Minimum Ultraviolet Light Dose Determination and Characterization of Stress Responses that Affect Dose for *Listeria monocytogenes* Suspended in Distilled Water, Fresh Brine, and Spent Brine

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ABSTRACT

Foodborne illnesses caused by *Listeria monocytogenes* have long been associated with ready-to-eat (RTE) meats contaminated after the primary thermal process has been applied. It is believed that brine solutions used to chill cooked RTE products may serve as a reservoir for *L. monocytogenes* becoming a potential point of post-processing contamination for RTE meats. Re-circulating ultraviolet light (UV) systems are being used to inactivate *L. monocytogenes* in chill brines; however very little has been reported on the dose response of healthy and stressed *L. monocytogenes* to UV in brine solutions. The objectives of this research were to determine 1) minimum dose of UV required to inactivate *L. monocytogenes* in distilled water, fresh brine, undiluted spent brine, and diluted spent brine, 2) if adaptation to food processing stresses affects the dose response, and 3) if the acquisition of antibiotic resistance mechanisms provides resistance to ultraviolet light 4) effect of stress adaptation on survival in brine solutions. After UV exposure, populations were reduced as follows from greatest to least: water > fresh brine > 5% spent brine > 35% spent brine > 55% spent brine > 100% spent brine ($P \leq 0.05$). There were no population differences between acid stressed and antibiotic resistant or healthy and heat shocked ($P > 0.05$). However, acid-stressed and sulfanilamide-resistant were more resistant to UV light than healthy and heat shocked *L. monocytogenes* ($P \leq 0.05$). Survival in brine solutions (no UV) followed the trend, from greatest to least ($P \leq 0.05$): sulfanilamide-resistant > acid-stressed > healthy > heat-shocked. Population estimates decreased from initial inoculation to final sampling for each cell type suspended in spent brine ($P \leq 0.05$), but only healthy and heat-shocked cells suspended in fresh brine were significantly reduced ($P \leq 0.05$). Knowledge of UV dosing required to control *L. monocytogenes* in brines used during RTE meat processing, and a greater understanding of the interactions that may influence dose will aid manufacturers in establishing appropriate food safety interventions for these products.

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CHAPTER I

INTRODUCTION and JUSTIFICATION

Listeria monocytogenes is a pathogen of concern in re-circulated brine solutions, commonly used to cool Ready-to-eat (RTE) meat products such as frankfurters and deli meats. *L. monocytogenes* is the causative agent of listeriosis, an invasive infection, which has a mortality rate of 20-30% in susceptible populations [1, 2]. Although *L. monocytogenes* is responsible for less than 0.1% of foodborne related illnesses, it accounts for 37% of the deaths [3]. Outbreaks of Listeriosis have prompted the United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) to implement a zero tolerance policy for this organism in RTE products [4], and the Food and Drug Administration (FDA) to set very strict standards [5]. Although easily killed during heat processing steps, *L. monocytogenes* is capable of surviving the cool temperatures and high salt conditions maintained in brine chillers, thereby increasing the possibility of post-processing contamination [6].

Ultraviolet light (UV) has been identified as a potential method to eliminate L. monocytogenes from brine solutions because it is economical, able to kill a variety of organisms and because it does not leave a residue or significantly affect the sensory or nutrient quality of food products [7-15]. UV has been successfully used to disinfect water [16, 17], processing surfaces, and fruit juices [10, 12]. However, brine solutions pose a unique problem due to the fact that they can be reused for up to four weeks [6]. As brine continues to be recycled, it becomes more turbid, the pH drops, and accumulating food particles can create microenvironments, which in turn may protect the organism from sterilization [18-20].

In order to effectively use UV for the disinfection of brine solutions, it is essential to know the actual amount of energy required to inactivate *L. monocytogenes* as well as the amount of energy being received by the microorganisms. Previous studies using gamma, long-wave ultraviolet, and UV to disinfect food and preparation surfaces have reported an extrapolated dose calculated by multiplying the time of exposure by the intensity of the bulb used to generate the energy [14, 21]. Although this provides a rough estimate of the dose, it does not take into account the loss of bulb intensity over time or the intrinsic factors of the food (turbidity, soluble solids, insoluble solids), which may affect the dose being received by each bacterial cell. A variety of studies have been conducted to determine the effectiveness of UV in eliminating *Listeria* and other organisms from different substances and surfaces [9-11, 13-15, 22-25], but no

information exists as to the amount required specifically to inactivate *L. monocytogenes* in a chilled brine solution. Knowing the minimum amount required to inactivate *L. monocytogenes* will allow a more effective use of UV treatment, and provide assurance that all cells have been eliminated.

Temperature, salt content, and equipment sterilization are intended to eliminate or inhibit the growth of microorganisms. However, under certain conditions, bacterial cells may adapt or become resistant to imposed stresses [26]. Because adaptation to certain stresses provides cross-protection against other stresses, it is necessary to determine if a similar relationship exists between common food processing stresses and UV treatment. Lou and Yousef determined that *L. monocytogenes* became more resistant to increased levels of sodium chloride (up to 25%) after a heat shock treatment of 45°C for one hour [26]. In addition, they also discovered an increased resistance to lethal amounts of acid (pH 3.5) after adaptation to mild acid conditions (pH 5.0) [26]. Similar studies have shown that starvation may increase the resistance of *L. monocytogenes* to electron beam irradiation [27]. Exposure to common industrial sanitizers may also increase the heat resistance of *L. monocytogenes* [28]. In order to effectively use UV energy to disinfect brine solution, the effect of stress adaptation on the dose required to inactivate *L. monocytogenes* must also be determined.

A recent study found that 73% of *L. monocytogenes* isolates from RTE meats, raw chicken and fresh produce were resistant to sulfonamide, and 8.5% to tetracycline [29]. Since the first antibiotic resistant strain of *L. monocytogenes* was isolated in 1988 [30], other resistant strains have been isolated [31, 32], including a strain resistant to trimethoprim, which is commonly used in combination with sulfamethoxazol for patients allergic to penicillin [30]. In a 1982 study, Meckes observed the effect of UV light on antibiotic resistant coliforms and determined that UV light selected for antibiotic resistant strains, and depending on the antibiotics, multi-resistant strains [33]. He found that the percentage of antibiotic resistant strains of coliforms was higher after UV disinfection of wastewater effluent than before [33]. Although occurrence of resistant strains of *L. monocytogenes* is still low in comparison to other species [32], it is likely that new strains will continue to emerge due to the ease with which resistant genes can be transferred from one species to another via transferable plasmids [34]. In addition, genes that grant antibiotic resistance for different antibiotics typically occur closely together on

the same plasmid, so bacteria may become resistant to multiple antibiotics with each transfer [34].

In order to appropriately apply ultraviolet light to the sterilization of brine solutions, the following objectives must be explored.

RESEARCH OBJECTIVES

1. To determine the minimal dose of ultraviolet light required to significantly reduce the amount of *Listeria monocytogenes* in brine solutions.
2. To determine if adaptation to common food processing stresses (acid and heat) affects the dose of ultraviolet light required to significantly reduce the amount of *Listeria monocytogenes* in brine solutions.
3. To determine if the acquisition of antibiotic resistance mechanisms provides resistance to ultraviolet light.
4. To determine the effect of stress and antibiotic resistance on the survivability of *Listeria monocytogenes* in brine solutions.

CHAPTER II

LITERATURE REVIEW

Listeria monocytogenes

The genus *Listeria* contains six species, two of which are pathogenic: *Listeria monocytogenes* (pathogenic to humans and animals), *L. ivanovii* (responsible for abortions in animals), *L. innocua , L. welshimeri, L. seeligeri,* and *L. grayi* [35]. *L. monocytogenes* was not recognized as a human pathogen until 1949 [36], and was not confirmed as a foodborne pathogen until an outbreak due to contaminated coleslaw occurred in Canada in 1981 [37] and in Mexican style cheese in both Switzerland and the United States during the mid 1980's [38, 39].

L. monocytogenes is a Gram-positive, facultatively anaerobic rod, which exhibits a tumbling motility at 20-25°C (due to several peritrichous flagella) [35] but not at its optimal growth temperature of 32-35°C . *L. monocytogenes* is capable of growth between 0-45°C [40, 41], pH 4.4-9.4 [42], and water activity as low as 0.90 [43], depending on the solute [2, 44]. Most mciroorganisms are inhibited at water activity below 0.93 [45]. *L. monocytogenes* is catalase positive, oxidase negative, and exhibits B-hemolysis on blood agar [35]. Cells are found as single cells, in short chains, or arranged in a V or Y pattern [35], and may display different morphological characteristics depending on the environment [46]. Viable cells have been isolated from soil, decaying vegetation [47], water, human and animal waste products [48, 49] and throughout food manufacturing environments [50]. This organism can be found in damp areas (where it may be aerosolized by high pressure cleaning) and on surfaces such as rubber, stainless steel and glass as sanitizer resistant biofilms [51].

L. monocytogenes exhibits heat resistance greater than other non-spore forming vegetative bacteria after exposure to sublethal stresses [52-54], and is capable of adapting to and growing in low or high pH [28, 39], high salinity, refrigeration temperatures [55-57] and on dry surfaces [58]. Exposure to sub-lethal processing stresses may actually help protect or acclimate *L. monocytogenes* to later stresses, making it more virulent and difficult to kill [59, 60].

Listeriosis

According to the Centers for Disease Control and Prevention (CDC) *L. monocytogenes* is responsible for over 2500 cases of Listeriosis each year, resulting in approximately 500 deaths [61]. Although rare (accounts for less than 0.1% of foodborne illnesses), Listeriosis is an invasive disease with a mortality rate between 20-30% [62]. Once ingested, the organism

penetrates the lining of the gastrointestinal tract, and is taken up by phagocytes [44, 63] which then move to different locations inadvertently transporting the organism throughout the body [42]. The onset of symptoms ranges from 2-3 weeks after ingestion [1]. The likelihood that the organism will penetrate the lining of the intestine depends on many factors, such as the number of cells ingested, virulence of the strain, and host susceptibility [1]. Risk factors for developing Listeriosis include age (over 65), pregnancy, HIV infection, and those suffering from diabetes, kidney disease or cancer [64]. In non-healthy adults or children, symptoms may include meningitis, encephalitis, septicemia [62]. Pregnant women may experience flu-like bacterimia leading to premature birth, stillbirth, or abortion [63]. In healthy non-pregnant adults, ingestion of *L. monocytogenes* cells may instead lead to noninvasive febrile gastrointestinal disease [65]. Eleven hours to one week after expose, symptoms such as fever, diarrhea, and flu-like symptoms may occur [65]. Although thirteen serotypes have been identified, 95% of human listeriosis infections are caused by serotype 4b, 1/2a and 1/2b [42, 66, 67].

Listeriosis infections are most commonly treated with penicillin G or ampicilin in combination with an aminoglycoside such as gentomycin [68]. For patients allergic to penicillin, trimethoprim is administered in combination with a sulfonamide [30].

Outbreaks

Listeria monocytogenes was first recognized as a foodborne pathogen during the 1980's, after separate outbreaks involving coleslaw and cheese occurred in Canada (18 deaths), Switzerland and the United States (48 deaths), respectively [37]. Since the foodborne link was confirmed, other outbreaks have been documented in Austria, England, Wales, Ireland, Denmark, Australia, France, Italy, and the United States [69]. Sources of these outbreaks have included contaminated dairy products (raw milk, butter, chocolate milk, cheeses), eggs, seafood, processed pork products, and vegetable salads [69]. Seafood [70] and dairy products [71] have also been implicated as sources of outbreaks of noninvasive febrile gastroenteritis disease in healthy adults.

Outbreaks in 1998 and 1999 due to contaminated frankfurters lead to 100 cases of listeriosis and 21 deaths [61], and in 2002 10 deaths occurred from the consumption of turkey deli meat [72]. Due to these and numerous other outbreaks in RTE products, the Food Safety Inspection Service (FSIS) currently maintains a "zero tolerance" policy for *L. monocytogenes* in

ready-to-eat (RTE) meat products [4], meaning that if two 25 gram samples test positive for *L. monocytogenes*, the product is considered adulterated [73]. Additionally, these outbreaks have prompted the FSIS to require that all producers of RTE meat products develop written protocols and procedures to control *L. monocytogenes* [4, 73]. The FDA is currently accepting public comments on proposed guideline changes. Among suggested changes are the classification of RTE products into two categories: those that support the growth of *L. monocytogenes* (0.04 CFU/g allowed) and those that do not (100 CFU/g allowed) [5]. Despite these regulations, outbreaks have continued to occur and products have continued to test positive for the organism [74].

Characteristics that Enhance the Survival of *Listeria monocytogenes*

The behavior of *L. monocytogenes* is often difficult to predict in a food system because of the complex relationship between temperature, pH, salinity water activity, and the overall food matrix [75]. Although it is impossible to assess the growth of *L. monocytogenes* in every food, it is possible to identify and characterize the factors responsible for its success [76, 77]. Ability to tolerate low temperature, high salinity, low water activity and pH, and its ability to adapt to stress allow *L. monocytogenes* to successfully grow in environments that are typically inhibitory to other microbes.

Ability to Tolerate Low Temperature

Temperature manipulation is a common and effective food preservation method used to decrease microbial spoilage and to control the growth of pathogens [78]. The effect of temperature, pH, or salinity on the growth of *L. monocytogenes* in RTE meat products is complicated by parameters such as individual strain characteristics [79], growth stage, and the food matrix [75]. *L. monocytogenes* is capable of survival and even growth at refrigeration temperatures [40, 41]. *L. monocytogenes* is inactivated by temperatures above 50°C [69], but can grow between 0°C and 45°C [40, 41] depending on the medium. The food medium or the presence of particular solutes (compatible osmolytes that help stabilize cells exposed to low temperature stress) can enhance growth at various temperatures [80]. Storing *L. monocytogenes* in chicken homogenate at -18°C for up to 84 days did not significantly reduce the viable cell count, illustrating the ability of rich media to enhance the survival of *L. monocytogenes* [81].

Buncic and colleagues found that serotype 1/2a survived better than serotype 4b when exposed to bacterocins at 4°C, but 4b survived post cold storage heat treatment better [79].

It has been estimated that up to 50% of RTE meat products are contaminated with *L. monocytogenes* [29, 82]. Although easily inactivated by temperatures above 50°C [37, 44], recontamination can occur during post-processing [83]. Because *L. monocytogenes* is able to survive and grow at refrigeration temperatures for extended periods, similar to the storage conditions of RTE meats products, it is possible that these products can be heavily contaminated by the time they reach consumers, increasing the likelihood that illness will occur. In addition, chilled brine solutions used to rapidly cool RTE meats, after heat processing, provide an ideal environment for *Listeria* species due to the low temperature and high salt concentration [6]. In an experiment using beef wieners, sixty-five percent were able to support the growth of *L. monocytogenes* under vacuum at 5°C after a seven-day lag. After 14 days, the populations had increased by an average of 1.26 log CFU/g [84]. Similarly, cheese brines maintained at 4°C were better able to support the growth of *L. monocytogenes* than at 12°C, despite the pH or salt content of the brine [85].

Ability to Tolerate High Salinity and Low Water Activity

A second factor that contributes to the success of *L. monocytogenes* in diverse environments is its ability to survive and grow under high salt, low moisture conditions, depending on the water activity (a_w) of the food as well as the primary solute present [2]. Most bacteria are inhibited at water activities below 0.93 [44, 45], however, experiments have shown that *L. monocytogenes* is capable of growth at lower water activities, with NaCl as the primary solute. Researchers have found that growth of *L. monocytogenes* is possible at 12% NaCl ($a_w \approx 0.92$) in brain heart infusion (BHI) broth [56] and trypticase soy broth (TSB) [43, 86]. Similarly, survival was possible up to 84 days at 4°C in fermented hard salami containing 5-7.8% NaCl and a water activity of 0.79-0.86 [82]. Farber and colleagues determined that *L. monocytogenes* was capable of growth in the presence of up to 14% salt [87]. *L. monocytogenes* was able to survive at least 132 days at 4°C in trypticase soy broth (TSB) containing 25.5% NaCl ($a_w \approx 0.83$) [88].

The ability to tolerate high salinity is strongly dependent on factors such as the temperature, pH, humectant, and bacterial strain [75]. Maximum growth in high salt conditions occurs at neutral pH [89] and lower temperatures [90]. *L monocytogenes* grown in TSB broth,

containing NaCl, survived longer at 4°C than at 22 or 37°C, and was ultimately able to survive for more than 200 days in commercial cheese brines held at 4°C [85]. Stenberg and Hammainen observed the growth of ten strains incubated at 20-24°C and found that *L. monocytogenes*: survived more than a year in 1% sucrose/10% NaCl, 24 days in 24% NaCl, 34-68 days in 12% NaCl [91]. In a similar study, *L. monocytogenes* grown in BHI medium, adjusted to equivalent water activity values using different solutes, exhibited the longest survival rate in glycerol ($a_w \approx 0.90$), NaCl ($a_w \approx 0.92$) was second, and propylene glycol third ($a_w \approx 0.97$) [6]. Shahamat and colleagues observed differences in survival of *L. monocytogenes* depending on salt concentration and storage temperature. *L. monocytogenes* in 25.5% NaCl gave the following results: cells incubation at 37°C lasted for 4 days, 22°C for 24 days, at 4°C lasted for 132 days. Decreasing the incubation temperature enhanced *L. monocytogenes* survival in concentrated salt solutions.

Ability to Tolerate Low pH

L. monocytogenes is able to grow from pH 4.4-9.6, optimally from 6.0-8.0 [44], depending on incubation temperature, type of acid, medium composition, and growth phase [37, 92]. In studies using *L. monocytogenes* isolates obtained from milk and from the 1985 Mexican cheese outbreak, both isolates were inhibited by 0.1% acetic, citric, or lactic acid as the incubation temperature was decreased [39]. *L. monocytogenes* Scott A grew to 10^8 CFU/ml in cabbage juice adjusted to 5.6 and incubated at 5°C, and was able to survive up to 35 days when stored in cabbage juice with a pH of 4.8 [93]. Using a "chemically defined medium", researchers were able to maintain two strains of *L. monocytogenes* at pH 3.5-4.0, cell death occurred below these values [92].

Bacterial Stress Response

L. monocytogenes thrives in cool moist conditions, like those encountered in processing plants and once established is extremely difficult to eradicate [94]. Not only does this organism survive and multiply under conditions that are normally lethal or inhibitory to other microbes [44], it is also able to adapt or become acclimated to these conditions [55]. Adaptation to stresses may create bacteria that are even more difficult to inactivate. *L. monocytogenes* can adapt to low pH, high salinity, low temperatures [55], alkaline sanitizers [28] and is capable of repairing cell damage under certain conditions [95]. Of particular interest to food processors is *L.*

monocytogenes ability to adapt to low pH and its heat shock responses, because of the residual effects and possible cross protection to other stresses.

Exposure to sub-lethal conditions may allow mciroorganisms to adapt to the stressful conditions, making them more resistance to other stresses as well [26]. Lou and Yousef found that stress resistance which occurred after adaptation was dependent on the type of stress and the lethal factor applied [26]. For example, they applied sub-lethal doses of ethanol, acid, hydrogen peroxide (H_2O_2), or NaCl to *L. monocytogenes* cells at the exponential phase. After heat shocking these cells for one hour at 45°C, the adapted cells were then exposed to lethal levels of NaCl, ethanol, H_2O_2, acid and starvation conditions. Adaptation to pH 4.5 or 5.0 increased the resistance to lethal doses of acid, ethanol, and H_2O_2. Heat shock and adaptation to ethanol increased the resistance to NaCl. [26]. Although acid adaptation induces an acid tolerance response (ATR) to heat and osmotic stress, heat shock does not elicit acid tolerance [96].

In a more recent study, acid adapted *L. monocytogenes* (incubated at pH 5.5 for two hours) showed an increased resistance to heat shock treatment at 52°C, osmotic shock (20-30% NaCl) and alcohol stress (15%) [92]. Heat adapted *L. monocytogenes* also exhibited an increased resistance to acid shock [92]. Maximum acid tolerance was obtained from 2-3 hours of adaptation, but prolonged adaptation produced weaker tolerance [92]. Acquired acid tolerance was maintained for several weeks if adapted cells were stored at 4°C [92].

Antibiotic Resistance: An Emerging Threat

Listeriosis infections are easily treated with antibiotics such as penicillin or ampicilin in combination with an aminoglycoside [68]. Since the first antibiotic resistant strain of *L. monocytogenes* was isolated in 1988 [30], other resistant strains have been isolated [31, 32], including a strain resistant to trimethoprim, which is commonly used in combination with sulfamethoxazol for patients allergic to penicillin [30] and strains resistant to tetracycline and erythromycin, also common treatments for Listeriosis infections [1, 30, 97, 98]. Although the occurrence of resistance strains of *L. monocytogenes* is still low in comparison to other species [32], it is likely that new strains will continue to emerge due to the ease with which resistant genes can be transferred from one species to another via transferable plasmids [34]. In addition, genes that grant antibiotic resistance for different antibiotics typically occur closely together on

the same plasmid, so bacteria may become resistant to multiple antibiotics with each transfer [34].

Using the agar diffusion method to determine minimum inhibitory concentrations (MIC) the susceptibility of 148 strains of *L. monocytogenes,* isolated from food, to common antibiotics was assessed. The strains showed resistance to four antibiotics, phosphomycin, lincomycin, and flumequine, and moderate sensetivilty to spiramycin, chloramemphenicol, tetracycline, and streptomycin. [31]

Resistance to ttetrcycline (6.7%) and penecilin (3.7%) was the most common among 1001 *L. monocytogenes* and *L. innocua* isolates obtained from 67 retail food samples. Resistance to more than one antibiotic was more common among *L. innocua* (19.5%) isolates than *L. monocytogenes* (0.6%). However, resistance in one species of *Listeria* increases the likelihood that transfer of resistance genes to *L. monocytogenes* will occur. [32]

It has been suggested that the possession of antibiotic resistance mechanisms may provide protection against ultraviolet light, disinfectants, and other stresses [34, 99] similar to the response observed by stress adapted cells. For example, Meckes determined that coliforms resistant to tetracycline or chloramphenicol were more likely to survive UV irradiation (253.7nm) than non-resistant coliforms [33]. In addition, he found that coliforms resistant to multiple antibiotics were twice as likely to survive UV irradiation than coliforms resistant to a single antibiotic [33].

Ready-to-eat Meat Products

Ready-to-eat meat products such as frankfurters and deli meats made from poultry, pork, and beef have been under close scrutiny due to Listeriosis outbreaks that have occurred over the past two decades [37, 38] and are currently under a zero tolerance policy by the Food Safety Inspection Service (FSIS) [4]. These products are of particular concern and present a high level of risk for harboring and transmitting *L. monocytogenes* because this microorganism is able to survive key processing steps.

Production

Frankfurter batter is a combination of salt, spices, curing ingredients, and either poultry, pork, or beef trimmings [78]. The ingredients are ground into an emulsion, pumped into a

cellulose casing, smoked and heated. After heat processing, the casing is removed and the frankfurters are either sprayed with cool water or dipped in a chilled brine solution in order to rapidly cool the product before they are vacuum packaged and stored at refrigeration temperatures until purchased by consumers. Cooking frankfurters for 70 minutes, or until the internal temperature reaches 160°F, is sufficient to kill *L. monocytogenes* [100]. If refrigerated, unopened packages should be consumed within two weeks of purchase, once opened, they should be consumed within a week [101]. RTE meat products can be safely stored in the freezer for up to 12 months.

Entry and Persistence of Listeria in the Processing Environment

L. monocytogenes may enter the food-processing environment through several routes, such as on the surfaces of raw meat [102] in soil, feed, animal feces [48] and through human carriers [102]. Once established, its ability to persist under harsh conditions makes it difficult to eradicate.

Studies have been conducted to assess the prevalence of *L. monocytogenes* in the processing environment. Samelis and Metaxopoulas found that 50% of raw pork and 60% of raw poultry is contaminated with *L. monocytogenes*, confirming an earlier study conducted by Ryser & Marth (1991) that post process contamination was largely due to microorganisms that survived the heat processes [102]. Wenger and colleagues determined that the peeler and conveyers used in the manufacturing of turkey frankfurters were heavily contaminated [103]. *L. monocytogenes* has also been isolated from 1.4-9.3% of environmental sites within dairy plants [104, 105] including around floor drains, conveyers, and damp areas, and has been shown capable of adhering to steel, rubber, and glass [106]. Grau and colleagues discovered that 53% of vacuum packed products are contaminated with *L. monocytogenes*, 4% of which had greater than 1000 CFU/g [107]. A 2006 survey of RTE meat products purchased from Florida supermarkets found that 3% were contaminated with *L. monocytogenes* [108].

Growth of *L. monocytogenes* in RTE products depends on the type of meat, pH, and the type and cell population of the competing flora [37]. Chicken supports the growth of *L. monocytogenes* better than most meats, such as roast beef and sausage [37].

Brine Chillers

Brine chilling systems are used to quickly cool thermally processed products [6], such as frankfurters, and to impart flavor to cheese [85] and some processed meat products. Based on hurdle technology principles, brine chilling systems use a combination of cold temperature and high salt concentrations (to lower water activity) to inactivate or inhibit the growth of microorganisms [6]. Commercial salt brines, used for meat products, contain approximately 13% salt, have a pH of 6.75, and are maintained at less than -2°C [85]. Because brine solutions are often reused, the USDA Food Safety and Inspection Service issued bulletin 83-16, a schedule and set of simple maintenance conditions that must be followed in order for the brine to be safely reused (Appendix A.1).

Brine chilling systems provide a risk to food manufacturers when the brine is reused, the longer the use, the higher the risk. As the products are dipped or sprayed with brine, nutrients and heat may leach into the solution, allowing psychotropic pathogens to grow. Using a model brine chilling system, Miller (1997) determined that growth of *L. monocytogenes* could be controlled if salt concentrations are maintained at 9% and temperatures below -2°C, but no conditions ensured death [6]. In fact, *L. monocytogenes* survived for 30 days at -12°C in 20% NaCl [6]. If contaminated brine is reused, *L. monocytogenes* could contaminate products. Since brine chilling takes place immediately before packaging, with no further processing, *L. monocytogenes* cells from the brine would not be inactivated. Storage at refrigeration temperatures, enclosed in a nutrient rich environment is an ideal place for *L. monocytogenes* to replicate and repair cells damaged by processing steps. Chawla and colleagues determined that cells can repair in 16-19 days while stored at 4°C [95]. An additional processing step is needed, to ensure that recycled brine is safe.

Ultraviolet Light

Commercial sterilization techniques are employed to ensure that food is safe, and to maintain the stability and quality of products. Non-thermal treatments are being evaluated because unlike thermal processes, the sensory and nutritional quality of food is not significantly altered during treatment. The effectiveness of any sterilization process is dependent on factors including the target organism, species or strain, growth stage, exposure to sub-lethal injury and stress history prior to treatment as well as the method itself.

Photochemical Theory

Light is a stream of electromagnetic energy, or photons (smallest form of radiant energy), that travel in the form of waves [8]. The efficiency of ultraviolet disinfection is governed by two common laws of Photochemistry. First, photons must have enough energy to break or form bonds [109]. Atoms and molecules will absorb the wavelength which provides enough energy to change their state [8]. Once enough energy has been absorbed, chemical changes take place. More specifically, once a molecule absorbs a photon, an electronically excited intermediate is formed [7]. Second, the photons must actually be absorbed in order to promote chemical reactions. Microorganisms absorb less than one percent of photons, because they are so small [109]. Reacting molecules and photons react on a one-to-one basis. Table A.2 (Appendix A) is a breakdown of the seven types of radiation, along with their wavelength and corresponding photon energies. The bond energies of biological molecules (370-850kJ/mole), such as microbial molecules, correspond to the photon energy in visible and UV radiation therefore would be the type absorbed by these molecules [7]. Microorganisms are capable of absorbing ultraviolet light between 200-300nm [7].

UV radiation can be further divided into vacuum ultraviolet (50-200nm) meaning it is absorbed by water and oxygen in the air, UVC (200-270nm), UVB (270-330nm) responsible for the sun-burn effect, and UVA (330-400m) which is responsible for sun tanning [109]. UVC, short-wave radiation, is referred to as the germicidal range because the nucleotides of microorganisms are capable of absorbing the photon energy (600-440kJ/Einstein) produced at this range, especially 254nm [7].

Mercury arc lamps are the most common systems used to generate germicidal UV. A small amount of elemental mercury and an inert gas are contained at opposite ends of a UV transmitting tube (usually quartz). An arc of energy established by electrodes at either end promotes mercury electrons to higher energy orbitals. As they return to their ground state, a photon is released [7].

UV Dose

Ultraviolet dose is defined as the amount of photons that a microorganism or microbial population has been exposed to and can be roughly calculated as the product of the exposure time and irradiance (UV intensity from all angles) [7]. Dose is complicated to estimate in a

circulating system because it is difficult to know how much radiation individual cells are receiving as they flow through the system. The dose required to inactivate target microorganisms depends on the initial microbial load, specific characteristics of the microorganism, and soluble solids present in the solution [12].

UV intensity, or light measurement, can be divided into chemical and physical quantification methods. The appropriate method is chosen based on the sample matrix, desired level of accuracy, detection limit, and delivery method (direct, flow through, thin film) [110, 111]. Regardless of the method, measurement is inherently difficult due to the nature of light. Light is difficult to physically collect [112].

Physical measurement is achieved through the use of radiometers or light sensors. Radiometers and sensors are limited by their sensitivity, able to respond to a narrow range of emitted wavelengths, and by their need for frequent calibration [112]. Physical measurement is more of an indication of source output, rather than UV dose.

Chemical measurement is achieved using a "chemical system (fluid, gas, solid) that undergoes a light induced reaction (at a certain wavelength) for which the quantum yield is accurately known" [113]. The quantum yield is defined as the "number of molecules changed, formed, or destroyed, divided by the number of absorbed photons of that particular wavelength in the same period of time"[113]. Actinometry measures the photon flux (movement of photons) of a chemical after exposure to light at a particular wavelength, and uses it to calculate the amount of light absorbed. The chemical and test conditions vary and are primarily dependent on the sample matrix [113]. Chemical actinometers require no specialized equipment, are less prone to systematic error, produce results that are more reproducible, and do not require recalibration [112].

For biological systems, uridine provides a model actinometer because it is not harmful to living organisms but is in fact a uracil with a ribose ring attached. Uridine undergoes a 5', 6' photohydration reaction upon exposure to UV light [114]. A water molecule replaces the 5', 6' double bond forming a photohydrate product. This product does not absorb radiation at 262nm, so can be used to calculate the amount of UV light absorbed by a sample [114, 115]. Jin and colleagues described a technique for the use of uridine in a flow through system [111]. Due to its low quantum yield, uridine was shown to have low sensitivity at low exposure times [111]. It was suggested that for the most accurate measurements, longer exposures to UV be evaluated.

Uridine was stable when used at a concentration of 1.2 x 10^{-3} M. Similar studies have suggested 10^{-4} M, and sodium chloride concentrations of no more than 90mg/L [110].

Physical detectors are of two basic types. Radiometers are instruments that measure the total power from a light source as it strikes the sensor [116]. Thermal radiometers consist of a black surface where all light that hits the surface it converted to heat [116]. A thermistor touching the back of the black surface and another black surface kept in the dark produce a current proportional to the power of the light striking the radiometer surface [116]. Thermal radiometers are not very sensitive. Photonic radiometers consist of a photocell with a UV-sensitive cathode that converts the photons that strike the sensor into a current [116]. Sensitivity varies with wavelength [116]. UV Sensors incorporate semiconductors, which only absorb in the UV range, such as diamond, Silicon carbide, and Gallium nitride, into electronic circuits [116]. Detector sensitivity may decrease with age or damage [113], and may require frequent calibration [112]. Physical detectors are also limited by their sensitivity, able to respond to a narrow range of emitted wavelengths, the resulting data more an indication of source output, rather than UV dose.

UV Induced Damage & Bacterial Repair Mechanisms

Short-wave ultraviolet irradiation (UVC), wavelength 254nm, can be absorbed by the nucleic acids and proteins of microorganisms, preventing the cells from reproducing [8]. Pyrimidine (thymine and cytosine) bases are ten times more sensitive to UV irradiation than purine (adenine and guanine) bases. UVC light causes damage by causing bonds to form between adjacent thymine in DNA chains. Pyrimidine is more susceptible to UVC, inducing the formation of covalently linked dimers between adjacent bases. If a thymine base absorbs a UV photon, a chemical bond can form between an adjacent thymine base. Dimers inhibit replication of DNA. If enough UV has been absorbed, so as to create enough dimers, DNA cannot replicate and the cells will die. The more radiation, the more dimers formed. [8].

Resistance to UV irradiation is a factor in the ability of the microorganism to repair damage. The two most common repair mechanisms are photoreactivation and dark repair [117]. Photoreactivation requires exposure to light between 310-490nm, to activate DNA photolyase [118], an enzyme that cleaves the pyrimidine dimers. The amount of photorepair that occurs is

dependent on the organism, the amount of UV absorbed, and the nutrient state of the organism [8].

Dark repair, which does not require the presence of light, accounts for 99% of UV damage repair. Excision repair, the most common type of dark repair, involves the following enzyme mediated steps: Repair endonuclease recognizes the damages DNA and cleaves the DNA strand, Exonuclease excises the damaged section, DNA polymerase rebuilds the damaged section, Polynucleotide ligase rejoins the severed strand.

Factors Affecting Microbial Susceptibility to UV and Limitations of UV Treatment

The effectiveness of ultraviolet treatment is dependent on a variety of factors, which can be divided into two categories: organism and solution related. Organism related factors that affect susceptibility to UV include the stage and physiological state of culture, growth medium, strain and its ability to repair damage, and initial microbial load at the time of disinfection. Yousef and Marth treated *L. monocytogenes* cultures on TSA plates to a 100μW/cm³ dose of germicidal UV for four minutes. They observed no difference between 24 and 48-hour cultures, but dry cells were more resistant to UV [14]. Increasing the dose of UV to 550 watts doubled the inactivation rate [14]. After starving *L. monocytogenes* isolates in 0.85% NaCl for eight days at 25°C, Mendonca (2004) found that starvation increased the microbes resistance to electron beam irradiation [27]. Earlier studies have shown that low water activity increases radiation resistance [119, 120]. Snowball and Hornsey (1988) found that cultures grown in rich organic media tended to be protected against UV radiation, possibly because rich media promotes the production of ribosomes within the cells, which in turn shield the DNA [16]. They also noticed that *E. coli* cells in lag phase were more resistant to UV than cells in log phase [16]. In vitro studies have also shown that the rate of dimer formation is faster below 25°C [121].

The level of suspended solids, turbidity or color (measurement of light scattering and absorbing properties), and the percent of UV transmittance (ratio of light entering sample to light exiting the sample), greatly determine the effectiveness of UV treatment [18, 122]. Table A.3 (Appendix A) is a list of acceptable water quality parameters that should be considered in order to appropriately use UV for disinfection of water and wastewater. The percent transmittance should not fall below 35%, with total suspended solids between 5-10 mg/l [18]. Higher suspended solids, turbidity and color lead to lower transmittance. Sharma et al (1996) determined

that background microorganisms, particulates, and organic matter interfere with the transmission of UV light, thereby decreasing the dose being applied. Similarly, HIV cells in tissue culture medium were inactivated in 10 minutes using a dose of 150-220μW/cm². HIV cells associated (not free floating) required 30 minutes, and HIV associated and suspended in whole human blood required 60 minutes for inactivation [123]. Microenvironments, created from pieces of food that fall into the brine during operation, may shield pathogens preventing uniform application.

Application of Ultraviolet Radiation

Although little research has been conducted to determine the specific conditions required to inactivate *L. monocytogenes* in brine solution using ultraviolet light, there has been a great deal conducted with other products and types of radiation. The most successful and extensive use of ultraviolet light has been for the disinfection of water and wastewater, despite the low penetrability of UV [10]. Ultraviolet light is used to disinfect the water used for the brewing industry, soft drinks, and cheesemaking. It is also being used for the disinfection of juices, surface treatment of produce and meats, manufacturing equipment, and brine solutions [10, 11, 13, 22].

Pure cultures of *L. monocytogenes*, as well as products such as chicken and shellfish contaminated with as high as 10^4 CFU/g have been successfully treated using 2 kGy gamma irradiation [124]. Long-wave ultraviolet light (UVA 365nm), at an intensity of 45 Wm^{-2} for 60 seconds, was effective at inactivating *L. innocua* and *E. coli* O157:H7 in cheese brines but fortunately did not kill the desirable yeasts [22]. Because of the high penetrability and selectivity of long wave irradiation, it is beneficial to use in situations were specific microflora survival is desired [22]. Reduction of *E. coli* in juices has been accomplished using thin film UV treatment. This technique produces a stream of liquid so thin that it is considered transparent, reducing the effect of color or solids content on the required dose. Thin film UV has been used to reduce *E. coli* numbers in orange and apple juice [10].

Ultraviolet light has also been used to reduce pathogens on the surfaces of produce and meat products. At an intensity of 1.5-24 mW/cm^2 of UVC, both *E. coli* an *Salmonella* were successfully reduced on the surface of apples (*E. coli*) and tomatoes (*Salmonella*) [13]. The greatest reduction was observed for the treatment of *E. coli* on apples, using the highest dose

[13]. Infrared (IR) pasteurization has been used to pasteurize the surface of turkey frankfurters. Infrared light heats the surface of the food, and is slowly transferred to the center by conduction. An average of 3.5-4.5 log reduction of *L. monocytogenes* were observed after IR pasteurization of turkey frankfurters [25]. Unfortunately, IR pasteurization is only appropriate for eliminating microbes on the outside immediately before packaging [25].

In one of the earliest studies which examined the effect of UV on pathogen populations, Yousef and Lou found that *L. monocytogenes* cultures grown on trypticase soy agar were able to be inactivated by 100 $\mu W/cm^2$ of UVC for 4 minutes [14]. More recently, goat's milk exposed to a UVC dose of 15.8 ±1.6 mJ/cm^2 resulted in a 5 log reduction of *L. monocytogenes* originally inoculated at 10^7 CFU/ml [15].

Summary

Listeria monocytogenes is a serious foodborne pathogen due to its ability to survive and grow in extreme food manufacturing conditions, and because of the high mortality rate associated in those who develop Listeriosis. During the processing of RTE meat products, *L. monocytogenes* has many opportunities to enter the food processing environment, and once established is difficult to eliminate. Food manufacturers are looking for methods to inactivate this microorganism in brine chilling systems that will not affect the quality or sensory characteristics of the food being conveyed through the chillers. Ultraviolet light has been suggested because of its successful application in water and juice disinfection. However, more research is needed to understand UV measurement and factors that might affect the dose required to reduce *L. monocytogenes* in brine chilling solutions.

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Chapter III
Dose of Ultraviolet Light Required to Inactivate *Listeria monocytogenes* in Distilled Water, Fresh Brine, and Spent Brine

Keywords: Ultraviolet light, *Listeria monocytogenes*, brine, spent brine
Note: This chapter formatted for submission to the Journal of Food Protection

ABSTRACT

Ultraviolet light (UV) may be an effective tool for control of *Listeria monocytogenes* in brines used to cool ready-to-eat (RTE) meat products. The purpose of this research was to establish the minimum dose of UV (peak: 253.7nm) required to inactivate *L. monocytogenes* in distilled water, fresh brine (9% NaCl), undiluted spent brine (20.86% NaCl), and diluted spent brine (5%, 35%, 55% spent brine) solutions using uridine as a chemical actinometer. *L. monocytogenes* strains N1-227 (hotdog batter), N3-031 (turkey franks), and R2-499 (RTE meat) were mixed in equal proportions and suspended in distilled water, fresh, undiluted spent brine, and diluted spent brine, each containing 10^{-4} M uridine. Fourteen ml of suspension was placed into a sterile quartz cell, and irradiated for 0, 5, 10, 15, 20, 25, or 30 minutes using an Oriel photoreactor (model 66901) fitted with a filter to allow only UV light in the 253.7 ± 10 nm range to pass to the sample. The sample was held at 8± 2°C and continuously stirred during UV exposure. Inactivation was evaluated by serially diluting samples in 0.1% peptone, surface plating onto Modified Oxford Agar (MOX) and Trypticase Soy Agar with Yeast Extract (TSAYE), and by enrichment in Brain Heart Infusion Broth (BHI); followed by incubation at 37°C for 24-48 hrs. For dose measurement, the absorbance of each sample was measured before and after irradiation, using a Shimadzu spectrophotometer. *L. monocytogenes* irradiated in water decreased to below the detection limit (1 log CFU/ml) at UV doses greater than 33.19 mJ/cm^2 (20 min), but was detected via enrichment after exposure of up to 46.78 mJ/cm^2 (30 min). *L. monocytogenes* irradiated in fresh brine decreased to below the detection limit after exposure to doses greater than 10.25 mJ/cm^2 (15 min) but was detected via enrichment after exposure to UV at doses greater than 23.70 mJ/cm^2 (30 min). *L. monocytogenes* irradiated in 5% spent brine decreased 3.8 and 4.0 logs CFU/ml on MOX and TSAYE, respectively, after exposure to doses of UV greater than 22.06 mJ/cm^2 (30 min). *L. monocytogenes* irradiated in 35% spent brine was reduced 3.8 and 3.6 log CFU/ml on MOX and TSAYE, after exposure to doses of UV greater than 18.00 mJ/cm^2 (30 min). *L. monocytogenes* irradiated in 55% spent brine was reduced 0.54 log and 1.3 CFU/ml on MOX and TSAYE, after exposure to doses of UV greater than 42.95 mJ/cm^2 (30 min). Uridine was undetectable in undiluted spent brine, making dose measurements impossible. There were differences observed in *L. monocytogenes* population numbers after UV

exposure based on solution ($P \leq 0.05$), although distilled water and fresh brine were not significantly different. After UV exposure, populations were reduced as follows from greatest to least depending on solution: water > fresh brine > 5% NaCl spent brine > 35% NaCl spent brine > 55% NaCl spent brine > spent brine. Time significantly affected population estimates; as time ($P \leq 0.05$) of exposure to UV light increased, UV intensity, and dose increased, thereby significantly decreasing populations in each solution. There were no differences between population estimates as determined on MOX or TSAYE in water, fresh brine, spent brine, or spent brine dilutions ($P > 0.05$). Knowledge of UV dosing required to control *L. monocytogenes* in brines used during RTE meat processing will aid manufacturers in establishing appropriate food safety interventions for these products.

INTRODUCTION

Brine chilling systems, used to quickly cool thermally processed meat products such as frankfurters and luncheon meats, use a combination of cold temperature and high salt concentration to inactivate or inhibit the growth of microorganisms [1]. Commercial salt brines, are used, reused, and maintained according to the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) bulletin 83-16 [2]. According to these guidelines, brine can be reused for up to 4 weeks if the salt concentration is maintained at 20% and the temperature at –12.2˚C [2]. These guidelines are based on in-plant experience, aerobic plate counts and coliform data, but have not been validated using pathogens [1]. As products are dipped or sprayed with brine, nutrients and heat may leach into the solution, allowing psychotropic pathogens to grow. Reused brine poses a risk to food manufacturers; the longer the reuse, the higher the risk of product contamination.

Although easily killed during heat processing steps, *Listeria monocytogenes* is capable of surviving the cool temperatures and high salt conditions maintained in brine chillers, increasing the possibility of post-processing contamination [3]. Even though *L. monocytogenes* is responsible for less than 0.1% of foodborne related illnesses, it accounts for 37% of the deaths [4]. Outbreaks of listeriosis have prompted the USDA FSIS) to establish a zero tolerance policy for this organism in RTE products [5], and the Food and Drug Administration (FDA) to set very strict standards [6].

Using a model brine chilling system, Miller (1997) determined that growth of *L. monocytogenes* could be controlled if salt concentrations are maintained at 9% and temperatures below -2°C, but no conditions ensured death [1]. In fact, *L. monocytogenes* survived for 30 days at -12°C in 20% NaCl [1]. Chilling of RTE products takes place immediately before packaging; the product receives no further processing. Use of contaminated brine can lead to contaminated products. Storage at refrigeration temperatures, enclosed in a nutrient rich environment is an ideal place for *L. monocytogenes* to replicate and repair cells damaged by processing steps. Chawla and colleagues determined that cells can repair in 16-19 days while stored at 4°C [7].

Ultraviolet light (UV) is being used to eliminate *L. monocytogenes* from brine solutions due to its ability to kill a wide range of microorganisms and because it does not leave a residue or significantly affect sensory or nutrient quality. To appropriately use UV for this purpose, it is

essential to know the amount of UV energy required to inactivate *L. monocytogenes* as well as the amount of energy being received by the microorganisms. Although studies have been conducted to determine the effectiveness of UV and other forms of light in eliminating *Listeria* and other microorganisms on different substances or surfaces [4, 5, 8-15], little, if any, information exists on the amount of UV energy required to inactivate *L. monocytogenes* in a chilled brine solution. Previous studies have reported an extrapolated dose calculated by multiplying the time of exposure by the intensity of the mercury bulb. Although this provides a rough estimate of the dose, it does not take into account the loss of bulb intensity over time or intrinsic factors of the solution (turbidity, soluble solids, insoluble solids), which may affect the dose applied to each bacterial cell.

A more accurate way of determining the dose is by using chemical actinometry. A chemical actinometer or dosimeter is a "chemical system (fluid, gas, solid) that undergoes a light induced reaction (at a certain wavelength) for which the quantum yield is accurately known" [16]. Actinometry measures the photon flux of a chemical after exposure to light at a particular wavelength, and uses it to calculate the amount of light absorbed. Uridine provides a model actinometer for biological systems because it is not harmful to living organisms, consisting of a uracil with a ribose ring attached. Uridine undergoes a 5', 6' photohydration reaction upon exposure to UV light. A water molecule replaces the 5', 6' double bond forming a photohydrate product. This product does not absorb radiation at 262nm, so it may be used to calculate the amount of UV light absorbed by a sample. [17, 18]

These experiments were designed to determine the minimum dose of UV light required to inactivate *L. monocytogenes* in water, fresh brine, and spent brine to provide a more effective use of ultraviolet treatment, and ultimately a greater assurance of process efficacy.

MATERIALS AND METHODS

Equipment and Facilities. Experiments were conducted at the Virginia Tech Food Science and Technology building, using an Oriel photoreactor (Model 66901, Newport Stratford, Stratford, CT). The photoreactor consisted of an aluminum arc lamp housing unit, 350-Watt mercury bulb (Model 6286, Newport Stratford, Stratford, CT), and a digital power supply unit (Appendix B).

Once the bulb was ignited, a reflector located parallel to the bulb focused the beam onto a condensing lens, which then produced a collimated (straight) beam directed horizontally into the UV chamber (Appendix B). The bulb was ignited at least 30 min prior to UV treatment, to allow the bulb to reach its maximum intensity and to stabilize. Mercury bulbs produce light between 200–2500 nm, therefore a 2 inch 253.7±10 nm directional filter (Model 56501, Newport Stratford, Stratford, CT) was attached to the end of the sample container to exclude all other wavelengths. Based on the distance from the bulb to the sample chamber (21 in), a maximum intensity of 350–400 mW/cm² was possible and was measured using a radiant power meter with thermopile sensor (Models 70260, 70263, Newport Stratford, Stratford, CT). Thermopile detectors work by absorbing radiation and measuring the resulting temperature increase making these detectors very sensitive to environmental temperatures. Accordingly, a fused silica filter (Model 70185, Newport Stratford, Stratford, CT) was mounted to the sensor to minimize drift in measurements. A digital power supply provided constant power to the bulb, displayed the wattage, and approximated the bulb hours (rated at 1000 hrs maximum).

A 24x12x12 in polystyrene chamber (Appendix B), lined with particleboard to prevent light penetration, was constructed to house the 4x4x2 in sample stand (Appendix B) during UV exposure. An opening for the condensing lens was made on one side and a pocket for the sensor directly opposite. The sample stand was placed directly in the path of the collimated beam. Samples were exposed to UV in cylindrical quartz containers (Model 14-385-930E Fisher Scientific, Suwanee, GA) with two ports located on the top (Appendix B). Before each use, each cell was rinsed with acetone (42324-0040 Fisher Scientific, Suwanee, GA) and autoclaved along with a 6x3x2 mm stir bar placed within the container. During UV treatments, a stirrer housed inside the sample stand was used to continuously stir the test liquid. Sample temperature was maintained at 10–15°C by re-circulating a 50/50 mix of water and ethylene glycol through the UV chamber and through the sample stand for one hour before and during UV application.

Culture Maintenance. The following *L. monocytogenes* strains, obtained from Dr. Kathryn Boor and Dr. Martin Wiedmann of Cornell University, were used: N1-227, serotype 4b, isolated from hotdog batter implicated in a 1998 outbreak; N3-031, serotype 1/2a, isolated from turkey franks; and R2-499, serotype 1/2a, isolated from a RTE meat product [19]. Upon arrival, each strain was transferred into 10 ml of Trypticase Soy Broth supplemented with 0.6% Yeast Extract

(TSBYE, 211825, Becton, Dickinson and Co, Sparks, MD) and incubated at 35°C for 48 hrs. Each strain was then swabbed onto multiple Modified Oxford Agar plates (MOX) prepared with Oxford Medium Base (222530, Becton, Dickinson and Co, Sparks, MD) supplemented with Modified Oxford Antimicrobic Supplement (6060973, Becton, Dickinson and Co, Sparks, MD), and incubated at 35°C for 48 hrs. Strains were confirmed, using *Listeria* API (10-300, Biomerieux, l'Etoile, France) and divided into multiple cryovials of Nutrient broth (23300, Becton, Dickinson and Co, Sparks, MD) supplemented with 15% glycerol (G33-1 Fisher Scientific, Suwanee, GA), for storage at -70°C.

Monthly, one cryovial of each strain was thawed at room temperature. Using a sterile prepackaged inoculating loop (13-070-3 Fisher Scientific, Suwanee, GA), 10µl of inoculum was transferred into 10 ml of TSBYE and incubated at 32°C for 48 hrs. Next, 10 µl was streaked onto MOX and incubated at 35°C for 48 hrs. A typical colony was then transferred into 10 ml of TSBYE and incubated at 35°C for 48 hrs. After incubation, a loopful of culture was streaked onto multiple TSAYE slants, incubated at 35°C for 48 hrs, and stored at 2-4°C for up to one month as stock cultures.

Solution Preparation. Distilled water was sterilized for 15 min at 121°C and stored at 2–4°C until use. Fresh brine (9% NaCl w/v) was prepared by adding 90g of Top-Flo evaporated salt (7559 Cargill, Minneapolis, MN) to a volumetric flask and bringing the volume up to 1000 ml with sterile, distilled water. Fresh brine was prepared within 24 hrs of use, filter sterilized (0.45 µm S66128 Nalgene, Rochester, NY) and stored at 2–4°C until use. Spent brine was received from a national RTE meat manufacturer and stored at 2–4°C until use. Because of the turbidity (0.32% Transmittance) and salinity (20.86%) of the spent brine, uridine could not be used for dose determination experiments. Consequently, spent brine was diluted with sterile distilled water to achieve final salt concentrations of 5%, 35%, and 55% spent brine, filter sterilized (S66128 Nalgene, Rochester, NY) and stored at 2–4°C until use.

Chemical actinometry, using uridine, was used to determine the dose of UV absorbed during exposure. A 10^{-2}M stock solution of uridine (Appendix B) (A15227 Alfa Aesar, Ward Hill, MD) was prepared by adding 1.22 g of uridine to a 500 ml volumetric flask, bringing up to volume with sterile distilled water, filter sterilized (09-740-22J Fisher Scientific, Suwanee, GA), and stored at 2–4°C for up to six months. A 10^{-4}M uridine working solution, made immediately

before use, was prepared by adding 1 ml of the 10^{-2} M stock solution to a 100 ml volumetric flask and bringing up to volume with sterile distilled water, fresh brine, or one of three dilutions of spent brine (spent brine diluted to 5%, 35%, and 55% spent brine).

Solution Characterization. Percent transmittance of distilled water, fresh brine, undiluted spent, and diluted spent brine solutions was measured at 253.7 nm using a Shimadzu spectrophotometer (Model UV-2101PC). Suspended solids were determined according to Standard Methods for the Examination of Water and Waste Water, method 2540 [20]. Sodium chloride concentration was established using Hach Aqua Check Dip-and-Read water quality test strips (23-292-765 Fisher Scientific, Suwanee, GA).

Inoculum Preparation. Each strain of *L. monocytogenes* was activated from stock cultures by three successive 24 hrs transfers in TSBYE, incubated at 35°C for 24 hrs. After the final incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before the pellet was re-suspended in 10 ml of the fluid being treated (distilled water, fresh brine or one of the three dilutions of spent brine; each prepared to contain 10^{-4} M uridine). Equal portions of each strain (3.3 ml) were added to a sterile 100 ml volumetric flask and brought up to volume using the fluid being treated (sterile water, fresh brine, undiluted brine or one of the three dilutions of spent brine). Each preparation was made the day of UV exposure and stored at 2-4°C.

Ultraviolet Treatment. One hour before treatment, the re-circulating refrigeration system was adjusted to 4–10°C and allowed to stabilize. Thirty minutes before treatment, the photoreactor was turned on, allowing the bulb to reach maximum intensity and for output to stabilize. UV intensity was recorded before and after UV exposure (without the sample cell). For each time period, 14 ml of inoculated test medium was aseptically transferred into a sterile quartz cell (14-385-930E Fisher Scientific, Suwanee, GA). Samples were exposed for 0, 5, 10, 15, 20, 25, or 30 minutes. In order to confirm that uridine would not interfere with the treatment conditions, water, fresh and spent brine samples were prepared without uridine and exposed to UV for 0, 5, 10, 15, 20, 25, 30 minutes.

Microbiological Evaluation. Before and after UV exposure, *L. monocytogenes* was enumerated by serially diluting in 0.1% peptone (211677 Becton, Dickinson and Co, Sparks, MD), spread plating 0.1 ml onto duplicate MOX and TSAYE, and incubating at 37°C for 48 hrs. A dual plating system was employed in order to determine the extent of cell injury. Additionally, 3 ml of each UV treated sample was enriched in 45 ml of Brain Heart Infusion broth (BHI, 237500 Becton, Dickinson and Co, Sparks, MD) and incubated at 37°C for 48 hrs. If turbid after 48 hrs, the BHI sample was streaked onto TSAYE and incubated at 37°C for 24–48 hrs for confirmation with *Listeria* API (10-300 Biomerieux, l'Etoile, France).

Quality Assurance and Control Procedures. Quality assurance and control procedures were performed according to Method 9020 of Standard Methods for the Examination of Water and Wastewater [20]. Waterbath, incubator, and refrigerator temperatures were monitored and recorded daily beginning a week before and continuing throughout the duration of survival experiments. Media lots were recorded with each use and tested using positive (*L. monocytogenes* Scott A) and negative (incubation at 35-37ºC for 48 hrs) controls. The suitability of distilled water was verified by periodically performing Heterotrophic Plate counts (TSA, 32ºC for 48 hours).

Chemical Actinometry. The remainder of each 14 ml sample (typically between 8–10ml) was filtered (0.45 µm 6780-2504 Whatman) to remove *Listeria* cells and stored between 2–4°C until all samples had been exposed to UV. The absorbance of an untreated sample (Ab262), as well as the absorbance after UV exposure of each sample (Aa262) was measured at 262 nm using a Shimadzu spectrophotometer (Model UV-2101PC). Dose received during each time period was calculated according to the model described by Jin *et al* (Appendix B)[6].

Statistical Analysis. Appendix C Table 1. Experimental parameters are presented in Appendix C. Differences between UV intensity ($\mu W/cm^2$), dose (mJ/cm^2), *Listeria* populations (log CFU/ml), solution (distilled water, fresh brine, spent brine, diluted spent brine), and media (MOX, TSAYE) were analyzed using the proc glm procedure in SAS version 9.1 (SAS Institute, Cary, NC). Significant factors ($P \leq 0.05$) were further distinguished using the least squared means procedure.

RESULTS

Water, Fresh and Spent Brine Characterization. Table 3.1. The percent transmission of water and fresh brine, measured at 253.7 nm, were both 91%. Undiluted spent brine had a percent transmission of 0.32%. With each successive dilution, the percent transmission and pH of spent brine increased: 1.60% (pH 5.55), 26.9% (pH 5.29) and 60.5% (pH 5.01). There were differences observed in *L. monocytogenes* population numbers after UV exposure based on solution ($P \leq 0.05$), although distilled water and fresh brine were not significantly different. After UV exposure, populations were reduced as follows from greatest to least depending on solution: water > fresh brine > 5% spent brine > 35% spent brine > 55% spent brine > undiluted spent brine. Time affected population estimates; as time ($P \leq 0.05$) of exposure to UV light increased, UV intensity, and dose increased, thereby significantly decreasing populations in each solution. There were no differences between population estimates as determined on MOX and TSAYE, therefore results for only TSAYE will be presented ($P > 0.05$). There were no differences between population estimates for *L. monocytogenes* grown with or without uridine as determined on MOX and TSAYE ($P > 0.05$).

Distilled Water. Table 3.2. Dose calculations, UV intensity, and population estimates on TSAYE, for distilled water, are presented in Table 3.2. Initial concentrations of *L. monocytogenes* were 7.55 log CFU/ml. *L. monocytogenes* fell below the limit of detection (1 log CFU/ml) after exposure to doses of UV greater than 33.19 mJ/cm^2 (20 min exposure), and was no longer detectable via enrichment at a dose of 46.78 mJ/cm^2 (30 min exposure). A 5-log reduction was achieved after exposure to 16.78 mJ/cm^2, corresponding to an exposure time of 10 min. As expected, time of UV exposure had a significantly ($P \leq 0.05$) influence on the inactivation of *L. monocytogenes*.

Fresh Brine. Table 3.3. Dose calculations, UV intensity, and population estimates on TSAYE, for fresh brine, are presented in Table 3.3. Initial concentrations of *L. monocytogenes* were 7.97 log CFU/ml. *L. monocytogenes* irradiated in fresh brine fell below the limit of detection after exposure to doses of UV greater than 10.35 mJ/cm^2 (15 min) After exposure to 23.70 mJ/cm^2

(30 min), the organism was not detected via enrichment. A greater than 5-log reduction in fresh brine was achieved after exposure to 8.06 mJ/cm^2 (10 min) UV. Time had a significant ($P \leq 0.05$) influence on the inactivation of *L. monocytogenes*.

Spent Brine. Tables 3.4. UV intensity and population estimates, as determined TSAYE, for undiluted spent brine are presented in Tables3.4. Because of the turbidity (0% Transmittance) and salinity (20.86%) of the spent brine, the brine was unable to be used as is for dose determination experiments. Consequently, spent brine was diluted with sterile distilled water to achieve final salt concentrations of 5%, 35%, and 55% spent brine. Media and time of UV exposure did not affect population estimates in spent brine ($P > 0.05$).

5% Spent Brine. Table 3.5. Dose calculations, UV intensity, and population estimates as determined on TSAYE, for 5% spent brine, are presented in Table 3.5. *L. monocytogenes* irradiated in spent brine (1.04% NaCl and 60% transmittance) did not fall below the limit of detection (1 log CFU/ml), but was reduced by 4 log CFU/ml after exposure to doses of UV greater than 22.06 mJ/cm^2 (30 min). Time significantly ($P \leq 0.05$) affected population estimates of *L. monocytogenes*.

35% Spent Brine. Table 3.6. Dose calculations, UV intensity, and population estimates as determined on TSAYE, for 35% spent brine, are presented in Table 3.6. *L. monocytogenes* irradiated in spent brine (5.22% NaCl and 25% transmittance) did not fall below the limit of detection, but was reduced by 3.8 log CFU/ml after exposure to doses of UV greater than 18.00 mJ/cm^2 (30 min). Time significantly ($P \leq 0.05$) affected population estimates of *L. monocytogenes*.

55% Spent Brine. Table 3.7. Dose calculations, UV intensity, and population estimates as determined on TSAYE, for 55% spent brine, are presented in Table 3.7. *L. monocytogenes* irradiated in spent brine (11.47% NaCl and 1.6% transmittance) did not fall below the limit of detection, but were reduced 1.3 log CFU/ml on TSAYE, respectively, after exposure to doses of UV greater than 42.95 mJ/cm^2 (30 min). Time significantly ($P \leq 0.05$) affected population estimates of *L. monocytogenes*.

DISCUSSION

Pathogenic bacteria, viruses, and yeast can be inactivated by UV (253.7 nm) doses as low as 10 mJ/cm^2 when suspended in clear solutions, but may require at least 40 mJ/cm^2 for as a 4-log reduction when suspended in cloudy or turbid substances [21, 22]. In one of the earliest studies, which examined the effect of UV on pathogen populations, *L. monocytogenes* cultures grown on trypticase soy agar were inactivated by 100 μW/cm^2 of UVC in 4 minutes [13]. No previous literature exists describing the appropriate dose of UV required to inactivate *L. monocytogenes* in distilled water or brine solutions. In the current study, the minimum dose for inactivation of *L. monocytogenes* suspended in distilled water and fresh brine, 46.78 mJ/cm^2 and 23.70 mJ/cm^2, respectively, are comparable to doses previously established for similar foodborne pathogens tested under similar conditions [22]. A study of the susceptibility of foodborne pathogens found *L. monocytogenes* to be among the most resistant: *L. monocytogenes* > *Staphylococcus aureus* >*Salmonella enteritidis* >*E.coli* >*Bacillus cereus* [23]. In the current studies, a 5-log reduction was observed in distilled water after 16.78 mJ/cm^2, and 8.06 mJ/cm^2 in fresh brine. Goat's milk exposed to a UVC dose of 15.8 ±1.6 mJ/cm^2 resulted in a 5-log reduction of *L. monocytogenes* originally inoculated at 10^7 CFU/ml [15].

The dose of ultraviolet light needed to inactivate or reduce a microorganism is highly dependent on the organism, growth temperatures and exposure to sub-lethal stress prior to UV exposure, and most importantly suspension solution. The present studies revealed that, as percent transmission and total suspended solids increased, so did the dose required to reduce *L. monocytogenes*. Fresh brine was the exception, requiring only 24.39 mJ/cm^2 to reduce population estimates, compared to 39.17 mJ/cm^2 in distilled water. It is possible that because percent transmission was still very high for fresh brine (91%), equal to that of the distilled water, that the salt and UV worked in combination to reduce *L. monocytogenes*. Conversely, percent transmission was greatly reduced for spent brine (Table 1) leading to an increase in the dose required to inactivate *L. monocytogenes.* Undiluted spent brine contained high levels of suspended solids, possibly consisting of minerals and protein rinsed from RTE meat products. Suspended particles scatter light, and shield bacteria, creating microenvironments [21, 24]. Nitrates, added to meat products to inhibit *Clostridium botulinum*, and dissolved organics may also leach into the brine, absorbing UV light and reducing the amount available for killing

microorganisms [25, 26]. Therefore, because of increased suspended solids and turbidity, the longer brine is used, the higher the UV dose required to inactivate target organisms. Tran and colleagues found that due to the high levels of suspended solids in orange juice, UV doses between 87±7 mJ/ cm^2 and 119±17 mJ/ cm^2 were required to reduce aerobes, yeast, and molds [12].

There were no differences between population estimates on MOX and TSAYE ($P < 0.05$), indicating that no sub-lethal cell injury occurred during UV treatment. This is unexpected because UV inactivation is theoretically gradual. Microorganisms have to absorb enough light to from dimers or new bonds between adjacent nucleotides [27]. Over time, more absorption of radiation leads to more dimers. Once enough dimers are formed, replication is halted resulting in cell death.

Uridine actinometry was an effective method for determining the dose of UV received by water, fresh brine and spent brine with less than 11% NaCl. Upon exposure to light, a unique and measurable change was induced, directly related to the chemical structure of uridine and the wavelength of UV light [17, 18, 28]. Uridine was not detectable in solutions with high turbidity, and low percent transmittance, so could not be used for dose determination in undiluted spent brine. As time of exposure was increased, dose and UV intensity naturally increased, leading to decreased population estimates. This is not unexpected because dose and UV intensity are directly related to time, longer exposure the UV light results in a higher absorbed UV dose.

Survival of *L. monocytogenes* in chill brines is an important potential cause of post-processing contamination in RTE meat products. Therefore, this study was undertaken to determine the relationship between ultraviolet light dose, as determined by uridine actinometry, and reductions in *L. monocytogenes* populations in water, fresh (9% NaCl), and spent (1%, 5%, 10% NaCl) brine. The results show that UV is effective in reducing *L. monocytogenes* by >7.5 log CFU/ml (i.e., to levels undetectable by enrichment) at doses of 39.17 mJ/cm^2 and 24 mJ/cm^2 in water and fresh brine, respectively. UV becomes less effective as the percent transmittance decreases. Prior to this study, there were no published reports describing the relationship between *L. monocytogenes* and UV dose in water or brines. Consequently, these results will aid food manufacturers in selecting UV processing conditions that are appropriate for adequate hazard control.

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Table 3.1. Characterization of distilled water, fresh brine, undiluted spent brine and three spent brine dilution

Test	Water	Fresh	Spent Brine	5% Spent Brine	35% Spent Brine	55% Spent Brine
pH	7.4	6.01	6.04	5.55	5.29	5.01
% NaCl	0.00%	9.00%	20.9%	1.04%	5.22%	11.47%
Total Suspended Solids (mg/l)	-	-	772.0	38.6	270.2	424.6
% Transmittance	91%	91%	0.32%	60.5%	26.9%	1.60%

Table 3. 2. Minimum ultraviolet light dose and intensity applied to *Listeria monocytogenes* suspended in distilled water, cell recovery via direct plating onto TSAYE, and enrichment in BHI

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	00.00	00.00	7.55
5	12.42	104.67	3.78
10	16.78	208.33	2.55
15	23.13	314.50	2.95
20	33.19	418.67	<1.00
25	41.41	525.83	II[d]
30	46.78	634.00	0[d]

[a]Minimum ultraviolet light (dose)

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

[d]Indicates number of enrichments (BHI; 37°C, 24 – 48 hrs) out of three in which L. monocytogenes was detected

Table 3.3. Minimum ultraviolet light dose and intensity applied to *Listeria monocytogenes* suspended in fresh brine (9% NaCl), cell recovery via direct plating onto TSAYE, and enrichment in BHI

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.97
5	2.51	109.50	3.91
10	8.06	218.00	2.46
15	10.35	353.17	1.70
20	12.09	399.33	II[d]
25	19.26	549.17	I[d]
30	23.70	662.00	0[d]

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs
[d]Indicates number of enrichments (BHI; 37°C, 24 – 48 hrs) out of three in which L. monocytogenes was detected

Table 3. 4. Minimum UV intensity applied to *Listeria monocytogenes* suspended in undiluted spent brine and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	-	0.00	7.72
5	-	88.12	7.65
10	-	172.97	7.72
15	-	261.45	7.63
20	-	340.00	7.56
25	-	421.42	7.23
30	-	514.80	7.44

[a]Minimum ultraviolet light (dose) unable to be calculated because uridine broken down by high NaCl concentration

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 3. 5. Minimum UV intensity and dose applied to *Listeria monocytogenes* suspended in 5% spent brine and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.47
5	2.98	83.61	7.04
10	5.78	170.98	6.30
15	8.28	252.00	3.43
20	12.50	343.12	2.73
25	21.2	417.85	2.34
30	22.06	514.86	3.53

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 3.6. Minimum UV intensity and dose applied to *Listeria monocytogenes* suspended in 35% spent brine and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.80
5	7.94	81.11	7.50
10	8.17	163.32	7.08
15	12.6	247.71	6.43
20	13.83	328.36	6.17
25	16.97	421.35	6.36
30	18.00	486.06	4.26

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 3.7. Minimum UV intensity and dose applied to *Listeria monocytogenes* suspended in 55% spent brine and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	8.15
5	6.57	82.65	7.50
10	9.63	166.00	7.53
15	13.72	246.30	7.19
20	26.76	332.00	6.83
25	34.91	411.50	7.24
30	42.95	480.30	6.84

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 –48 hrs

Chapter IV
The Effect of Acid Stress, Antibiotic Resistance and Heat Shock on the Minimum Dose of Ultraviolet Light Required to Reduce *Listeria monocytogenes* in Distilled Water and Fresh Brine

Keywords: Ultraviolet light, *Listeria monocytogenes*, brine, heat shock, acid stress, antibiotic resistance Note: This chapter formatted for submission to the Journal of Food Protection

ABSTRACT

Exposure to sub-lethal processing treatments can stimulate bacterial stress responses, resulting in bacteria that are more resistant to the original processing stress as well as other unrelated stresses. The purpose of this research was to determine if adaptation to common food processing stresses affects the dose of ultraviolet light (UV) required to significantly reduce the amount of *Listeria monocytogenes* in water and a 9% NaCl solution using uridine as a chemical actinometer. In addition, the relationship between antibiotic resistance and UV treatment was also explored. *L. monocytogenes* strains N1-227 (hotdog batter), N3-031 (turkey franks), and R2-499 (Ready-to-eat meat) were used to produce acid stressed (35ºC for 3 hrs in Trypticase Soy Broth with Yeast Extract, TSBYE, acidified to pH 5.0), heat shocked (Brain Heart Infusion, BHI, at 48ºC for 1 hour), and antibiotic resistant (512 µg/ml sulfanilamide) cells which were mixed in equal proportions and suspended in distilled water and 9% NaCl solution, each containing 10^{-4} M uridine. Fourteen milliliters of suspension was placed into a sterile quartz cell, and irradiated for 0, 5, 10, 15, 20, 25, or 30 minutes using an Oriel photoreactor fitted with a filter to allow only UV light in the 253.7 +/- 10 nm range to pass to the sample. The sample was held at 8±2°C and continuously stirred during UV exposure. Inactivation was evaluated by serially diluting samples in 0.1% peptone, surface plating onto Modified Oxford Agar (MOX) and TSAYE, and by enrichment BHI; followed by incubation at 37°C for 24-48 hrs. The absorbance of each sample was measured before and after irradiation, using a Shimadzu spectrophotometer (model UV-2101PC) to calculate the dose of UV. Acid stressed *L. monocytogenes* irradiated in water decreased by 5.81 log CFU/ml on MOX and 5.98 log CFU/ml on TSAYE and after exposure of 43.89 mJ/cm^2 (30 min). Acid stressed *L. monocytogenes* irradiated in 9% NaCl decreased by 4.45 log CFU/ml on MOX and 5.95 log CFU/ml on TSAYE after UV exposure greater than 32.57 mJ/cm^2 (30 min). Heat shocked *L. monocytogenes* irradiated in water decreased to below the detection limit (1 log CFU/ml) at UV doses greater than 13.14 mJ/cm^2 (20 min) but was detected via enrichment after exposure of up to 24.78 mJ/cm^2 (30 min). Heat Shocked *L. monocytogenes* irradiated in 9% NaCl decreased to below the detection limit after exposure to doses greater than 20.41 mJ/cm^2 (30 min). Antibiotic resistant *L. monocytogenes* irradiated in water decreased by 5.92 log CFU/ml on MOX and 5.94 log CFU/ml on TSAYE after UV

exposure greater than 70.33 mJ/cm^2 (30 min). Antibiotic resistant *L. monocytogenes* irradiated in 9% NaCl decreased by 5.55 log CFU/ml on MOX and 5.36 log CFU/ml on TSAYE after UV exposure greater than 136.66 mJ/cm^2 (30 min). Time significantly affected population estimates ($P \leq 0.05$); as time of exposure to UV light increased, UV intensity, and dose increased, thereby significantly decreasing populations in each solution. There were no differences between population estimates based on media (MOX or TSAYE) or solution (distilled water or fresh brine) ($P > 0.05$). There were no differences between population estimates of acid stressed and antibiotic resistant or healthy and heat shocked. However, acid stressed and antibiotic resistant were more resistant to UV light than healthy and heat shocked *L. monocytogenes* ($P \leq 0.05$).

INTRODUCTION

Food processing treatments intended to inactivate bacteria may actually make them stronger. Bacteria which survive sub-lethal exposure to acid, heat, extreme cold and osmotic shock can become more resistant to later applications of the same stress or to unrelated stresses [1-7]. This phenomenon, know as the bacterial stress response, has the potential to enhance bacterial survival in processed foods [3, 8].

Listeria monocytogenes, a psychotrophic bacteria capable of growth in refrigerated, acidic and high salt foods can even multiply within the human immune system [9]. Due to this ability to persist and quickly adapt to its environment, *Listeria* infections account for 37% of all foodborne related deaths in the United States [10]. Lou and Yousef determined that *L. monocytogenes* became more resistant to increased levels of sodium chloride (up to 25%) after a heat shock treatment of 45°C for one hour [5]. Conversely, adaptation to high osmotic conditions increased thermotolerance [11]. They also discovered an increased resistance to lethal amounts of acid (pH 3.5) after adaptation to mild acid conditions (pH 5.0) [5]. Similar studies also found an increase in heat resistance after exposure to common industrial sanitizers [12].

Adaptation to sub-lethal stresses has been well characterized for *L. monocytogenes* [3-5, 8], however, the relationship between exposure to sub-lethal stress and ultraviolet light (UV) has not. UV is being used to eliminate *L. monocytogenes* from food products because it is economical, able to kill a variety of organisms, and because it does not leave a residue or significantly affect the sensory or nutrient quality of food products [13-20].A 2004 study found that starvation may increase the resistance of *L. monocytogenes* to electron beam irradiation [21].

It has been suggested that antibiotic resistance mechanisms may provide cross protection against sub-lethal stress, similar to the protection or susceptibility that is exhibited by bacterial stress responses [22]. In a 1982 study, Meckes observed the effect of UV light on antibiotic resistant coliforms and determined that UV light selected for antibiotic resistant strains, and depending on the antibiotics, multi-resistant strains [23]. He found that the percentage of antibiotic resistant strains of coliforms was higher after UV disinfection of wastewater effluent than before [23]. Although occurrence of resistant strains of *L. monocytogenes* is still low in comparison to other species [24], it is likely that new strains will continue to emerge due to the ease with which resistant genes can be transferred from one species to another via transferable

plasmids [25]. In addition, genes that grant antibiotic resistance for different antibiotics typically occur closely together on the same plasmid, so bacteria may become resistant to multiple antibiotics with each transfer [25].

The purpose of this research was to determine if adaptation to common food processing stresses (acid and heat) affects the dose of ultraviolet light required to significantly reduce the amount of *L. monocytogenes* in brine solutions and to determine if antibiotic resistance translates into resistance to ultraviolet light.

MATERIALS AND METHODS

Equipment and Facilities. Experiments were conducted at the Virginia Tech Food Science and Technology building, using an Oriel photoreactor (Model 66901, Newport Stratford, Stratford, CT). The photoreactor consisted of an aluminum arc lamp housing unit, 350-Watt mercury bulb (Model 6286, Newport Stratford, Stratford, CT), and a digital power supply unit (Appendix B). Once the bulb was ignited, a reflector located parallel to the bulb focused the beam onto a condensing lens, which then produced a collimated (straight) beam directed horizontally into the UV chamber (Appendix B). The bulb was ignited at least 30 min prior to UV treatment, to allow the bulb to reach its maximum intensity and to stabilize. Mercury bulbs produce light between 200–2500 nm, therefore a 2 inch 253.7±10 nm directional filter (Model 56501, Newport Stratford, Stratford, CT) was attached to the end of the sample container to exclude all other wavelengths. Based on the distance from the bulb to the sample chamber (21 in), a maximum intensity of 350–400 mW/cm² was possible and was measured using a radiant power meter with thermopile sensor (Models 70260, 70263, Newport Stratford, Stratford, CT). Thermopile detectors work by absorbing radiation and measuring the resulting temperature increase making these detectors very sensitive to environmental temperatures. Accordingly, a fused silica filter (Model 70185, Newport Stratford, Stratford, CT) was mounted to the sensor to minimize drift in measurements. A digital power supply provided constant power to the bulb, displayed the wattage, and approximated the bulb hours (rated at 1000 hr maximum).

A 24x12x12 in polystyrene chamber (Appendix B), lined with particleboard to prevent light penetration, was constructed to house the 4x4x2 in sample stand (Appendix B) during UV

exposure. An opening for the condensing lens was made on one side and a pocket for the sensor directly opposite. The sample stand was placed directly in the path of the collimated beam. Samples were exposed to UV in cylindrical quartz containers (Model 14-385-930E Fisher Scientific, Suwanee, GA) with two ports located on the top (Appendix B). Before each use, each cell was rinsed with acetone (42324-0040 Fisher Scientific, Suwanee, GA) and autoclaved along with a 6x3x2 mm stir bar placed within the container. During UV treatments, a stirrer housed inside the sample stand was used to continuously stir the test liquid. Sample temperature was maintained at 10–15°C by re-circulating a 50/50 mix of water and ethylene glycol through the UV chamber and through the sample stand for one hour before and during UV application.

Culture Preparation and Maintenance. The following strains, obtained from Dr. Kathryn Boor and Dr. Martin Wiedmann of Cornell University, were used: N1-227, serotype 4b, isolated from hotdog batter implicated in a 1998 outbreak; N3-031, serotype 1/2a, isolated from turkey franks; and R2-499, serotype 1/2a, isolated from a RTE meat product [26]. Upon arrival, each strain was transferred into 10 ml of Trypticase Soy Broth supplemented with 0.6% Yeast Extract (TSBYE, 211825, Becton, Dickinson and Co, Sparks, MD) and incubated at 35°C for 48 hrs. Each strain was then swabbed onto multiple Modified Oxford Agar plates (MOX) prepared with Oxford Medium Base (222530, Becton, Dickinson and Co, Sparks, MD) supplemented with Modified Oxford Antimicrobic Supplement (6060973, Becton, Dickinson and Co, Sparks, MD), and incubated at 35°C for 48hrs. Strains were confirmed, using *Listeria* API (10-300, Biomerieux, l'Etoile, France) and divided into multiple cryovials of Nutrient broth (23300, Becton, Dickinson and Co, Sparks, MD) supplemented with 15% glycerol (G33-1 Fisher Scientific, Suwanee, GA), for storage at -70°C.

Monthly, one cryovial of each strain was thawed at room temperature. Using a sterile prepackaged inoculating loop (13-070-3 Fisher Scientific, Suwanee, GA), 10 µl of inoculum was transferred into 10 ml of TSBYE and incubated at 32°C for 48 hrs. Next, 10 µl was streaked onto MOX and incubated at 35°C for 48 hrs. A typical colony was then transferred into 10 ml of TSBYE and incubated at 35°C for 48 hrs. After incubation, a loopful of culture was streaked onto multiple TSAYE slants, incubated at 35°C for 48 hrs, and stored at 4°C for up to one month as stock cultures.

Solution Preparation. Distilled water was sterilized for 15 min at 121°C and stored in at 2-4°C until use. Fresh brine (9% NaCl w/v) was prepared by adding 90 g of Top-Flo evaporated salt (0.45 μm 7559 Cargill, Minneapolis, MN) to a volumetric flask and bringing the volume up to 1000 ml with sterile, distilled water. Fresh brine was prepared within 24 hrs of use, filter sterilized (S66128 Nalgene, Rochester, NY) and stored at 2–4°C until use. Percent transmittance of distilled water and fresh brine was measured at 253.7 nm using a Shimadzu spectrophotometer (Model UV-2101PC).

Chemical actinometry, using uridine, was used to determine the dose of UV absorbed during exposure. A 10^{-2} M stock solution of uridine (Appendix B) (A15227 Alfa Aesar, Ward Hill, MD) was prepared by adding 1.22 g of uridine to a 500 ml volumetric flask, bringing up to volume with sterile distilled water, filter sterilized (09-740-22J Fisher Scientific, Suwanee, GA), and stored at 2–4°C for up to six months. A 10^{-4} M uridine working solution, made immediately before use, was prepared by adding 1 ml of the 10^{-2} M stock solution to a 100 ml volumetric flask and bringing up to volume with sterile distilled water or fresh brine.

Acid Stressed Cells. Each strain of *L. monocytogenes* was acid stressed, separately, by first activating healthy cells, from stock cultures, with three successive 24 hrs transfers in TSBYE at 35°C. After the final incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before each pellet was re-suspended in 10 ml of TSB acidified with 85% lactic acid (A162-500 Fisher Scientific, Suwanee, GA) to a pH of 5.0 [27]. Each strain was incubated for 3 hrs at 30°C [27]. After incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water and resuspended in 10 ml of the fluid being treated (distilled water or fresh brine; each prepared to contain 10^{-4} M uridine). Equal portions of each strain (3.3 ml) were added to a sterile 100 ml volumetric flask and brought up to volume using sterile distilled water or fresh brine. Each preparation was made the day of UV exposure and stored at 2-4°C.

Antibiotic Resistant Cells. Sulfanilamide, a derivative of sulfonamide, was chosen for antibiotic resistance experiments due to high levels of sulfonamide resistance observed in retail RTE food isolates in multiple Florida grocery stores [28]. Antibiotic resistant cells of each strain

were created by first activating healthy cells, from stock cultures, with three successive 24 hrs transfers in TSBYE at 35°C. According to the method described by Blackburn and Davies, ten microliters of each strain was transferred into 10 ml of TSBYE and incubated at 35°C for 24 hrs. The cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, the pellet washed with distilled water, and re-suspended in sterile distilled water. Cells resistant to sulfanilamide (86060, Fisher Scientific, Suwanee, GA) were selected for by transferring 10 µl of the culture into 100 ml of TSB and incubating at 37°C for 24 hrs. Following incubation, the culture was added to 100 ml of TSB, containing 1024 µg/ml of sulfanilamide (twice the desired antibiotic resistance). After incubation at 37°C for 48 hrs, 0.1 ml of culture was spread plated onto TSA plates containing 512 µg/ml sulfanilamide (Sigma, Atlanta, GA) and incubated again at 37°C for 48 hrs. Antibiotic resistant strains were streaked onto TSA slants, and maintained at 2-8°C. Resistance was verified before use by streaking onto TSA containing 512 µg/ml sulfanilamide. [29].

Antibiotic cells were prepared for UV treatment by inoculating 10 ml TSBYE containing 512 µg/ml sulfanilamide and incubating at 37°C for 48 hrs [29]. After incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile distilled water. This process was repeated twice before the pellet was re-suspended in 10 ml of the fluid being treated (distilled water or fresh brine; each prepared to contain 10^{-4} M uridine). Equal portions of each strain (3.3 ml) were added to a sterile 100 ml volumetric flask and brought up to volume using the fluid being treated (sterile water or fresh brine). Each preparation was made the day of UV exposure and stored at 2-4°C.

Healthy Cells. Each strain of *L. monocytogenes* was activated from stock cultures by three successive 24 hr transfers in TSBYE, incubated at 35°C for 24 hrs. After the final incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before the pellet was re-suspended in 10 ml of the fluid being treated (distilled water or fresh brine; each prepared to contain 10^{-4} M uridine). Equal portions of each strain were added to a sterile 100 ml volumetric flask and brought up to volume using sterile water or fresh brine, and stored at 2-4°C.

Heat Shocked Cells. Heat shocked cells were prepared according to the method described by McMahon and colleagues [30]. To produce stationary phase cells, shown to be more resistant to many types of stress [31], 10 µl of each strain was transferred into separate flasks containing 30 ml of brain heart infusion broth (BHI, 237500 Becton, Dickinson and Co, Sparks, MD) and incubated at 30°C for 24 hrs. Next, 1 ml of each culture was transferred to 30 ml of fresh BHI and incubated at 30°C for 18 hrs. Following the second incubation, 3 ml of each culture was resuspended in 27 ml of BHI preheated and maintained at 48±0.2°C for 60 min in a waterbath. After heat shock, cells were chilled in ice water for 1 min [30], and then centrifuged for 5 min at 10,000 x g. This process was repeated twice before each pellet was re-suspended in 10 ml of the fluid being treated (distilled water or fresh brine; each prepared to contain 10^{-4} M uridine). Equal portions of each strain (3.3 ml) were added to a sterile 100 ml volumetric flask and brought up to volume using sterile distilled water or fresh brine. Each preparation was made the day of UV exposure and stored at 2-4°C.

Ultraviolet Treatment. One hour before treatment, the re-circulating refrigeration system was adjusted to 4–10°C and allowed to stabilize. Thirty minutes before treatment, the photoreactor was turned on, allowing the bulb to reach maximum intensity and to stabilize output. UV intensity was recorded before and after UV exposure (without the sample cell). For each time period, 14 ml of inoculated test medium was aseptically transferred into a sterile quartz cell. Samples were exposed for 0, 5, 10, 15, 20, 25, or 30 minutes. In order to confirm that uridine would not interfere with the treatment conditions, water, fresh and spent brine samples were prepared without uridine and exposed to UV for 0, 5, 10, 15, 20, 25, 30 minutes.

Microbiological Evaluation. Before and after UV exposure, *L. monocytogenes* were enumerated by serially diluting in 0.1% peptone (211677 Becton, Dickinson and Co, Sparks, MD), spread plating 0.1 ml onto duplicate MOX and TSAYE, and incubating at 37°C for 48 hrs. A dual plating system was employed in order to determine the extent of cell injury. Additionally, 3ml of each UV treated sample was enriched in 45 ml of Brain Heart Infusion broth (BHI, 237500 Becton, Dickinson and Co, Sparks, MD) and incubated at 37°C for 48 hrs. If turbid after

48 hrs, the BHI sample was streaked onto TSAYE and incubated at 37°C for 24–48 hrs for confirmation with *Listeria* API (10-300 Biomerieux, l'Etoile, France).

Quality Assurance and Control Procedures. Quality assurance and control procedures were performed according to Method 9020 of Standard Methods for the Examination of Water and Wastewater [32]. Waterbath, incubator, and refrigerator temperatures were monitored and recorded daily beginning a week before and continuing throughout the duration of survival experiments. Media lots were recorded with each use and tested using positive (*L. monocytogenes* Scott A) and negative (incubation at 35-37ºC for 48 hrs) controls. The suitability of distilled water was verified by periodically performing Heterotrophic Plate counts (TSA, 32ºC for 48 hrs).

Chemical Actinometry. The remainder of each 14 ml sample (typically between 8–10ml) was filtered (6780-2504 Whatman) to remove cells and stored between 2–4°C until all samples had been exposed to UV. The absorbance of an untreated sample (Time 0=Ab262), as well as the absorbance after UV exposure of each sample (Aa262) was measured at 262 nm using a Shimadzu spectrophotometer (Model UV-2101PC). Dose received during each time period was calculated according to the procedure proposed by Jin *et al* (Appendix B)[6].

Statistical Analysis. Appendix C Table 2. Experimental parameters are presented in Appendix C. Differences between UV intensity ($\mu W/cm^2$), dose (mJ/cm^2), *Listeria* populations (log CFU/ml), solution (distilled water and fresh brine), and media (MOX, TSAYE) were analyzed using the proc glm procedure in SAS version 9.1 (SAS Institute, Cary, NC). Significant factors ($P \leq 0.05$) were further distinguished using the least squared means procedure.

RESULTS

Solution Characterization. The average percent transmittance, measured at 253.7 nm, was 91% for both solutions (distilled water, fresh brine). There were no differences between population estimates as determined on MOX and TSAYE, therefore results for only TSAYE will be presented ($P > 0.05$). No differences were detected for population estimates based on solution (distilled water or fresh brine), suggesting that salt concentration did not influence inactivation (P

> 0.05). Time affected population estimates; as time ($P \leq 0.05$) of exposure to UV light increased, UV intensity, and dose increased, thereby significantly decreasing overall populations in each solution. There were no population differences between acid stressed and antibiotic resistant or healthy and heat shocked. However, acid stressed and antibiotic resistant were significantly more resistant to UV light than healthy and heat shocked *L. monocytogenes* ($P \leq 0.05$).

Acid Stressed. Table 4.1 and 4.2. Dose calculations, UV intensity, and population estimates for acid stressed cells suspended in distilled water and fresh brine are presented in Tables 4.1 and 4.2. Initial concentrations of *L. monocytogenes* in distilled water were 7.58 and 7.60 log CFU/ml as determined on MOX and TSAYE. Estimates decreased by 5.81 log CFU/ml on MOX and 5.98 log CFU/ml on TSAYE after UV exposure equivalent to 43.89 mJ/cm^2 (30 min). In fresh brine, initial concentrations of *L. monocytogenes* as determined on MOX and TSAYE were 7.44 and 7.45 log CFU/ml. After UV exposure greater than 32.58 mJ/cm^2 (30 min), *L. monocytogenes* decreased by 4.45 log CFU/ml on MOX and 5.95 log CFU/ml on TSAYE.

Antibiotic Resistant. Tables 4.3 and 4.4. Dose calculations, UV intensity, and population estimates for acid stressed cells suspended in distilled water and fresh brine are presented in Tables 4.3 and 4.4. Initial concentrations in distilled water were 7.35 and 7.49 log CFU/ml on MOX and TSAYE, respectively. Populations were reduced 5.92 and 5.95 log CFU/ml on MOX and TSAYE after exposure to 70.33 mJ/cm^2 (30 min). In fresh brine, initial concentrations of *L. monocytogenes* as determined on MOX and TSAYE were 7.63 and 7.49 log CFU/ml. After UV exposure greater than 136.66 mJ/cm^2 (30 min), *L. monocytogenes* decreased by 5.55 log CFU/ml on MOX and 5.35 log CFU/ml on TSAYE.

Healthy Cells. Tables4. 5 and 4.6. Dose calculations, UV intensity, and population estimates for acid stressed cells suspended in distilled water and fresh brine are presented in Tables 4.5 and 4.6. Initial concentrations of *L. monocytogenes* on MOX and TSAYE were 7.37 and 7.55 log CFU/ml, respectively. *L. monocytogenes* fell below the limit of detection (1 log CFU/ml) after exposure to doses of UV greater than 18.80 mJ/cm^2 (20 min exposure), and was no longer

detectable via enrichment at a dose of 39.17 mJ/cm^2 (30 min exposure). A 5-log reduction was achieved after exposure to 11.89 mJ/cm^2, corresponding to an exposure time of 10 minutes. Initial concentrations of *L. monocytogenes* on MOX and TSAYE were 7.86 and 7.97 log CFU/ml, respectively. *L. monocytogenes* irradiated in fresh brine fell below the limit of detection after exposure to doses of UV greater than 12.47 mJ/cm^2 (15 min) After exposure to 24.39 mJ/cm^2 (30 min), the organism was not detected via enrichment. A greater than 5-log reduction in fresh brine was achieved after exposure to 7.99 mJ/cm^2 (10 min) UV.

Heat Shocked. Tables 4.7 and 4.8. Dose calculations, UV intensity, and population estimates, as determined on MOX and TSAYE, are presented in Tables 4.7 and 4.8. Heat shocked *L. monocytogenes* irradiated in water decreased to below the detection limit (1 log CFU/ml) at UV doses greater than 13.14 mJ/cm^2 (20 min) but was detected via enrichment after exposure of up to 24.78 mJ/cm^2 (30 min). After 12.95 mJ/cm^2 (15 min) of exposure, more than a 5-log reduction was achieved on MOX and TSAYE. Heat Shocked *L. monocytogenes* irradiated in fresh brine decreased to below the detection limit after exposure to doses greater than 20.41 mJ/cm^2 (30 min). A greater than 5-log reduction, below the limit of detection (1 log CFU/ml) was achieved after UV exposure greater than 12.76 mJ/cm^2 (15 min) on MOX and 17.14 mJ/cm^2 (20 min) on TSAYE.

DISCUSSION

The food matrix is an unfriendly environment for microorganisms, containing complex substrates for growth, competition from other bacteria, lack of moisture, and acidic by-products produced by other organisms. Processing treatments exert further stress. Microorganisms go from their natural environment (optimum conditions) to food environment (stress because of low water activity, low pH, nutrients unsuitable for the microorganisms to metabolize) then potentially onto a host (increased stress). In order to survive, complex changes, or stress responses, may take place in cell composition (changes in the membrane and lipid composition)

and gene expression (heat or cold chock proteins, acid tolerance response) [33]. Such responses enable microorganisms to maintain the physiology of the cell and potentially survive and grow under extreme conditions. Specific stress responses have been identified for exposure to high and low temperatures, acid, high salt, and preservatives [1-8, 34-37]. In general, when exposed to a mild stress, microorganisms may adapt to the stress, developing tolerance or resistance to greater amounts of the stress or to different unrelated stresses.

Based on the current experiments, it was determined that sulfanilamide resistant and acid stressed *L. monocytogenes* were more resistant to UV light than heat shocked and healthy unstressed cells regardless of suspension solution (distilled water or fresh brine). There were no differences between population estimates based on media ($P > 0.05$) (MOX or TSAYE) or solution ($P > 0.05$) (distilled water or fresh brine) indicating that no sub-lethal cell injury occurred and that addition of NaCl did not significantly influence inactivation. Time affected population estimates; as time ($P \leq 0.05$) of exposure to UV light increased, UV intensity, and dose increased, thereby significantly decreasing overall populations in each solution.

Bacteria survive acidic environments because of their ability to regulate their internal pH close to neutrality (by controlled movement of cations across their membranes) [2]. Upon exposure to mildly acidic conditions, an Acid Tolerance Response (ATR) may occur. Due to the expression of different levels of proteins, bacteria acquire the ability to survive lethal acid concentrations, and protection against thermal and osmotic stresses [33, 38]. According to previously published literature, exposure to the present experimental conditions is sufficient to induce an acid tolerance response in *L. monocytogenes* [33]. Increased resistance to heat shock 52ºC and osmotic shock (25-30%) was observed in *L. monocytogenes* acid stressed at pH 5.5 for 2 hrs [39]. Lou and Yousef exposed *L. monocytogenes* to sub-lethal doses of acid (pH 4.0, 5.0), NaCl (7%) and heat shock (45ºC) for 1 hr, after which they were exposed to lethal amounts to acid (pH 3.5) and NaCl (25%). Exposure to acid (pH 5.0) increased *L. monocytogenes* resistance to lethal doses of acid, ethanol, and hydrogen peroxide. Heat shock increased the resistance to ethanol and NaCl. There experiments also highlighted the highly dependent relationship between the type of stress and subsequent lethal factors [5]. In another study, three clinical isolates of *L. monocytogenes* acid adapted for 1 hr (citric, acetic, and lactic) *L. monocytogenes* were inoculated into seafood salad and exposed to various levels of gamma irradiation (0.59-0.72 kGy) *[40]*.

Compared to non-adapted cells, the 1 hr acid adapted cells showed the greatest difference in resistance to irradiation *[40]*.

In the current study, rather than protecting, heat shock at 48°C for 60 min made *L. monocytogenes* more vulnerable to UV. A 2004 study found that heat shocking *L. monocytogenes* at 45ºC for 1 hr or 48ºC for 10 min had the potential to produce opposite stress responses. Heat shocking at 45ºC for 1 hr increased tolerance to 25% NaCl, .01% crystal violet, and 18% ethanol [4]. Heat shocking at 48ºC for 10 min increased resistance to 25% NaCl, but reduced resistance to crystal violet and ethanol [4]. Heat adapted cells also had increased resistance to acid shock [39]. Heat shock response varies with strain, heat shock treatment, and type of subsequent stress [4].

Clinical and food strains of *L. monocytogenes* resistant to chloramphenicol, erythiromycin, streptomycin, tetracycline, vancomycin, and trimethoprim have been characterized in recent literature [41-45]. Research suggests that *L. monocytogenes* is slowly acquiring antibiotic resistance genes from other Gram-positive bacteria [41, 42]. Susceptibility testing performed on 167 *L. monocytogenes* isolates recovered from RTE meats, raw chicken, and produce from the Florida and Washington DC areas exhibited a high resistance to common clinical antibiotics [28]. Seventy three percent of the isolates were resistant to sulfonamide (60% of RTE isolates), 8% to tetracycline (15% of RTE isolates), and 1.8% to Ciprofloxacin (1% of RTE isolates) [28]. Walsh and colleagues examined the potential relationship between antibiotic resistance and resistance to heat. Compared to wild type strains (no antibiotic resistance), strains resistant to streptomycin were found to be no more resistant to heat (55ºC for 25 min) after heat shock treatment at 48ºC for 10 min than wild type strains [46]. Cells resistant to sulfanilamide were more resistant to UV in both solutions. In fresh brine, an unusually high UV dose was observed for antibiotic resistant cells, for unknown reasons. The current study suggests that antibiotic resistance mechanisms do however provide cross-protection against UV light. This information provides a basis for further investigation, because only one antibiotic within a single group of available drugs was tested.

Bacteria encounter many stressful conditions in food products and in the processing environment, as a result complex changes may take place in cell composition and regulation. Such changes enable microorganisms to maintain the physiology of the cell and potentially survive and grow. Stress responses have been identified for exposure to temperature, acid,

osmolarity, and preservatives. However, the physiology, biochemistry, and genetic mechanisms have not been outlined for all stress responses. In general, when exposure to a mild stress, microorganisms may adapt to the stress, developing tolerance or resistance to greater amounts of the stress. Exposure to low levels of stress protects microorganisms against the lethal effects of higher levels of the same stress or of different stresses. Under the current experimental conditions, *L. monocytogenes* became more resistant to UV exposure after exposure to acid. In addition, it was determined that antibiotic resistant cells also became more resistant to UV exposure. Heat shocking as described made *L. monocytogenes* more susceptible. Further research is needed in order to fully understand the complex relationship between stress responses, antibiotic resistance and UV treatment.

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Table 4. 1. Minimum ultraviolet light dose and intensity applied to acid stressed *Listeria monocytogenes* suspended in distilled water and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.60
5	2.40	80.20	5.56
10	4.30	159.20	3.88
15	11.17	237.35	3.36
20	12.76	318.27	3.19
25	38.84	402.00	3.01
30	43.89	482.40	1.62

[a]Minimum ultraviolet light (dose)

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 4.2. Minimum ultraviolet light dose and intensity applied to acid stressed *Listeria monocytogenes* suspended in fresh brine (9% NaCl) and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.45
5	1.39	78.65	5.49
10	3.31	156.57	3.56
15	19.83	236.67	2.83
20	24.66	315.87	3.33
25	30.43	387.83	2.49
30	32.57	458.50	1.50

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 4.3. Minimum ultraviolet light dose and intensity applied to antibiotic resistant *Listeria monocytogenes* suspended in distilled water and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.49
5	11.58	81.95	6.50
10	31.64	163.77	2.96
15	44.24	245.20	3.09
20	50.70	327.33	2.39
25	63.27	407.67	1.38
30	70.33	491.20	1.72

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 4. 4. Minimum ultraviolet light dose and intensity applied to antibiotic resistant *Listeria monocytogenes* suspended in fresh brine (9% NaCl) and cell recovery via direct plating TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.49
5	42.48	80.45	6.00
10	74.74	160.90	3.58
15	98.58	241.13	2.69
20	125.60	322.20	2.68
25	-	405.63	2.45
30	136.66	483.75	2.13

[a]Minimum ultraviolet light (dose)

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 - 48 hrs

Table 4. 5. Minimum ultraviolet light dose and intensity applied to healthy *Listeria monocytogenes* suspended in distilled water, cell recovery via direct plating onto TSAYE, and enrichment in BHI

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.55
5	12.42	104.67	3.78
10	16.78	208.33	2.55
15	23.13	314.50	2.95
20	33.19	418.67	<1.00
25	41.41	525.83	II[d]
30	46.78	634.00	0[d]

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); at 37°C for 24 - 48 hrs
[d]Indicates number of enrichments (BHI; 37°C, 24 – 48 hr) out of three in which *L. monocytogenes* was detected

Table 4. 6. Minimum ultraviolet light dose and intensity applied to healthy *Listeria monocytogenes* suspended in fresh brine (9% NaCl), cell recovery via direct plating onto TSAYE, and enrichment in BHI

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.97
5	2.51	109.50	3.91
10	8.06	218.00	2.46
15	10.35	353.17	1.70
20	12.09	399.33	II[d]
25	19.26	549.17	I[d]
30	23.70	662.00	0[d]

[a]Minimum ultraviolet light (dose)
[b]UV Intensity calculated from radiometer measurement
[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); at 37°C for 24 - 48 hrs
[d]Indicates number of enrichments (BHI; 37°C, 24 – 48 hr) out of three in which *L. monocytogenes* was detected

Table 4. 7. Minimum ultraviolet light dose and intensity applied to heat shocked *Listeria monocytogenes* suspended in distilled water, cell recovery via direct plating onto TSAYE, and enrichment in BHI

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	6.99
5	5.65	103.5	3.45
10	4.91	209.33	3.35
15	12.95	314.50	1.72
20	13.14	411.33	II[e]
25	22.57	532.50	II[e]
30	24.78	624	II[e]

[a]Minimum ultraviolet light (dose)

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); incubated at 37°C for 24 – 48 hrs

[d]Indicates number of enrichments (BHI; 37°C, 24 – 48 hr) out of three in which *L. monocytogenes* was detected

Table 4. 8. Minimum ultraviolet light dose and intensity applied to heat shocked *Listeria monocytogenes* suspended in fresh brine (9% NaCl) and cell recovery via direct plating onto TSAYE

Time (minutes)	UV[a] (mJ/cm^2)	Intensity[b] ($\mu W/cm^2$)	TSAYE[c] (log CFU/ml)
0	0.00	0.00	7.36
5	4.46	81.66	4.22
10	9.82	159.70	3.66
15	12.76	244.88	2.48
20	17.14	329.00	2.11
25	17.57	403.44	1.46
30	20.41	494.40	<1.00

[a]Minimum ultraviolet light (dose)

[b]UV Intensity calculated from radiometer measurement

[c]Trypticase Soy Agar + 0.6% Yeast Extract; direct plating (0.1 ml); at 37°C for 24 –48 hrs

Chapter V
The Effect of Acid Stress, Antibiotic Resistance and Heat Shock on the Survival of *Listeria monocytogenes* in Fresh and Spent Brine Used for Cooling Ready-to-Eat Meat Products

Keywords: *Listeria monocytogenes*, brine, survival studies
Note: This chapter formatted for submission to the Journal of Food Protection

ABSTRACT

Listeria monocytogenes survives in brine solutions used to rapidly cool Ready-to-eat (RTE) meat products. Minimal research has been conducted to determine how sub-lethal processing stress and brine conditions affect survival. The purpose of this research was to determine the survivability of antibiotic resistant, acid stressed, healthy, and heat shocked *L. monocytogenes* in fresh and spent brine solutions. *L. monocytogenes* strains N1-227 (hotdog batter), N3-031 (turkey franks), and R2-499 (Ready-to-eat meat) were used to produce acid stressed (35ºC for 3 hrs in Trypticase Soy Broth with Yeast Extract, TSBYE, acidified to pH 5.0), healthy (Brain Heart Infusion, BHI, 37ºC for 24 hrs) heat shocked (BHI, at 48ºC for 1 hr), and antibiotic resistant (512 µg/ml sulfanilamide) cells which were mixed in equal proportions and suspended in fresh brine (9% NaCl) or spent brine (20.86% NaCl) obtained from a commercial meat processor. Prepared in triplicate for each cell type and solution combination and stored at 4ºC, fresh brine was sampled once daily for seven days and spent brine for twenty-eight days. Samples were serially diluted in 0.1% peptone, plated onto MOX and TSAYE, and incubated at 37ºC for 48 hrs. After seven days, acid stressed *L. monocytogenes* declined by an average of 0.27 CFU/ml and 0.15 log CFU/ml on MOX and TSAYE. Antibiotic resistant cells decreased by 0.01 log CFU/ml as determined on MOX and increased by 0.44 log CFU/ml on TSAYE. Healthy *L. monocytogenes* declined by an average of 0.25 log CFU/ml and 0.20 CFU/ml log as determined on MOX and TSAYE. Heat shocked cells declined by 0.25 log CFU/ml and 0.52 log CFU/ml on MOX and TSAYE. In spent brine, acid stressed *L. monocytogenes* declined by an average of 0.56 CFU/ml and 0.45 log CFU/ml and on MOX and TSAYE. Antibiotic resistant cells decreased by 0.57 log CFU/ml as determined on MOX and increased by 0.64 log CFU/ml on TSAYE. Healthy *L. monocytogenes* declined by an average of 1.46 log CFU/ml and 1.01 CFU/ml log as determined on MOX and TSAYE. Heat shocked cells declined by 3.79 log CFU/ml and 3.62 log CFU/ml on MOX and TSAYE. Stress and time significantly influenced the survival of *L. monocytogenes* in fresh and spent brine. In each solution, survival followed the trend, from greatest to least ($P \leq 0.05$): antibiotic resistant >acid stressed > healthy >heat shocked. Population estimates decreased significantly from initial inoculation to final sampling for each cell type suspended spent brine ($P \leq 0.05$), but only for healthy and heat shocked cells suspended in fresh brine. Plate counts on TSAYE (CFU/ml) were higher than estimates on MOX (CFU/ml), except for acid stressed cells suspended in fresh brine, indicating significant cell

injury ($P \leq 0.05$). This research highlights the impact that sub-lethal stress can have on the survival of *L. monocytogenes* in brine solutions and emphasizes the ability of this organism to persist in extreme environments.

INTRODUCTION

Brine chilling systems, used to quickly cool thermally processed meat products such as frankfurters and luncheon meats, use a combination of cold temperature and high salt concentration to inactivate or inhibit the growth of microorganisms [1, 2]. Commercial salt brines, are used, reused, and maintained according to the United States Department of Agriculture Food Safety and Inspection Service bulletin 83-16 (Appendix 3) [3]. According to these guidelines, brine can be reused for up to 4 weeks if the salt concentration is maintained at 20% and the temperature at –12.2˚C [3]. Established using aerobic plates counts and coliform data, these guidelines have not been fully validated using pathogens [1].

Reused brine poses a risk to food manufacturers, the longer the use the greater the risk. As products are dipped or sprayed with brine, nutrients and heat may leach into the solution, allowing psychotropic pathogens to grow. In addition, exposure to sub-lethal processing stress, treatments that are only slightly less than lethal, allowing some cells to survive, may illicit bacterial stress responses. Resulting changes in bacterial cell physiology and gene expression can lead to resistance to the previously imposed stress as well as resistance to unrelated stresses [4].

L. monocytogenes, a Gram-positive aerobe, is capable of growing at refrigeration temperatures and high salt contents [5, 6]. Although responsible for less than 0.1% of foodborne related illnesses, it accounts for 37% of the deaths [7]. Outbreaks of listeriosis have prompted the United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) and the Food and Drug Administration (FDA) to establish a zero tolerance policy for this organism in Ready-to-eat (RTE) products, such as those cooled by chill brine systems [8].

The purpose of this research was to determine the survivability of antibiotic resistant, acid stressed, healthy, and heat shocked *L. monocytogenes* in fresh and spent brine solutions.

MATERIALS AND METHODS

Culture Preparation and Maintenance. Cocktails of the following strains were used for each experiment: N1-227, serotype 4b, isolated from hotdog batter implicated in a 1998 outbreak; N3-031, serotype 1/2a, isolated from turkey franks; and R2-499, serotype 1/2a, isolated from a RTE meat product [9]. Upon arrival, each strain was transferred into 10 ml of Trypticase Soy Broth supplemented with 0.6% Yeast Extract (TSBYE, 211825, Becton, Dickinson and Co, Sparks,

MD) and incubated at 35°C for 48 hrs. Each strain was then swabbed onto multiple Modified Oxford Agar plates (MOX) prepared with Oxford Medium Base (222530, Becton, Dickinson and Co, Sparks, MD) supplemented with Modified Oxford Antimicrobic Supplement (6060973, Becton, Dickinson and Co, Sparks, MD), and incubated at 35°C for 48hrs. Strains were confirmed, using *Listeria* API (10-300, Biomerieux, l'Etoile, France) and divided into multiple cryovials of Nutrient broth (23300, Becton, Dickinson and Co, Sparks, MD) supplemented with 15% glycerol (G33-1 Fisher Scientific, Suwanee, GA), for storage at -70°C.

Monthly, one cryovial of each strain was thawed at room temperature. Using a sterile prepackaged inoculating loop (13-070-3 Fisher Scientific, Suwanee, GA), 10 µl of inoculum was transferred into 10 ml of TSBYE and incubated at 32°C for 48 hrs. Next, 10 µl was streaked onto MOX and incubated at 35°C for 48 hrs. A typical colony was then transferred into 10 ml of TSBYE and incubated at 35°C for 48 hrs. After incubation, a loopful of culture was streaked onto multiple TSAYE slants, incubated at 35°C for 48 hrs, and stored at 2-4°C for up to one month as stock cultures.

Acid Stressed Cells. Each strain of *L. monocytogenes* was acid stressed, separately, after first activating healthy cells, from stock cultures, with three successive 24 hrs transfers in TSBYE at 35°C. After the final incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before each pellet was re-suspended in 10 ml of TSB acidified with 85% lactic acid (A162-500 Fisher Scientific, Suwanee, GA) to a pH of 5.0 [10]. Each strain was incubated for 3 hrs at 30°C [10]. After incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water and re-suspended in the fluid being treated (fresh or spent brine). Equal portions of each strain were added to a sterile volumetric flask and brought up to volume using fresh or spent brine.

Antibiotic Resistant Cells. Sulfanilamide, a derivative of sulfonamide, was chosen for antibiotic resistance experiments due to high levels of sulfonamide resistance observed in retail RTE food isolates in multiple Florida grocery stores [11]. Antibiotic resistant cells of each strain were created by first activating healthy cells, from stock cultures, with three successive 24 hr transfers in TSBYE at 35°C. According to the method described by Blackburn and Davies, ten microliters

of each strain was transferred into 10 ml of TSBYE and incubated at 35°C for 24 hrs. The cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, the pellet washed with distilled water, and re-suspended in sterile distilled water. Cells resistant to sulfanilamide (86060, Fisher Scientific, Suwanee, GA) were selected by transferring 10 µl of the culture into 100 ml of TSB and incubating at 37°C for 24 hrs. Following incubation, the culture was added to 100 ml of TSB, containing 1024 µg/ml of sulfanilamide (twice the desired antibiotic resistance). After incubation at 37°C for 48 hrs, 0.1 ml of culture was spread plated onto TSA plates containing 512 µg/ml sulfanilamide (Sigma, Atlanta, GA) and incubated again at 37°C for 48 hrs. Antibiotic resistant strains were streaked onto TSA slants, and maintained at 2-4°C. Resistance was verified before use by streaking onto TSA containing 512 µg/ml sulfanilamide. [12]

Antibiotic cells were prepared for UV treatment by inoculating 10 ml TSBYE containing 512 µg/ml sulfonamide and incubating at 37°C for 48 hrs [12]. After incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile distilled water. This process was repeated twice before the pellet was re-suspended in 10 ml of the fluid being treated (fresh or spent brine). Equal portions of each strain were added to a sterile volumetric flask and brought up to volume using fresh or spent brine.

Heat Shocked Cells. Heat shocked cells were prepared according to the method described by McMahon and colleagues [13]. To produce stationary phase cells, shown to be more resistant to many types of stress [14], ten microliters of each strain were transferred into separate flasks containing 30 ml of BHI broth (BHI, 237500 Becton, Dickinson and Co, Sparks, MD) and incubated at 30°C for 24 hrs. Next, 1ml of each culture was transferred to 30ml of fresh BHI and incubated at 30°C for 18 hrs. Following the second incubation, 3 ml of each culture was re-suspended in 27 ml of BHI preheated and maintained at 48±0.2°C for 60 min in a waterbath. After heat shock, cells were chilled in ice water for 1 min [13], and then centrifuged for 5 min at 10,000 x g. This process was repeated twice before each pellet was re-suspended in 10 ml of the fluid being treated (fresh or spent brine). Equal portions of each strain were added to a sterile volumetric flask and brought up to volume using fresh or spent brine.

Healthy Cells. Each strain of *L. monocytogenes* was activated from stock cultures by three successive 24-hour transfers in TSBYE incubated at 35°C for 24 hrs. After the final incubation, the cultures were centrifuged for 5 min a 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before the pellet was re-suspended in 10ml of the fluid being treated (fresh or spent brine). Equal portions of each strain were added to a sterile volumetric flask and brought up to volume using fresh or spent brine.

Solution and Inoculum Preparation. Fresh brine (9% NaCl w/v) was prepared by adding 90g of Top-Flo evaporated salt (7559 Cargill, Minneapolis, MN) to a volumetric flask and bringing the volume up to 1000ml with sterile, distilled water. Spent brine was received from a national RTE meat manufacturer and stored at 2–4°C until use. Each solution was filter sterilized (S66128 Nalgene, Rochester, NY), dispensed into twelve Nalgene bottles (225 ml fresh brine, 450 ml spent brine), and stored at 4°C for 24 hrs to allow the solutions to equilibrate. Twenty-five milliliters of *L. monocytogenes* cocktail was dispensed into fresh brine, in triplicate for each type of stress. Fifty milliliters of *L. monocytogenes* cocktail was dispensed into spent brine, in triplicate for each type of stress. On TSAYE, initial concentrations ranged from 10^7 to 10^8 CFU/ml.

Sampling and Enumeration. Fresh brine was sampled at 0, 1, 2, 3, 4, 5, 6, and 7 days. Spent brine was sampled at 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 19, 21, 26, and 28 days. *L. monocytogenes* was enumerated by serially diluting in 0.1% peptone (211677 Becton, Dickinson and Co, Sparks, MD) and spread plating 0.1 ml onto duplicate MOX and TSAYE, followed by incubation at 37°C for 48 hrs. A dual plating system was employed in order to determine the extent of cell injury.

Quality Control Procedures. Quality assurance and control procedures were performed according to Method 9020 of Standard Methods for the Examination of Water and Wastewater [15]. Waterbath, incubator, and refrigerator temperatures were monitored and recorded daily beginning a week before and continuing throughout the duration of survival experiments. Media lots were recorded with each use and tested using positive (*L. monocytogenes* Scott A) and negative (incubation at 35-37ºC for 48 hrs) controls. The suitability of distilled water was verified by periodically performing Heterotrophic Plate counts (TSA, 32ºC for 48 hrs).

Statistical Analysis. Appendix C Table 3. Experimental parameters are presented in Appendix C. Differences between UV intensity ($\mu W/cm^2$), dose (mJ/cm^2), *Listeria* populations (log CFU/ml), solution (distilled water and fresh brine), and media (MOX, TSAYE) were analyzed using the proc glm procedure in SAS version 9.1 (SAS Institute, Cary, NC). Significant factors ($P < 0.05$) were further distinguished using the least squared means procedure.

RESULTS

Stress and time significantly influenced the survival of *L. monocytogenes* in fresh and spent brine. In each solution, survival followed the trend, from greatest to least ($p < 0.05$): antibiotic resistant >acid stressed > healthy >heat shocked. Population estimates decreased from initial inoculation to final sampling for each cell type suspended in spent brine ($P \leq 0.05$), but only healthy and heat shocked cells suspended in fresh brine were significantly reduced ($P \leq 0.05$). Plate counts on TSAYE (CFU/ml) were higher than estimates on MOX (CFU/ml), except for acid stressed cells suspended in fresh brine, indicating significant cell injury ($P \leq 0.05$).

Fresh Brine. Figure 5.1. Figure 5.1 is a visual representation of the population changes that occurred in fresh brine over a seven day sampling period. After seven days, acid stressed *L. monocytogenes* declined by an average of 0.27 CFU/ml and 0.15 log CFU/ml and on MOX and TSAYE. Antibiotic resistant cells decreased by 0.01 log CFU/ml as determined on MOX and increased by 0.44 log CFU/ml on TSAYE. Healthy *L. monocytogenes* declined by an average of 0.25 log CFU/ml and 0.20 CFU/ml log and as determined on MOX and TSAYE. Heat shocked cells declined by 0.25 log CFU/ml and 0.52 log CFU/ml MOX and TSAYE. There were significant differences between plate counts on MOX and TSAYE for antibiotic resistant, healthy, and heat shocked cells. Populations decreased from day 0 to day seven for heat shocked and healthy cells ($P \leq 0.05$), but not for acid stressed or antibiotic resistant. Survival followed the trend, from highest to least ($P \leq 0.05$): antibiotic resistant >acid stressed > healthy >heat shocked.

Spent Brine. Figure 5.2. Figure 5.2 is a visual representation of the population changes that occurred in spent brine over a twenty-eight day sampling period. Acid stressed *L. monocytogenes*

declined by an average of 0.56 CFU/ml and 0.45 log CFU/ml and on MOX and TSAYE. Antibiotic resistant cells decreased by 0.57 log CFU/ml as determined on MOX and increased by 0.64 log CFU/ml on TSAYE and. Healthy *L. monocytogenes* declined by an average of 1.46 log CFU/ml and 1.01 CFU/ml log and as determined on MOX and TSAYE. Heat shocked cells declined by 3.79 log CFU/ml and 3.62 log CFU/ml on MOX and TSAYE. Populations decreased significantly from day 0 to day twenty-eight for each cell type ($P \leq 0.05$), but not necessarily between each time period. There were significant differences between plate counts on TSAYE and MOX for antibiotic resistant, acid stressed, healthy, and heat shocked cells ($P \leq 0.05$). Survival followed the trend, from highest to least ($P \leq 0.05$): antibiotic resistant >acid stressed > healthy >heat shocked.

DISCUSSION

The current studies indicate that not only can *L. monocytogenes* survive in brine solutions but exposure to certain types of sub-lethal stress may enhance survival. Exposure to acid stress and acquisition of antibiotic resistance mechanisms allowed *L. monocytogenes* to persist longer, or grow (antibiotic resistant in fresh brine), in fresh and spent brine compared to heat shocked and healthy cells. Although in both fresh and spent brine, acid stressed, antibiotic resistant, and healthy cell populations were ($P \leq 0.05$) reduced over the course of sampling, overall reductions were less than 1.5 log CFU/ml. Heat stressed cells were the most significantly reduced in both fresh and spent brines ($P \leq 0.05$). Despite the fact that initial populations were significantly higher (log 7-8 CFU/ml) than would be observed in real life scenarios, these experiments illustrate the impact that sub-lethal stress can have on the survival of *L. monocytogenes* and emphasize the ability of this organism to persist in extreme environments.

Survival of *L. monocytogenes* in high salt solutions and low temperatures is well documented. Larson and colleagues determined that *L. monocytogenes* could survive in commercial cheese brine for up to 259 days, though no growth was observed [16]. Survival did not correlate with pH, salt, mineral or nitrogen content. In general, greater survival was observed at 4ºC than at 12ºC [16]. A 2003 study which used both a test tube and simulated re-circulating brine system to test *L. monocytogenes* survivability at a range of pH (5, 6, 7) and free chlorine (0. 3, 5, 10 ppm) values found that these parameters had little effect on survival [17]. Although brine temperature and salt content were maintained at -12ºC and 20%, *L. monocytogenes* was

still detected in both systems for the duration of the sampling period [17]. Using a model brine chilling system, Miller and colleagues evaluated the growth, injury, and survival potential of *L. monocytogenes* incubated for up to 30 days at temperatures from –12 to 28ºC and 0.5 to 20% NaCl, using BHI as the substrate. Growth was observed at both 5ºC and 5% NaCl and 12ºC in 9% NaCl, and was controlled if salt concentrations were maintained at 9% and temperature below -2°C, but no conditions ensured death. In fact, *L. monocytogenes* survived for 30 days at -12°C in 20% NaCl. Unlike the current experiments, no significant injury was detected based on a dual plating system, perhaps due to the rich nutrient composition of the BHI used as the suspension substrate. [1].

Limited research has been conducted to determine the relationship between antibiotic resistance and response to processing treatments. Walsh and colleagues examined the potential relationship between antibiotic resistance and resistance to heat. Compared to wild type strains (no antibiotic resistance), strains resistant to streptomycin were found to be no more resistant to heat (55ºC for 25 minutes) after heat shock treatment at 48ºC minutes for 10, than wild type strains [18].

FSIS brine reuse guidelines were established based on in plant experience, aerobic plates counts and coliform data [1]. The current study indicates that sub-lethal stress can enhance survival in brine solutions, suggesting the need for guideline re-evaluation and incorporation of additional intervention steps, to eliminate *L. monocytogenes* from chill brines.

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Figure 5.1. Survival of acid stressed, antibiotic resistant, healthy, and heat shocked *Listeria monocytogenes* in fresh brine after 7 days

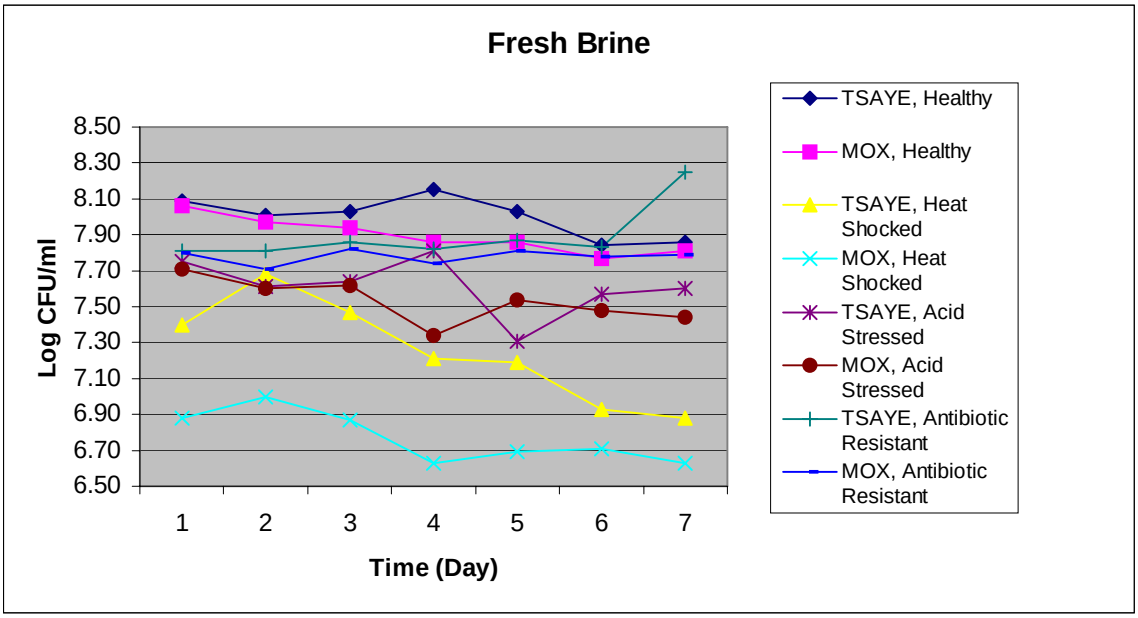
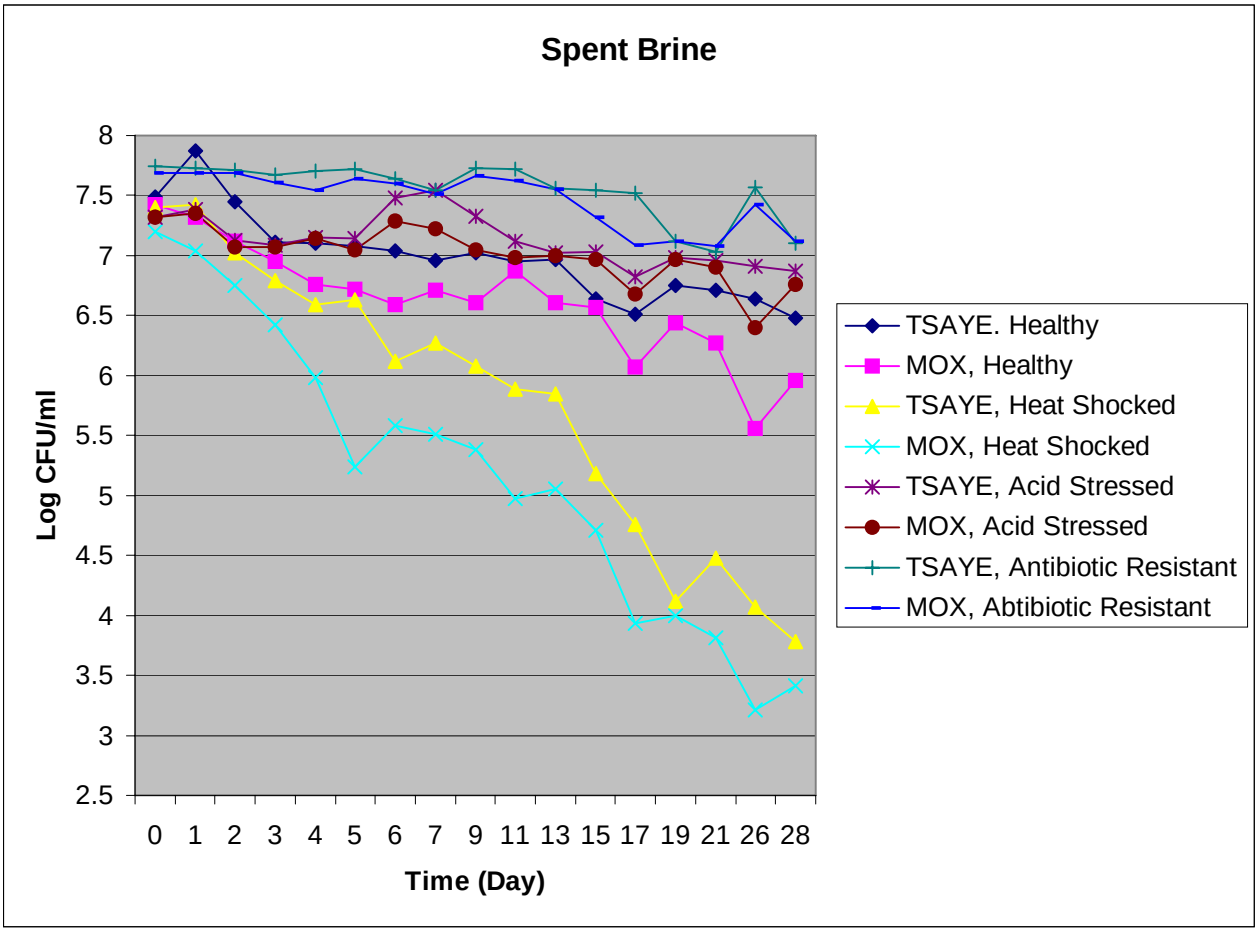


Figure 5. 2. Survival of acid stressed, antibiotic resistant, healthy, and heat shocked *Listeria monocytogenes* in spent brine after 28 days



SUMMARY

Invasive infections due to *Listeria monocytogenes* require strict guidelines to ensure that contamination of vulnerable products does not occur. To that end, research to develop new processing strategies and a greater understanding of interactions that take place between this organism and its environment (food matrix, processing stress) are necessary.

Ultraviolet light is an effective method for eliminating *L. monocytogenes* from brine solutions, under controlled conditions. The minimum dose of UV required to reduce *L. monocytogenes* to below detection limits is comparable to other foodborne microorganisms (10-40 mJ/cm²) and is highly dependent on turbidity, suspended solids, and exposure of organisms to sub-lethal stress prior to UV exposure. Sub-lethal processing stress may also enhance *L. monocytogenes* survival in brine solutions.

Future research should address the following issues, to appropriately apply UV to the sterilization of brine solutions:

1. Modeling UV inactivation of *L. monocytogenes* as the brine matrix changes over time (increase in turbidity and suspended solids, decrease in pH).
2. Characterization of suspended particles that may negatively influence UV inactivation.
3. Validation of chemical actinometry for use in production facilities.
4. Expansion of test parameters, to include additional antibiotics and a variety of sub-lethal treatments.

APPENDIX A

Factors Affecting the Effectiveness of Ultraviolet Light When Applied to Brine Solutions

Table A.1 USDA Food Safety Inspection Service Brine Reuse Schedule

	Solution Maintenance Conditions	
Reuse duration	Minimum Salt Concentration (%)	Maximum temperature
One Shift	None	Undefined
Up to 24 Hours	5	4.4°C
Up to 1 week	9	-2.2°C
Up to 4 weeks	20	-12.2°C

Table A. 2. Types of Radiation

Radiation Type	Wavelength, nm	Photon Energy (kJ/Einstein)
Gamma Rays (Irradiation)	<0.1	$>10^6$
Radiographs	≈0.1-50	≈10^6-2,400
UV	≈50-400	≈2,400-300
Visible	400-700	300-170
IR	700-10^7	170-.01
Microwaves	10^7-10^8	.01-.001

Table A.3. Parameters affecting UV disinfection of wastewater

Parameters	Acceptable values
% Transmittance	35-65
Total suspended solids (TSS)	5-10 mg/l
Particle size	10-40 μm
Iron	< 0.3 mg/l

APPENDIX B

Equipment Used for the Ultraviolet Treatment of Brine Solutions

Figure B.1. Oriel photoreactor, arc lamp housing unit, condensing tube

Figure B.2. Interior view of arc housing unit, mercury bulb, condensing lens

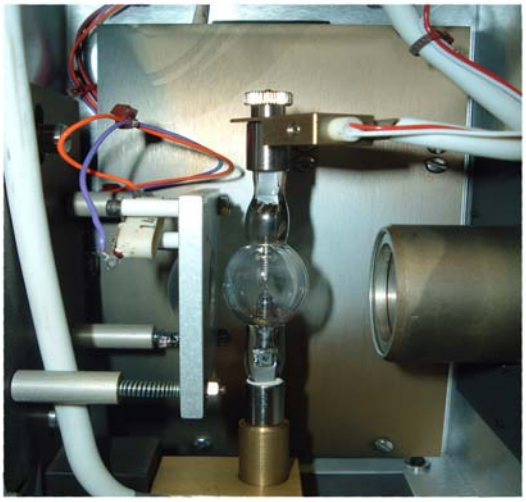


Figure B. 3. Polystyrene sample chamber with circulating refrigerant

Figure B.4. Sample stand with magnetic stir bar

Figure B. 5. Cylindrical Quartz Sample Cell, Part Number 14-385-930E

Figure B. 6. Chemical Structure of Uridine

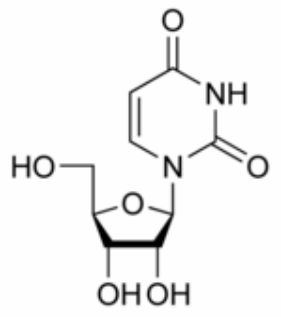


Table B.1. Parameters for calculation of ultraviolet light dose, using uridine actinometry

Fluence=	$\frac{\text{Ln (Ab262/Aa262) X } \mathbf{U} \text{ X } \mathbf{t}}{2.303 \text{ X } 1{,}000 \text{ X } \boldsymbol{\varepsilon} \text{ X } \boldsymbol{\Phi} \text{ X } \mathbf{t}}$

Ab262 = Absorbance at 262 nm, before UV exposure

Aa262 = Absorbance at 262 nm, after UV exposure

ε = molar absorption coefficient of uridine at 254 nm (10,185 M^{-1} cm^{-1})

Φ = Quantum yield (0.02 mol/E)

t = Exposure time, seconds

U = UV energy, mJ/E (conversion factor)

APPENDIX C

Experimental Parameters for the Completion of Research Objectives

Table C.1. Experimental parameters for minimum ultraviolet dose determination for *Listeria monocytogenes* in distilled water, fresh brine, undiluted spent brine (20.86%NaCl), 5% spent brine 35% spent brine and 55% spent brine

Solution	Distilled Water	Fresh Brine	Spent Brine	5% Diluted Spent Brine (1.04% NaCl)	35% Diluted Spent Brine (5.22% NaCl)	55% Diluted Spent Brine (11.47% NaCl)
Stress	Healthy	Healthy	Healthy	Healthy	Healthy	Healthy
Time	0	0	0	0	0	0
	5	5	5	5	5	5
	10	10	10	10	10	10
	15	15	15	15	15	15
	20	20	20	20	20	20
	25	25	25	25	25	25
	30	30	30	30	30	30
Media	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX
Count	X 2	X 2	X 2	X 2	X 2	X 2
Rep	3	3	3	3	3	3

Table C.2. Experimental parameters for the effect of stress on minimum dose of ultraviolet light required to inactivate *Listeria monocytogenes* in distilled water and fresh brine

	Distilled Water				Fresh Brine			
Stress	H[a]	AS[b]	AR[c]	HS[d]	H	AS	AR	HS
Time	0	0	0	0	0	0	0	0
	5	5	5	5	5	5	5	5
	10	10	10	10	10	10	10	10
	15	15	15	15	15	15	15	15
	20	20	20	20	20	20	20	20
	25	25	25	25	25	25	25	25
	30	30	30	30	30	30	30	30
Media	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX	TSAYE MOX
Count	X 2	X 2	X 2	X 2	X 2	X 2	X 2	X 2
Rep	3	3	3	3	3	3	3	3

[a]Healthy
[b]Acid stressed
[c]Antibiotic resistant
[d]Heat shocked

Table C.3. Experimental parameters for the survivability of healthy, acid stressed, antibiotic resistant, and heat shocked *Listeria monocytogenes* in fresh and spent brine

Fresh Brine				Undiluted Spent Brine			
H[a]	AS[b]	AR[c]	HS[d]	H	AS	AR	HS
1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2
3	3	3	3	3	3	3	3
4	4	4	4	4	4	4	4
5	5	5	5	5	5	5	5
6	6	6	6	6	6	6	6
7	7	7	7	7	7	7	7
-	-	-	-	9	9	9	9
-	-	-	-	11	11	11	11
-	-	-	-	13	13	13	13
-	-	-	-	15	15	15	15
-	-	-	-	17	17	17	17
-	-	-	-	19	19	19	19
-	-	-	-	21	21	21	21
-	-	-	-	26	26	26	26
-	-	-	-	28	28	28	28
TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3	TSAYE MOX X 2 3

[a]Healthy
[b]Acid stressed
[c]Antibiotic resistant
[d]Heat shocked