kb	0.7473	0.8271	0.099	0.4006	0.0583	0.0478*
Lbp	0.8804	0.6928	0.5367	0.8001	0.6563	0.922
Lyz	0.4743	0.5911	0.3309	0.7418	0.706	0.3998
Rela	0.4823	0.6304	0.6129	0.7763	0.6893	0.9342
Cd14	0.1756	0.051	0.1152	0.8855	0.3872	0.1954
Tlr4	0.7906	0.8014	0.4276	0.5798	0.6131	0.1981
Nod2	0.9226	0.2902	0.7006	0.2243	0.7042	0.2536
Il8	0.7194	0.5797	0.3114	0.8995	0.5427	0.5821
Slpi	0.7908	0.8637	0.8188	0.9821	0.7258	0.7692

*Values with an asterisk are considered statistically significant with a p-value < 0.05

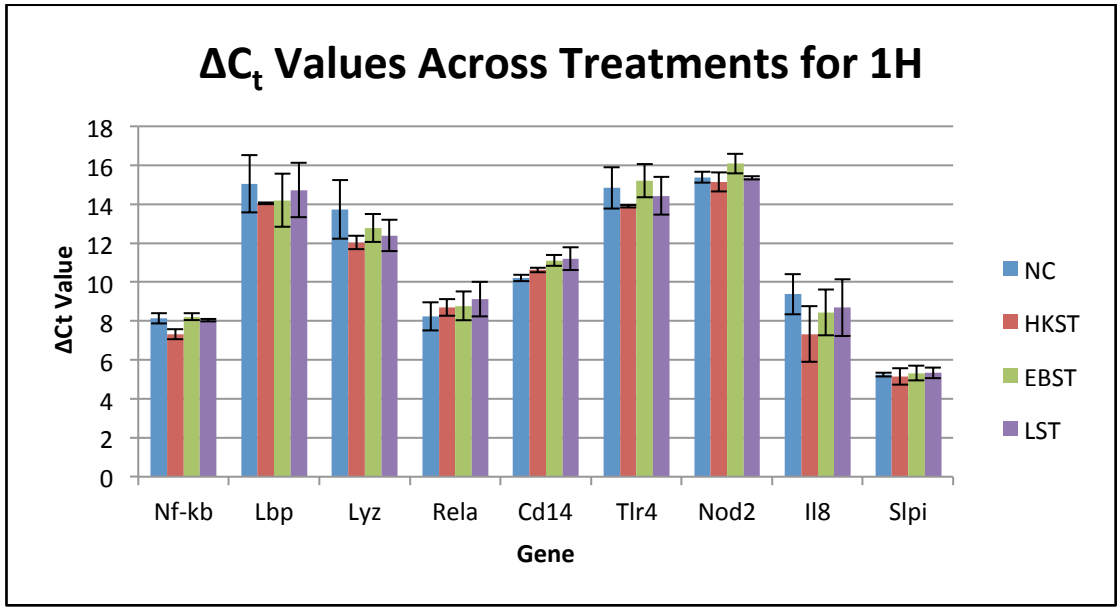

Figure 6. ΔC_t Values Across Treatments for 1H. Average delta C_t values of the target HCT-8 genes for each treatment after 1 hour of infection. NC represents the negative control where host cells were not infected with *Salmonella*, whereas HK, EB, and LST represent the host cells that were infected with heat-inactivated, e-beam inactivated, and live *Salmonella* respectively.

Comparison of Average Delta C_t Values of HCT-8 Cells When Exposed to Live, Heat-inactivated, and E-beam Inactivated *Salmonella* After 4 Hours of Infection

The delta C_t values for all target host genes, under each treatment and the 24-hour time point are listed in Table 6. The *p*-values for each gene were also obtained to determine if treatment comparisons were significant (Table 7). Treatment comparison values that contain an asterisk are considered statistically different. A column graph of the average delta C_t values for the target host genes and their corresponding standard deviation bars for each treatment was generated (Figure 7). EBST infected host cell expression of *Il-8* was significantly higher than the expression induced by non-infected cells. For genes *Nf-kb, Lbp, Lyz, Cd14, Tlr4,* and *Nod2* there was a significant level of differential gene expression when comparing the 1-hour LST infected host cells to the EBST infected host cells. HKST infected host cells had a higher expression of *Lyz* that LST infected host cells.

Table 6. Average delta C_t values of the target host genes for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 4 hours of infection. The first column lists the host genes, and the remaining columns represent the different treatments.

Gene	NC	HK	EB	LST
Nf-kb	7.977	7.309	7.229	8.045
Lbp	10.87	11.12	10.18	14.72
Lyz	9.341	9.193	8.478	12.39
Rela	9.158	10.09	9.445	9.129
Cd14	9.314	10.07	9.247	9.314
Tlr4	11.35	12.23	9.89	14.43
Nod2	14.76	15.02	14.1	15.35
Il8	7.203	6.31	5.949	8.69
Slpi	5.291	6.083	5.622	5.335

Table 7. *P*-values for each gene for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 4 hours of infection.

Gene	LSTvs NC	EBvs NC	HKvs NC	EBvs LST	HKvs LST	EBvs HK
Nf-kb	0.5165	0.0418*	0.0751	0.0314*	0.0568	0.8355
Lbp	0.0936	0.5552	0.8448	0.0321*	0.0704	0.1387
Lyz	0.0580	0.3534	0.8843	0.0089*	0.0287*	0.2264
Rela	0.9791	0.6343	0.1627	0.7464	0.3494	0.0414
Cd14	0.0995	0.9287	0.3667	0.0364*	0.1680	0.1179
Tlr4	0.0535	0.0797	0.5382	0.0103*	0.2195	0.1188
Nod2	0.5780	0.5407	0.8223	0.0028*	0.5057	0.1286
Il8	0.3669	0.0150*	0.1292	0.1398	0.1954	0.5464
Slpi	0.8849	0.0971	0.0339*	0.3952	0.1065	0.1629

*Values with an asterisk are considered statistically significant with a p-value < 0.05

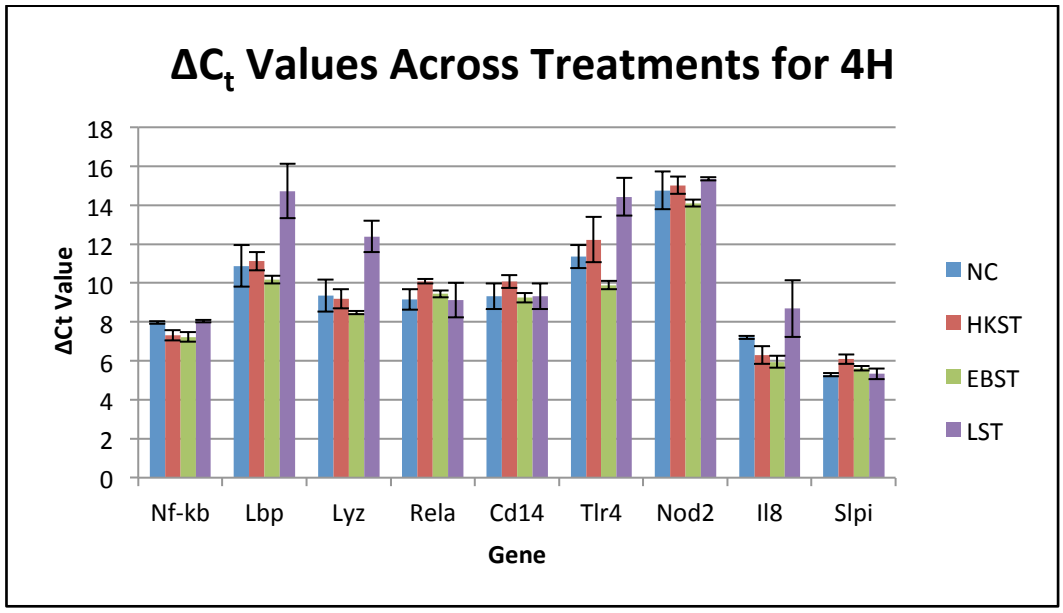

Figure 7. ΔC_t Values Across Treatments for 4H. Average delta C_t values of the target HCT-8 genes for each treatment after 4 hours of infection. NC represents the negative control where host cells were not infected with *Salmonella*, whereas HK, EB, and LST represent the host cells that were infected with heat-inactivated, e-beam inactivated, and live *Salmonella* respectively.
*LST delta C_t values are those for the 1-hour time point

Comparison of Average Delta C_t Values of HCT-8 Cells When Exposed to Live, Heat-inactivated, and E-beam Inactivated *Salmonella* After 24 Hours of Infection

The delta C_t values for all target host genes, under each treatment and the 24-hour time point are listed in Table 8. The *p*-values for each gene were also obtained to determine if treatment comparisons were significant (Table 9). Treatment comparison values that contain an asterisk are considered statistically different. A column graph of the average delta C_t values for the target host genes and their corresponding standard deviation bars for each treatment was generated (Figure 8). The gene expression levels of *Nf-kb* were significantly higher in EBST and HKST infected host cells when

compared to non-infected host cells. Higher levels of gene expression of *Il-8* were observed in EBST and HKST infected HCT-8 cells when compared to non-infected HCT-8 cells.

Table 8. Average delta C_t values of the target host genes for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 24 hours of infection. The first column lists the host genes, and the remaining columns represent the different treatments.

Gene	NC	HK	EB	LST
Nf-kb	8.023	7.581	7.558	8.045
Lbp	16.35	15.55	19.29	14.72
Lyz	14.08	13.84	11.46	12.39
Rela	8.709	8.481	8.401	9.129
Cd14	12.19	12.55	10.9	11.2
Tlr4	16.07	14.99	12.7	14.43
Nod2	20.52	19.81	17.89	15.35
Il8	6.317	5.371	5.652	8.69
Slpi	7.031	7.112	6.978	5.335

Table 9. *P*-values for each gene for negative control (NC), heat-killed (HK), e-beam irradiated (EB) and live (LST) *Salmonella* challenged host cells after 24 hours of infection.

Gene	LSTvs NC	EBvs NC	HKvs NC	EBvs LST	HKvs LST	EBvs HK
Nf-kb	0.8805	0.0414*	0.0317*	0.0145*	0.0064*	0.8477
Lbp	0.3745	0.5011	0.4728	0.3318	0.6105	0.3960
Lyz	0.1694	0.1637	0.8525	0.6018	0.3300	0.2454
Rela	0.6999	0.5703	0.6557	0.4686	0.5100	0.6443
Cd14	0.1658	0.1460	0.008*	0.7605	0.0829	0.0830
Tlr4	0.3690	0.1489	0.5578	0.3615	0.7189	0.2597
Nod2	0.0024*	0.1555	0.4788	0.1234	0.0011*	0.2441
Il8	0.1812	0.0476*	0.0141*	0.1081	0.0871*	0.3360
Slpi	0.0034*	0.7515	0.4677	0.0062	0.0035	0.5000

*Values with an asterisk are considered statistically significant with a p-value < 0.05

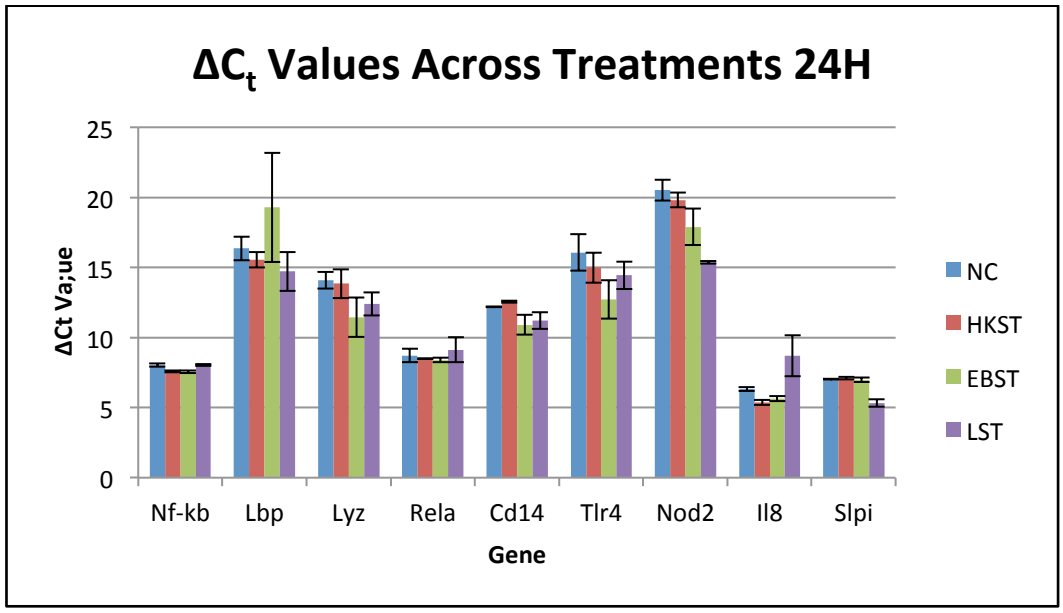

Figure 7. ΔC_t Values Across Treatments 24H. Average delta C_t values of the target HCT-8 genes for each treatment after 24 hours of infection. NC represents the negative control where host cells were not infected with *Salmonella*, whereas HK, EB, and LST represent the host cells that were infected with heat-inactivated, e-beam inactivated, and live *Salmonella* respectively.
*LST delta C_t values are those for the 1-hour time point

Average Host Gene Expression Comparison of E-beam Inactivated Versus Heat-inactivated *Salmonella* Treatment at 1 Hour

Gene expression presented in fold change of e-beam irradiated *Salmonella* challenged host cells was compared to heat-inactivated *Salmonella* challenged host cells at the 1-hour time point. Table 10 contains the average fold change of gene expression for each gene with their corresponding standard error values. Target gene expression with *p*-values less than 0.05 were considered significant, or statistically different between the groups. A graphical representation of the fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 1 hour of

infection was generated (Figure 9). The only significant differential gene expression observed at this time point was the higher level of *Cd14* expression in HKST infected HCT-8 cells. Overall, there was not a significant difference between EBST infected HCT-8 cells and HKST infected HCT-8 cells when comparing the expression levels of the target genes.

Table 10. Comparison of target gene expression presented as fold-change ± S.D. in host cells when exposed to e-beam inactivated *Salmonella* versus heat-inactivated *Salmonella* after 1 hour of infection.

Gene	*p*-value	HKST	EBST
Nf-kb	0.1183	1.81 ± 0.3026	0.9609 ± 0.1076
Lbp	0.8714	3.777 ± 1.764	4.555 ± 3.86
Lyz	0.6444	5.785 ± 2.785	3.839 ± 2.736
Rela	0.7176	0.7568 ± 0.156	0.6928 ± 0.05255
Cd14	0.0247*	0.7502 ± 0.02782	0.5422 ± 0.05237
Tlr4	0.1667	2.744 ± 1.148	0.7961 ± 0.123
Nod2	0.3716	1.552 ± 0.7912	0.7183 ± 0.2492
Il8	0.1489	4.605 ± 1.404	2.008 ± 0.3853
Slpi	0.9467	0.9864 ± 0.1855	1.01 ± 0.2736

*Values with an asterisk are considered statistically significant with a *p*-value < 0.05

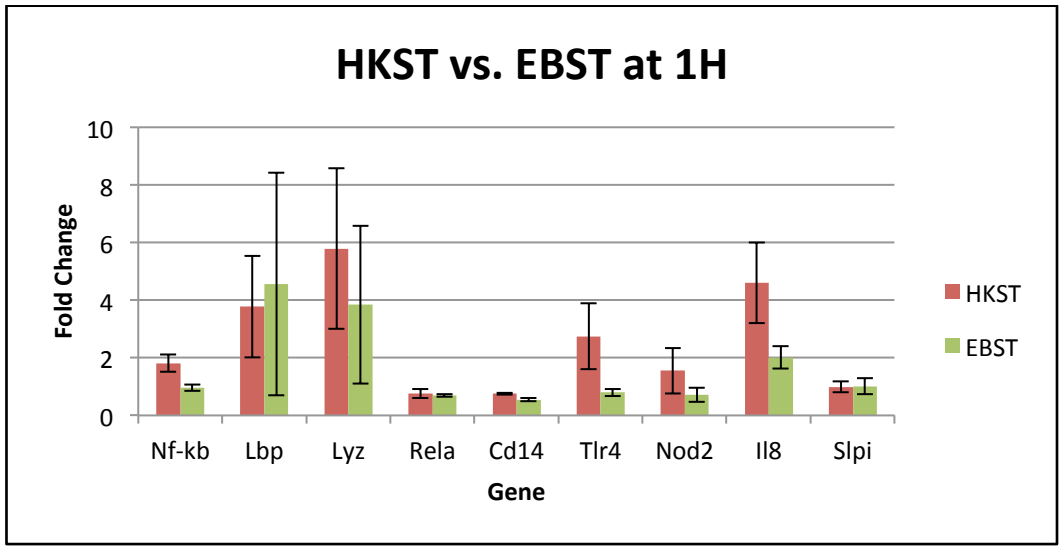

Figure 8. Average fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 1 hour of infection. HK and EB represent the host cells that were infected with heat-inactivated, e-beam inactivated *Salmonella* respectively.
*LST delta C_t values are those for the 1-hour time point

Average Host Gene Expression Comparison of E-beam Inactivated Versus Heat-inactivated *Salmonella* Treatment at 4 Hours

Gene expression presented in fold change of e-beam irradiated *Salmonella* challenged host cells was compared to heat-inactivated *Salmonella* challenged host cells at the 4-hour time point. Table 11 contains the average fold change of gene expression for each gene with their corresponding standard error values. Target gene expression with *p*-values less than 0.05 were considered significant, or statistically different between the groups. A graphical representation of the fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 24 hours of infection was generated (Figure 10). Overall, there was not a significant difference

between the levels of gene expression in the target genes for EBST infected HCT-8 cells when compared to HKST infected HCT-8 cells.

Table 11. Comparison of target gene expression presented as fold-change ± S.D. in host cells when exposed to e-beam inactivated *Salmonella* versus heat-inactivated *Salmonella* after 4 hours of infection.

Gene	*p*-value	HKST	EBST
Nf-kb	0.7811	1.623 ± 0.228	1.759 ± 0.3955
Lbp	0.4704	1.275 ± 0.5485	2.867 ± 1.922
Lyz	0.4096	1.21 ± 0.3118	2.427 ± 1.285
Rela	0.3087	0.5674 ± 0.1411	0.8852 ± 0.2334
Cd14	0.3741	0.6671 ± 0.2005	1.29 ± 0.5901
Tlr4	0.1073	0.986 ± 0.4606	2.963 ± 0.8372
Nod2	0.4808	1.202 ± 0.5008	2.196 ± 1.178
Il8	0.7429	2.146 ± 0.8507	2.489 ± 0.4747
Slpi	0.2220	0.5926 ± 0.08921	0.8123 ± 0.1232

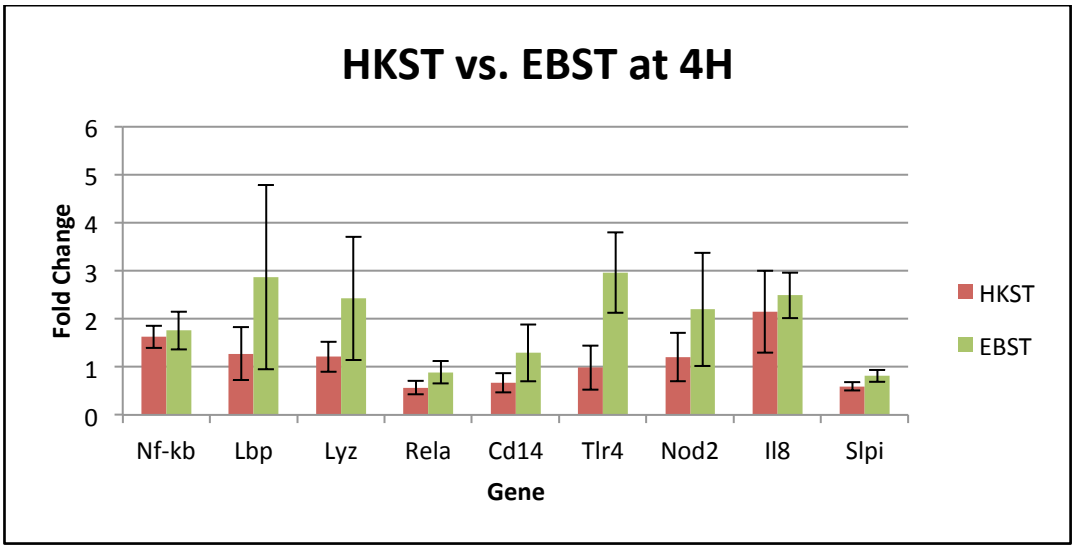

Figure 9. Average fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 4 hours of infection. HK and EB represent the host cells that were infected with heat-inactivated, e-beam inactivated *Salmonella* respectively.

Average Host Gene Expression Comparison of E-beam Inactivated Versus Heat-inactivated *Salmonella* Treatment at 24 Hours

Gene expression presented in fold change of e-beam irradiated *Salmonella* challenged host cells was compared to heat-inactivated *Salmonella* challenged host cells at the 24-hour time point. Table 12 contains the average fold change of gene expression for each gene with their corresponding standard error values. Target gene expression with *p*-values less than 0.05 were considered significant, or statistically different between the groups. A graphical representation of the fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 24 hours of infection was generated (Figure 11). HKST infected HCT-8 cells have a higher level of expression when compared to EBST infected host cells. There is an observed trend of higher level of expression in *Lyz, Tlr4,* and *Nod2* when comparing EBST and HKST treatments, but the differences were not statistically significant. EBST infections and HKST infections were observed to have similar effects on the HCT-8 cell gene expression when compared.

Table 12. Comparison of target gene expression presented as fold-change ± S.D. in host cells when exposed to e-beam inactivated *Salmonella* versus heat-inactivated *Salmonella* after 24 hours of infection.

Gene	*p*-value	HKST	EBST
Nf-kb	0.8698	1.364 ± 0.08967	1.387 ± 0.0939
Lbp	0.3269	2.003 ± 0.7749	0.9593 ± 0.5229
Lyz	0.3315	1.467 ± 0.6429	13.99 ± 11.33
Rela	0.8271	1.312 ± 0.4167	1.48 ± 0.5909
Cd14	0.2226	0.7819 ± 0.05455	3.214 ± 1.685
Tlr4	0.2873	2.265 ± 0.5651	18.84 ± 13.51
Nod2	0.2846	1.69 ± 0.2886	19.7 ± 14.59
Il8	0.0342*	1.928 ± 0.06708	1.589 ± 0.08355
Slpi	0.4634	0.9487 ± 0.05934	1.048 ± 0.1071

*Values with an asterisk are considered statistically significant with a *p*-value < 0.05

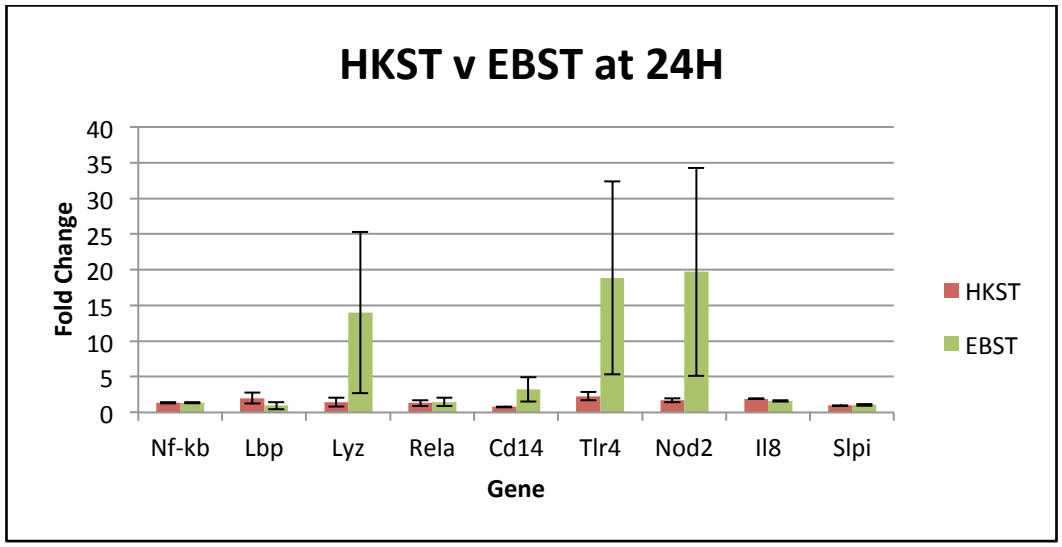

Figure 10. Average fold change for each target gene under heat-inactivated and e-beam inactivated *Salmonella* treatment after 24 hours of infection. HK and EB represent the host cells that were infected with heat-inactivated, e-beam inactivated *Salmonella* respectively.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Epithelial cells at mucosal surfaces are the first line of defense against microbial pathogens. *Salmonella* invade non-phagocytic cells like intestinal epithelial cells by inducing membrane deformation and rearrangement. This research was conducted to determine if e-beam irradiated *Salmonella* maintains the capacity to invade human host cells *in vitro*. The *Bac*Light staining assays were conducted to confirm that membrane integrity of *Salmonella* after e-beam inactivation at a target dose of 7 kGy is maintained. Then, infectivity assays were conducted to analyze the ability of e-beam irradiated *Salmonella* to infect human host cells. Visualization of internalized *Salmonella* in the host epithelial cells was attempted. Real-time quantitative reverse transcription PCR was performed to analyze differential host gene expression between non-treated, live, heat-inactivated, and e-beam inactivated *Salmonella* infection of the host cells.

Confirmation of Membrane Integrity of E-beam Irradiated *Salmonella*

Visualization of live *Salmonella* cells stained using the LIVE/DEAD® *Bac*Light Viability Kit served as the comparison to e-beam irradiated *Salmonella,* which was also stained with the *Bac*Light. Live bacterial cells with intact membranes are expected to fluoresce green and dead bacterial cells are expected to fluoresce red after the staining procedure. SYTO 9® stain, penetrates all bacterial membranes and stains the cells green, while Propidium iodide only penetrates cells with damaged membranes. The combination of the two stains produces red fluorescing cells (96, 97). Propidium iodide,

the red fluorescent stain used in the *Bac*Light staining procedure, can only permeate through bacterial cells whose membrane structure has been damaged in which it labels these bacteria red by staining the DNA and DNA containing organelles (96-98). The images captured of live *Salmonella* stained with *Bac*Light clearly show the bacteria fluorescing green. These results confirm that live *Salmonella* maintains its membrane integrity as expected. Images of heat-inactivate *Salmonella* stained with *Bac*Light showed the bacteria fluorescing red, which indicated that heat-killed *Salmonella* does not maintain its membrane integrity after the lethal heat treatment. It was observed that *Salmonella* that had been e-beam irradiated at 7 kGy also fluoresced green. These results indicate and confirm that *Salmonella* that has been e-beam irradiated at a lethal dose of 7 kGy maintains its membrane integrity. Other forms of microbial inactivation, such as heat-killing, cause damage to the bacterial membrane (98). Auty et. al confirmed that heat-killed *L. paracasei* fluoresces red when stained with *Bac*Light. Another study utilized the *Bac*Light stain to observe the differences in *E. coli, S.* Typhimurium, and *Shigella flexneri* that had been exposed to different artificial doses of UVA irradiation (99). In this research it was observed that e-beam irradiated *Salmonella* maintained its membrane integrity after irradiation; however, there are claims that other forms of ionizing radiation, such as X-ray, cause membrane damage and malfunction (100) The authors observed the viable *S.* Typhimurium bacterial populations demonstrated strong green fluorescence, while those samples that were exposed to UVA exposure demonstrated a strong red fluorescence (99). The authors concluded that certain doses of UVA irradiation would cause membrane damage in *S.* Typhimurium as well as other

gram-negative bacteria that were tested. If *Salmonella* maintains its membrane integrity but not the ability to replicate it is possible that e-beam inactivated *Salmonella* could serve as a suitable vaccine so long as it is no replication occurs. In this study, e-beam irradiated *Salmonella* did not replicate after being irradiated at a lethal dose of 7 kGy. The maintenance of membrane structures after microbial inactivation would be ideal, because the bacteria would be more immunogenic than bacteria whose membrane structure has been compromised.

Visualization of Internalized Bacteria in Host Cell Lysates

HCT-8 cells were challenged with live and e-beam irradiated *Salmonella* for 1 hour. The host cells were lysed with 1% Triton X-100 and stained with *Bac*Light. The purpose of this assay was to visualize those bacteria that had invaded the host cells. By lysing the host cells it was hypothesized that the internalized bacteria would be released into the lysate where it would be stained the *Bac*Light stain. The cell lysate containing the internalized bacteria were viewed under a fluorescence microscope to determine if the internalized live and e-beam irradiated bacteria could be detected. Unfortunately, the images captured could not definitively provide evidence that the material, which was giving off fluorescence, was that of bacteria that had invaded the host cell. The image of LST challenged HCT-8 cells (Figure 3) shows what is thought to be host cell debris potentially containing internalized bacteria. The same result was observed in the EBST challenged HCT-8 cell lysate. Based off of the images obtained, it cannot be concluded that the material giving off green fluorescence in the images is bacteria that has invaded the host cells. Both the host cell lysate and bacterial cells stained and fluoresced green,

therefore the results from this assay were inconclusive. There are no current published studies that have used the *Bac*Light staining method to stain bacterial infected eukaryotic cell lysate.

Real-time Quantitative Reverse Transcription PCR Analysis

The gene expression of the human adenocarcinoma cell line, HCT-8, was analyzed after being infected *in vitro* with live (LST), heat-inactivated (HKST), and e-beam inactivated (EBST) *Salmonella* over 1, 4, and 24 hours of infection. Live, heat-inactivated, and e-beam inactivated infections will be referred to as LST, HKST, and EBST infections respectively. The negative control was non-infected HCT-8 cells, while the positive control was LST infected host cells for 1 hour. Host cells infected with LST for 1 hour were used as a positive control comparison for all time points, because HCT-8 cells could not withstand 4 or 24 hours of co-incubation with LST. Preliminary studies not included in this work showed that HCT-8 cells subjected to LST infection for longer than 3 hours would result in the cell monolayers losing adherence capabilities to the cell culture flask or wells. In this scenario, the infected host cell samples would be lost in the repeated wash steps, thus losing the samples for further downstream assays. *Salmonella* infected HCT-8 cells were collected and real-time PCR assays were conducted to determine if there was differential gene expression in LST, heat-inactivated HKST, and EBST treatments over time.

Nf-kB Gene Expression Analysis

The average delta C_t values of the gene *Nf-kB* were analyzed over the 1, 4 and 24 hour time periods to determine if there was a significant difference in gene expression

over time. Treatments at each time point were also compared to each other to determine if there were any significant differences present. At 1 hour, *Nf-kB* delta C_t values were compared between treatments and no significant differences in gene expression were observed. At 4 hours, a significant difference (*p*-value= 0.0418) between EBST infected cells versus the negative control was observed. The EBST infected host cell gene expression of *Nf-kB* was higher than the negative control as indicated by its lower delta C_t value. This suggests that EBST transfection of host cells caused an up-regulated expression of *Nf-kB,* which is due to the host cell recognizing extracellular and intracellular bacteria. When the host cell recognizes extracellular and intracellular bacteria, *Nf-kB* is activated via protein signaling cascades. It is then activated and transcribes genes that code for inflammatory mediators in response to recognized bacteria (68). When comparing the 1-hour LST infection to the 4-hour EBST infection a significant difference (*p*-value= 0.0314) in the gene expression was found. EBST infected cells had a higher gene expression level than the 1-hour LST infected cells suggested by its lower delta C_t value. However, this could not be considered a true difference in gene expression, as the time course of infection was not the same for which treatment.

At 24 hours, a significant difference (*p*-value= 0.0414) between EBST infected cells and non-infected cells were observed. EBST infected cells had a higher gene expression level of *Nf-kB* than non-infected cells as indicated by its lower delta C_t value. When comparing the HKST infected cells to the non-infected cells a significant difference (*p*-value= 0.0317) was also observed. HKST infection induced a higher gene

expression level of *Nf-kB* than the non-infected cells. It was confirmed that HKST and EBST infections of host cells caused up-regulation of *Nf-kB*, further confirming the host cells can detect surrounding inactivated bacteria. When comparing the 24-hour EBST infection to the 1-hour LST infection a significant difference (*p*-value= 0.0145) was notice. EBST infected cells had a higher level of gene expression than the 1-hour LST infected cells. This same trend was seen in the comparison between HKST infected cells and 1-hour LST infected cells (*p*-value= 0.0064). Gene expression was higher in HKST infected cells when compared to 1-hour LST infected cells suggested by its lower delta C_t value. As previously indicated, these gene expression levels could not be considered a true difference in gene expression, as the time course of infection was not the same for each treatment.

Rela Gene Expression Analysis

There were no statistical differences observed in the expression levels of *Rela* between treatments for 1, 4, or 24 hours of infection as indicated by their *p*-values. When comparing the delta C_t values for each individual treatment across time points, there did not seem to be significant variation. Based off of the data collected, the transfection of human host cells with LST, EBST, and HKST caused no difference in gene expression levels of *Rela*. Because *Rela* couples with *Nf-kB* to regulate transcription of genes in response to stress, up-regulation of *Rela* was expected (71). These results may have been due to low primer binding efficiency or too low of an MOI during the transfection protocol. If the MOI was increased from 10 to 100, the expression levels of *Rela* may have been more robust.

Lbp Gene Expression Analysis

At the 1-hour time point there was no statistical difference in gene expression levels of *Lbp* between non-infected, LST, HKST, and EBST infected cells.

At 4 hours, a significant difference (*p*-value= 0.0321) between EBST infected cells and 1-hour LST infected cells was detected. EBST infected cells had a higher gene expression level of *Lbp* than the 1-hour LST infected cells as indicated by it lower delta C_t value. The difference of gene expression levels that were observed could not be a true comparison considering the time course of infection was the not same for each treatment.

At 24 hours, there was a difference in delta C_t values for *Lbp* across treatments; however, a statistical difference in gene expression levels between treatment comparisons was not observed as suggested by their *p*-values. Even though the differences were not significant, there was a clear observation of increased expression of *Lbp* in host cells that had been infected with EBST when compared to the negative control and heat-killed *Salmonella* treatments. When the *Lbp* gene is transcribed the host cell will secrete lipopolysaccharide (LPS) binding protein, which binds LPS from Gram-negative bacteria (69). The results indicate that host cells respond to EBST at a higher level than HKST when observing *Lbp* gene expression, which could potentially mean that LPS on EBST may be more intact and recognizable by the host cells than HKST. This further confirms that e-beam irradiation at 7 kGy does not cause damage to the membrane of *Salmonella*.

Lyz Gene Expression Analysis

After 1 hour of infection, there were no statistical differences observed in *Lyz* gene expression levels across treatment comparisons. After 4 hours, there was a significant difference (*p*-value= 0.0089) observed between EBST infected cells when compared to 1-hour LST infected cells. The 4-hour EBST infection treatment induced higher gene expression of *Lyz* than the 1-hour LST infection of host cells suggested by its lower delta C_t value. The same trend was observed when comparing HKST infected cells to the 1-hour LST infected cells. HKST infected cells had a significantly higher (*p*-value= 0.0287) level of gene expression of *Lyz* when compared to 1-hour LST infected cells as indicated by the C_t value. However, these differences could not be considered true differences in gene expression, as the time course of infection was not the same for each treatment comparison. There were no statistical differences of *Lyz* gene expression levels observed at the 24-hour time point across treatment comparisons. Lysozyme is an anti-microbial agent secreted by human host cells in response to bacterial invasion (70). The *Lyz* gene was not considered up-regulated in response to LST infection of host cells, which may have been due to the MOI not being high enough. If the MOI were increased to 100, there may have been a more robust and consistent level of *Lyz* expression.

Cd14 Gene Expression Analysis

Gene expression levels for *Cd14* at the 1-hour time point showed no significant differences when comparing the delta C_t values across infection treatments. This was indicated by the *p*-values for each treatment comparison. After 4 hours of infection, there was a significant difference (*p*-value= 0.0364) between EBST infected host cell gene

expression and 1-hour LST infected host cell gene expression of *Cd14*. EBST infected cells had a higher gene expression level than the 1-hour LST infected cells suggested by its lower delta C_t value. However, this could not be considered a true difference in gene expression, as the time course of infection was not the same for each treatment. When comparing HKST infection to the non-infected after 24 hours of infection a significant difference (*p*-value= 0.008) in the gene expression was found. HKST infected cells had a higher gene expression level than the non-infected cells suggested by its lower delta C_t value. It is important to note that the expression levels of *Cd14* were highest in EBST infected cells followed by LST infected cells. There was not a significant difference observed in the gene expression level comparison between EBST and LST infected cells, suggesting EBST infection was inducing similar gene expression of *Cd14* as the 1-hour LST infected cells. *Cd14* is a co-receptor with *Tlr4* that is responsible for the detection of extracellular LPS from gram-negative bacteria (72, 73). The gene expression results suggest that host cells can recognize e-beam irradiated *Salmonella* LPS, which further confirms that LPS is most likely not damaged after irradiation.

Tlr4 Gene Expression Analysis

At 1 hour, there were no significant differences of *Tlr4* expression levels between treatment comparisons as indicated by their delta C_t values and corresponding *p*-values. There were significant differences of *Tlr4* expression values observed at 4 hours. Continuing with the same trend, EBST infected cell gene expression of *Tlr4* was significantly (*p*-value= 0.0103) higher than the 1-hour LST infected cell gene expression as suggested by its delta C_t value. However, the difference in gene expression cannot be

considered a true comparison, as the time course of infection was the not same for each treatment in this particular case. No significant differences of *Tlr4* expression levels were detected at 24 hours between any of the infection treatment comparisons. *Tlr4* couples with *Cd14* to detect LPS from Gram-negative bacteria to mediate signal transduction pathways (72, 73). Even though the differences were not significant, it was observed that both *Cd14* and *Tlr4* were expressed at higher levels in EBST infected host cells than HKST infected host cells at 24 hours. These gene expression results suggest that the host cells can recognize e-beam irradiated *Salmonella* LPS, which further confirms that LPS is most likely not damaged after irradiation. If the MOI was increased from 10 to 100 there may have been a more robust gene expression response and lower standard error, which would have indicated a significant difference of expression levels of these target genes.

Nod2 Gene Expression Analysis

At 1 hour, there were no significant differences of *Nod2* expression levels between treatment comparisons as indicated by their delta C_t values and corresponding *p*-values.

At 4 hours, a significant difference (*p*-value= 0.0028) in *Nod2* gene expression when comparing EBST infected cells to the 1-hour LST infected cells was observed. The EBST infected cells had a higher level of gene expression in *Nod2* than the 1-hour LST infected cells as depicted in the delta C_t values. While a significant difference was observed, this difference could not be considered a true comparison, as the time course of infection was not the same for each treatment.

At 24 hours, a significant difference (*p*-value= 0.0024) between LST infected cells and non-infected cells were observed. The 1-hour LST infected host cells had a higher level of expression of *Nod2* than non-infected host cells as indicated by the C_t value. This difference in expression was expected. There was also a significant difference (*p*-value= 0.0011) in HKST infected cells versus the 1-hour LST infected cells. HKST infected cells had a higher gene expression level of *Nod2* than the 1-hour LST infected host cells. This comparison could also not be considered a true comparison between gene expression levels, because the time course of infection was not the same for each treatment.

Although there were no comparable significant differences between the gene expression levels of *Nod2,* there was an observed trend of higher levels of *Nod2* expression in host cells infected with EBST at 4 and 24 hours when compared to HKST infected host cells. This is important to note, because *Nod2* is an intracellular pattern recognition receptor that recognizes LPS and peptidoglycan from Gram-negative bacteria that have invaded the host cell (75). If EBST infected host cells showed a considerably significant increased expression of *Nod2,* then it could be stated that EBST has the capability to invade human intestinal epithelial cells.

Il-8 Gene Expression Analysis

At 1 hour, there were no significant differences of *Il-8* expression levels between treatment comparisons as indicated by their delta C_t values and corresponding *p*-values.

At 4 hours, a significant difference (*p*-value= 0.0150) was observed between EBST infected cells and non-infected cells. EBST infected cells had higher levels of *Il-8* expression than non-infected cells suggested by the delta C_t value.

At 24 hours, both EBST and HKST infections are significantly different (*p*-value= 0.0476 and 0.0141 respectively) than the non-infected cells. EBST and HKST infected cells had a higher level of *Il-8* gene expression than non-infected cells. A significant difference was not detected when comparing EBST infection to HKST infection, so it can be assumed that these two treatments have similar effects on the host cell *Il-8* expression.

Slpi Gene Expression Analysis

At 1 hour, there were no significant differences of *Slpi* expression levels between treatment comparisons as indicated by their delta C_t values and corresponding *p*-values.

A significant difference (*p*-value= 0.0339) was observed between HKST infected cells and non-infected cells at the 4-hour time point. The non-infected host cells had higher gene expression levels of *Slpi* than HKST infected cells as indicated by the C_t values. This could be a result of a low MOI when conducting the invasion assay.

At 24 hours, a significant difference (*p*-value= 0.0034) was observed between LST infected cells and non-infected cells as expected. LST infected cells had a higher level of expression for *Slpi* than non-infected cells. Both EBST and HKST infected cells had significantly (*p*-value= 0.0062 and 0.0035 respectively) lower gene expression of *Slpi* than LST infected cells, which was expected.

Spli is a secreted inhibitor that provides antibiotic activity to epithelial cells in response to bacterial invasion (77). The *Spli* gene was not considered up-regulated in response to LST infection of host cells, which may have been due to the MOI not being high enough. If the MOI were increased to 100, there may have been a more robust and consistent level of *Spli* expression.

EBST Treatment Compared to HKST Treatment

Gene expression levels, presented in fold change values, were compared between the EBST infected cells and HKST infected cells at the 1, 4, and 24 hour time points of infection. At 1 hour the only significant difference observed between expressions in the target genes was for *Cd14*. However, this does not hold true for the 4, and 24-hour time points. At 4 hours, there was no significant difference in the gene expression for any target genes when comparing the two treatments. At 24 hours, expression of *Il-8* for HKST infected host cells was significantly higher (p-value= 0.0342) than EBST infected host cells. This suggests that HKST infections induced a higher anti-inflammatory response than EBST infections through up-regulating *Il-8*, the chemoattractant responsible for mediating inflammatory responses. All other target genes were not significantly different between EBST and HKST infected cells.

Analysis of Results

It was confirmed that e-beam irradiated *Salmonella* maintains its membrane integrity after a lethal dose of irradiation. The host cell lysate staining assay conducted to detect internalized *Salmonella* yielded inconclusive results. The HCT-8 host gene expression between treatments was compared to determine if EBST infection was

significantly different than LST and HKST infections as well as non-infected host cells. It can be concluded that EBST infection of host cells induced higher gene expression in *Nf-kB* at 4 hours and 24 hours when compared to non-infected host cells. Both EBST and HKST infections resulted in significantly higher levels of expression of *Nf-kB* at the 24-hour time point. Because there was not a significant difference in gene expression levels of *Nf-kB* when comparing EBST and HKST infected cells, it can be concluded that the EBST and HKST infections have similar effects on *Nf-kB* expression. *Nf-kB* regulates the expression of genes involved in defense and immune processes, and is typically activated following extracellular stimulation in response to threatening pathogens (101, 102). Published studies have confirmed that traditional transfection of host epithelial cells with live bacteria will induce *Nf-kB* activation (102, 103). Because the EBST and HKST both induced significant differential gene expression of *Nf-kB* when compared to non-infected cells, we can make the claim that EBST and HKST make a have similar effects on the host epithelial cells as LST infection.

Il-8 expression was also significantly higher in EBST infected cells than non-infected cells for the 4 and 24-hour time points. Because there was not a significant difference in gene expression levels of *Il-8* when comparing EBST and HKST infected cells, it can be concluded that the EBST and HKST infections have similar effects on *Il-8* expression. When EBST and HKST infected host cells were compared as a whole, there was not a significant difference observed. Chemokines are a family of small polypeptides, which have chemoattractant properties for inflammatory cells (104). *Il-8,* which is secreted by several cell types including endothelial cells, is one of the most

extensively studied members of this group (59, 104, 105). Studies have proven that intestinal epithelial cells secrete *Il-8* after exposure to invasive bacteria (59, 105).

Eckmann *et al*. investigated the ability of T84 colonic intestinal epithelial cells to provide an inflammatory response through the release of chemotactic cytokines such as *Il-8* (59, 106). They proved epithelial cells secrete *Il-8* in response to invasive bacteria, such as *S*. Typhimurium (ATCC 14028), and that bacterial entry is required to produce increased levels of *Il-8* (59). Eckmann analyzed the effects of bacterial invasion on Caco-2 and HT-29 colonic epithelial cells as well, in which *Il-8* was produced at higher levels when exposed to bacterial invasion (105). Because *Il-8* gene expression was significantly higher in EBST and HKST infection of HCT-8 cells when compared to non-infected cells, and no significant difference when compared to LST infected host cells, it can be concluded that they have a similar effects on host cells that are infected with live, invasive bacteria.

At 24 hours of infection EBST infected host cell gene expression *appeared* to be different for *Lyz, Tlr4,* and *Nod2* when compared to HKST infected host cells; however, because the standard error of the expression levels of these genes was large these values were not considered significantly different. The innate immune response involves a number of constitutively expressed and inducible humoral factors including antimicrobial peptides like lysozyme (107, 108). These antimicrobial peptides are produced and secreted when pathogenic bacteria invade and infect host cells (108). Even though the gene expression of *Lyz* in the EBST infected cells was not significant, there was an obvious trend of increased expression at 24 hours. Additional invasion assays

and gene expression analysis must be conducted to definitively state that *Lyz* is significantly upregulated during EBST infection.

The *Tlr4* gene codes for toll-like receptor 4 proteins, which detect lipopolysaccharide (LPS) from gram-negative bacteria (109). Studies have confirmed that *Tlr4* is expressed at low levels in intestinal epithelial cells under normal conditions (110). Although the gene expression of *Tlr4* in the EBST infected cells was not significant, there was an obvious trend of increased expression at 24 hours. Only few studies have investigated pathogen-associated molecular patterns, like LPS and peptidoglycan, and their receptors in mediating human cell activation using whole bacteria (111-113). Elson *et al*. defined the host cell receptors that predominately mediated cell activation to bacterial pathogen-associated molecular patterns when presented to cells as whole bacteria (114). This study similarly utilized invasion assays on human epithelial cells to study the change in expression profiles of similar genes used in this research. This study also challenged human host cells with heat-killed and antibiotic killed bacteria as treatments for their invasion assay. They specifically assessed the effect of heat inactivation on the integrity of pathogen-associated molecular patterns and their ability to stimulate an immune response. They were able to conclude that heat-killed bacteria and antibiotic (gentamicin)-killed bacteria produced equivalent levels of cellular activation when analyzing *Tlr4, Il-8,* and *Cd14*, where heat-killed and antibiotic-killed bacteria caused higher levels of expression of those specific genes when compared to their negative control. (114). One of the limitations Elson *et al*. faced was similar to a limitation in this research; live bacterial transfection of the host cells

produced varied results, and host cells could not withstand prolonged periods of transfection incubation periods with the bacteria (114).

There was a considerably higher level of expression of *Tlr4* in host cells infected with HKST, which was consistent with Elson's findings. EBST infected host cells had an even higher level of expression of *Tlr4* suggesting the host cells could better recognize EBST through its pattern-recognition receptor, *Tlr4*. These findings could also support the evidence that EBST maintains its membrane integrity after irradiation.

Studies have similarly found that *Nod2* expression levels are significantly up-regulated in epithelial cells upon stimulation with LPS, which results in the activation of *Nf-kB* (115). Nod2 is a protein that directly recognizes bacterial molecules like peptidoglycans and lipopolysaccharides, which are components of the *Salmonella* bacterial cell wall (70). It is an intracellular surveillance protein that will detect internalized bacteria within the host cell (116). Studies have proven that a loss of activity by Nod2 can result in the inability of local responses in the intestinal mucosa to control bacterial infection (70). Keestra *et al*. discovered that *S*. Typhimurium (ATCC 14028) infection of HeLa intestinal epithelial cells causes the activation of the protein Nod2, which contributes to the activation of Nf-kB (116, 117).

Keestra's findings can be compared to those in this study, because a human intestinal epithelial cell line was used to analyze the expression and production of Nod2 in response to *S*. Typhimurium infections. Similar to Keestra's findings, both *Nf-kB* and *Nod2* expression levels were higher in LST infections of host cells, and there was an observed increased level of expression of both genes in EBST infected host cells. These

results suggest EBST infection has similar effects on the host cells when compared to LST infection. While there was an observed trend of increased expression of the *Nod2* gene at 24 hours for EBST infected cells, the observation was not considered significant. This can be attributed to the large standard error in the data. To confirm that EBST infection causes increased gene expression of *Nod2*, additional invasion assays and gene expression studies must be conducted. If *Nod2* host cell gene expression can be proven to be significantly upregulated during EBST infection it could help prove EBST has the ability to invade human host cells.

The first objective of this study – to determine if e-beam irradiated *S*. Typhimurium has the ability to invade human cells – was not definitively achieved in that e-beam *Salmonella* could not be visualized inside the human host cells. However, it was proven that e-beam inactivated *S*. Typhimurium infection of human intestinal epithelial cells does cause differential gene expression.

Future Directions

The next measure that would be taken to attempt to visualize intracellular bacteria of the infected host cells would be to utilize immunofluorescence microscopy. Double immunofluorescence staining techniques are used to differentiate between extracellular and intracellular bacteria in host cells grown in cell culture monolayers (118). Infected monolayers are fixed and stained with antibacterial antibodies followed by labeling with a secondary fluorescent label such as fluorescein. The monolayers are then permeabilized and relabeled with antibacterial bodies with a secondary fluorescent label that is different than the one used previously (118, 119). Since the eukaryotic cell

membrane prevents penetration of antibodies until it is permeabilized, the extracellular bacteria will have been tagged with both labels, while the intracellular will have been tagged with only one fluorescent label. The ingested or intracellular bacteria can then be distinguished from the bacterial cells attached to the surface of the host cells when being visualized under a fluorescence microscope.

Host gene expression was analyzed between non-infected, HKST, EBST and LST infected human epithelial cells at 1, 4, and 24-hour time points. One of the main limitations of this assay was the ability to compare LST infections to the other treatments over long periods of time. Elson *et al*. also expressed this as a main limitation in their infection protocol and further down-stream assays (114). The host cells could not withstand long periods of exposure to LST. Consequently, this resulted in an untrue comparison of gene expression between LST and other treatments for 4 and 24 hours. A potential solution to this would be to transfect the host cells for 1 hour and proceed with the gentamicin protection assay. After the gentamicin treatment, the LST infected host cells would be allowed to incubate for an allotted amount of time until recovery of host cells was desired for gene expression analysis. This would allow the intracellular bacteria to replicate within the host cell for 4 and 24 hours, where the host cells could then be collected for gene expression analysis and be compared to the other treatments across time points. For results with less standard error, the gentamicin protection assay would need to be conducted again where the host cells would be infected at an MOI of 100. Studies have shown that intestinal epithelial cells subjected to pathogenic bacteria at an MOI of 100 results in a more robust and consistent response of target gene

expression (120-122). Ultimately, further studies will be required to confirm that e-beam irradiated *Salmonella* has the capability to invade human host cells; however, it can be definitively concluded that EBST transfection of host cells does in fact cause differential gene expression and trends of increased gene expression in extracellular and intracellular pattern recognition receptors in human intestinal epithelial host cells.

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