MECHANISMS OF INACTIVATION AND MICROBIAL INACTIVATION KINETICS IN THE PULSED LIGHT TREATMENT OF FOODS

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Aaron Reid Uesugi

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Aaron Reid Uesugi, Ph. D.

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Pulsed Light technology utilizes high powered pulses of broad spectrum light to reduce microorganisms on food and food contact surfaces. While previous research has shown Pulsed Light to be effective at reducing various microorganisms on a variety of substrates, a better understanding of inactivation kinetics, pairing Pulsed Light with other antimicrobial treatments, and the cellular effect of Pulsed Light treatment will provide better insight into potential opportunities for future use of this technology. Inactivation of *Listeria innocua* in liquid suspensions and on the surface of stainless steel showed pronounced tailing. The utilization of the Weibull model accurately predicted inactivation in clear liquids but overestimated Pulsed Light's effectiveness on stainless steel surfaces where complex surface properties as well as inoculum size were of significant influence. The combination of Pulsed Light with the antimicrobial nisin was able to reach reductions of 4 to 5 log CFU/sausage of *Listeria* on the surface of sausages, extending storage from 8 day for individual treatments to 28 to 48 days suggesting that Pulsed Light in combination with additional antimicrobials can effectively reduce surface contamination of ready-to-eat foods. To evaluate the potential for repeated Pulsed Light exposure to lead to changes in growth and resistance behavior of survivors, *L. monocytogenes, L. innocua,* and *E. coli* were exposed to both low (1.1 J/cm^2) and high (10.1 J/cm^2) levels of Pulsed Light and

survivors were recovered and subsequently treated for 10 cycles of exposure and recovery. Isolates of all three organisms did not show changes in growth kinetics or resistance after multiple exposures when compared to untreated cells at either low or high fluence levels. Reduction levels of 3-4 and 5-6 log CFU/ml were obtained after exposure to 1.1 and 10.1 J/cm^2, respectively, for the untreated control and the repeatedly treated and recovered isolates. Whole genome microarray analysis showed increased transcription levels for stress related proteins, motility, and transcriptional regulators following both Pulsed Light and UV light. Removing the UV spectrum of Pulsed Light lead to no differences between treated and untreated samples and showed a downregulation in motility and cell membrane associated genes as well as no increases in stress response genes following exposure, suggesting that the mechanism of inactivation is related to the UV portion of Pulsed Light and the visible and NIR spectrum do not play a role in cell inactivation.

BIOGRAPHICAL SKETCH

Aaron Reid Uesugi was born on the side of the road on the way to the hospital in Pacifica, California. Apparently, bottoming out the car in the excitement of getting to the maternity ward is a very effective method of speeding through labor. He followed his older sister's footsteps as Valedictorian at Terra Nova High School and then onto the University of California, Davis. Being terribly bored by lifting a piece of metal to determine the angle at which the sand would start sliding, Aaron left Civil and Environmental Engineering to the applied world of Food Science. After making sauerkraut for class and consumption at a department party, he was hooked on Food Science. During his junior year, Aaron developed an interest in food microbiology, both by the positive and negative aspects of these microorganisms. After receiving his Bachelor of Science in Food Science and minor in Environmental Toxicology, Aaron spent a year working for the Department of Food Science at UC Davis as a teaching assistant for a variety of classes, food microbiology lab assistant, and a research assistant. In the fall of 2001, he joined the research laboratory of Dr. Linda J. Harris and earned his Master's of Science in Food Science at UC Davis in 2004. His thesis was titled "The growth and survival of *Salmonella enterica* serovar Enteritidis phage type 30 on almond hulls and shells." He stayed in the Harris lab as a Staff Researcher continuing to study almonds and *Salmonella* as well as working with watermelons, strawberries, and oranges. Still feeling that there were experiences in higher education not yet encountered, he made his way across the country to the snow and four seasons of Ithaca to the Department of Food Science at Cornell University where he joined the of research group of Dr. Carmen I. Moraru to begin his doctoral studies, working above the pilot plant and too close to the Dairy Bar and Dairy Store. Upon completing his dissertation, Aaron will finally leave the academic world and begin his career as a Scientist at Kraft Foods and return to the world of almonds and nuts.

To my family for their love, support, advice, and encouragement.

To Keriann, for being my present and future.

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CHAPTER 1

INTRODUCTION

Pulsed Light technology has been actively investigated in recent years as an alternative to thermal treatment for killing pathogenic and spoilage microorganisms in foods. This technology uses short, high power pulses of broad-spectrum light that have the capacity to inactivate bacteria, yeasts, molds, and viruses on the surfaces of foods, food contact materials and medical devices and in clear liquids. The US Federal and Drug Administration (FDA) has recognized the use of Pulsed Light for the decontamination of food or food contact surfaces using light pulses of wavelengths between 200-1000 nm, with a pulse width not exceeding 2 ms and a cumulative treatment of less than 12 J/cm^2 (FDA Code 21CFR179.41).

Generation of Pulsed Light

Pulsed Light is generated by converting electricity into light pulses of short duration with high peak energy. Pulsed Light equipment may vary from manufacturer to manufacturer, but all Pulsed Light systems consist of several common components (Figure 1.1). A flash lamp filled with an inert gas, typically Xenon, is the critical component of the system. A high voltage power supply provides electrical power to the storage capacitor, which stores electrical energy for the flash lamp. The pulse-forming network determines the pulse shape and spectrum characteristics. A trigger signal initiates discharging of the electrical energy to the flash lamp.

The Xenon gas discharge flash lamp then converts 45% to 50% of the input electrical energy to pulsed radiant energy (29). The power supply is standard line voltage AC power, such as 220V at 50Hz, which feeds into the control module where the line power is converted into high voltage DC power. The energy is stored in the capacitor for a relatively long period of time (milliseconds), and is then rapidly

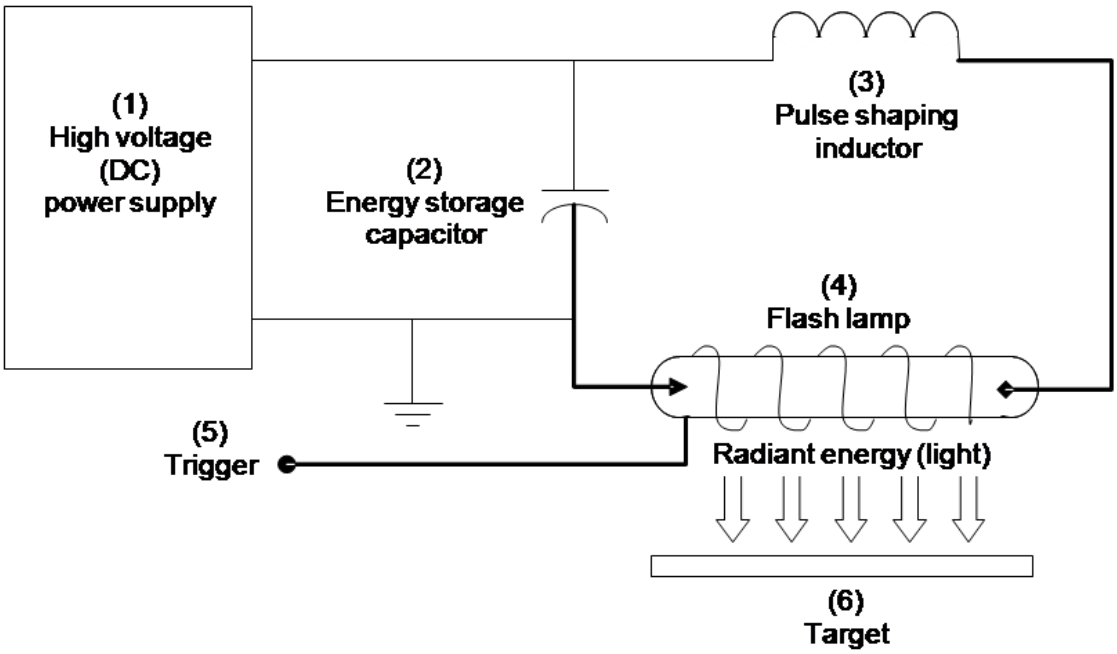

Figure 1.1. Pulsed Light schematic setup. (29).

released (microseconds) to the lamp causing the inert Xenon gas in the lamp to become excited and emit the short, intense light pulse which typically has a pulse width of a few hundred microseconds. The flash lamps used to generate the broad spectrum light are available in a variety of shapes, such as linear or circular, which allows them to uniformly treat substrates of different shapes and sizes. The lamps are typically enclosed in a lamp housing which often includes a quartz panel to protect the lamp. Pulsed Light units may also have a cooling system to reduce any heat buildup in the treatment area and reflectors may also be incorporated in the treatment chamber in order to redirect light to the sample. The flashes of light may be applied at a rate of 1 to 20 flashes per second for most applications.

The treatment dose is quantified as "fluence," which is the dose of total radiant energy of all wavelengths that reaches the substrate surface, and is expressed in Joules/cm^2. The Code of Federal Regulations (Title 21, Section 179.41) defines the conditions for the safe treatment of foods using Pulsed Light. These conditions include (1) The radiation sources consist of xenon flash lamps designed to emit broadband radiation consisting of wavelengths covering the range of 200 to 1,100 nm, and operated so that the pulse duration is no longer than 2 ms. (2) The treatment is used for surface microorganism control. (3) Foods treated with Pulsed Light shall receive the minimum treatment necessary to produce the intended effect. (4) The total cumulative treatment should not exceed 12.0 Joules/cm^2.

Pulsed Light systems can be designed for batch or continuous treatments. In batch configurations, such as those developed by the US Company Xenon Corporation (Waltham, MA), samples are placed within a chamber with lamps located along the ceiling of the chamber. The simplest designs include a single lamp located above the sample and an adjustable tray or a shelf to hold the samples. More complex designs may incorporate up to eight lamps within a chamber along with a quartz stand to hold

the sample and allow a 360° exposure and treatment. The French company Claranor has developed a wide range of Pulsed Light equipment for the food-processing and pharmaceutical industries: static equipment for laboratories and technical centers (*Tecum*), treatment units for unwrapped or packaged products on flat conveyor belts (*Gratia*), on spool-bars (*Plena*) or in tunnels (*Dominus*), as well as in-line treatment units for caps, pre-formed packaging, films, or jars (*Ventris*). In these systems, a flash lamp or a number of flash lamps are located above a moving conveyor belt. Other components of the equipment include a hood to contain the lamp, reflectors to redirect light to the sample, and a quartz panel that has the role to protect both the flash lamp and the workers from being exposed to the treatment. The same company also built a reactor for in-line treatment of clear liquids and water (*Maria*), in which liquids are moved through a cylindrical reactor that is equipped with a lamp that is placed in the center of the reactor. The system can be integrated into the manufacturing line as a single treatment reactor, or several reactors could be coupled in series in order to increase the Pulsed Light exposure and thus treatment efficiency (17).

In addition to its use to treat food, Pulsed Light equipment has been developed for other applications. Pulsed Light equipment could be installed within the heating, ventilating, and air conditioning air ducts to reduce airborne microorganisms – either for processing facilities or for offices or even residential buildings. These air purification systems can use Pulsed Light to reduce and destroy biological agents that may be introduced in a bio-terrorism attack upon a facility through the building's ventilation system. Other facilities that may require cleaned air include hospital operating and isolation rooms, clean rooms in manufacturing or pharmaceutical production, or for general air quality improvement. Pulsed Light treatment units have also been developed for the treatment of medical prosthetics and implants (such as the *Mulieribus* system from Claranor). Xenon Corporation is commercializing equipment

for Pulsed UV curing processes such as optical disc coatings, wood coatings, plastic bonding, or medical electrodes (17).

Antimicrobial effects of Pulsed Light

Pulsed Light sources generate light over a broad spectral range from 200 – 1100 nm, which includes light from the UV to the NIR range. The UV spectrum can be further subdivided into long wave UV (UVA) between 315 and 400 nm, medium wave UV (UVB) from 280 to 315 nm, and short wave UV (UVC) that is between 200 and 280 nm (14).

Due to the high UV content of Pulsed Light, it is generally agreed that the primary cellular target of the treatment is represented by nucleic acids, since it is known that DNA is the target molecule for UV wavelengths. UV radiation in the range of 250-260 nm, with a maximum at 254 nm, has been proven lethal to many microorganisms (Figure 1.2). This is due to the formation of pyrimidine (cytosine and thymine) dimers from adjacent bases (3) (Figure 1.3). These dimers typically form in the following preference: T-T, C-T, and C-C, since thymine has a greater absorbance than cytosine in the germicidal UV range, although in organisms with a high C-T ratio this preference may not be followed (7). The formation of these structures prevents the DNA from unzipping, therefore blocking replication. Without proper DNA repair, mutations, impaired replication and transcription and eventual cell death may occur. For the UVC treatment of bacterial spores, single and double strand breaks, pyrimidine dimers, and the "spore photoproduct" 5-thyminyl-5,6-dihydrothymine were reported (23). Long wave UV radiation (UVA and UVB) have a lesser impact upon living cells but still may cause lethal effects. The mode of action for these longer wavelengths is believed to be membrane damage or reactions with photosensitizing pigments (3). The low lethality of UVA against microorganisms

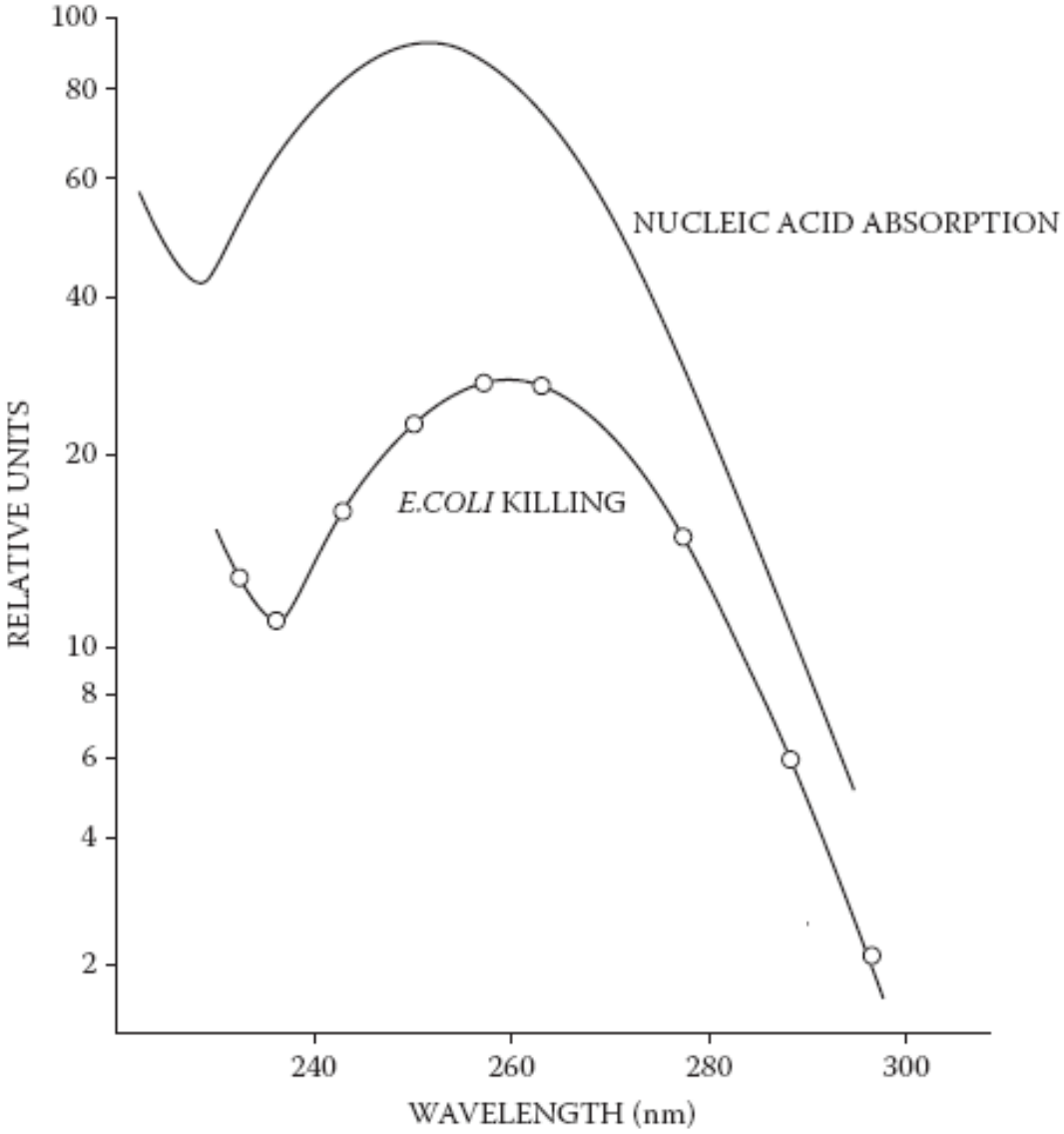

Figure 1.2. Similarity of the action spectrum for inactivation of *E. coli* cells to the absorption spectrum of nucleic acids (7).

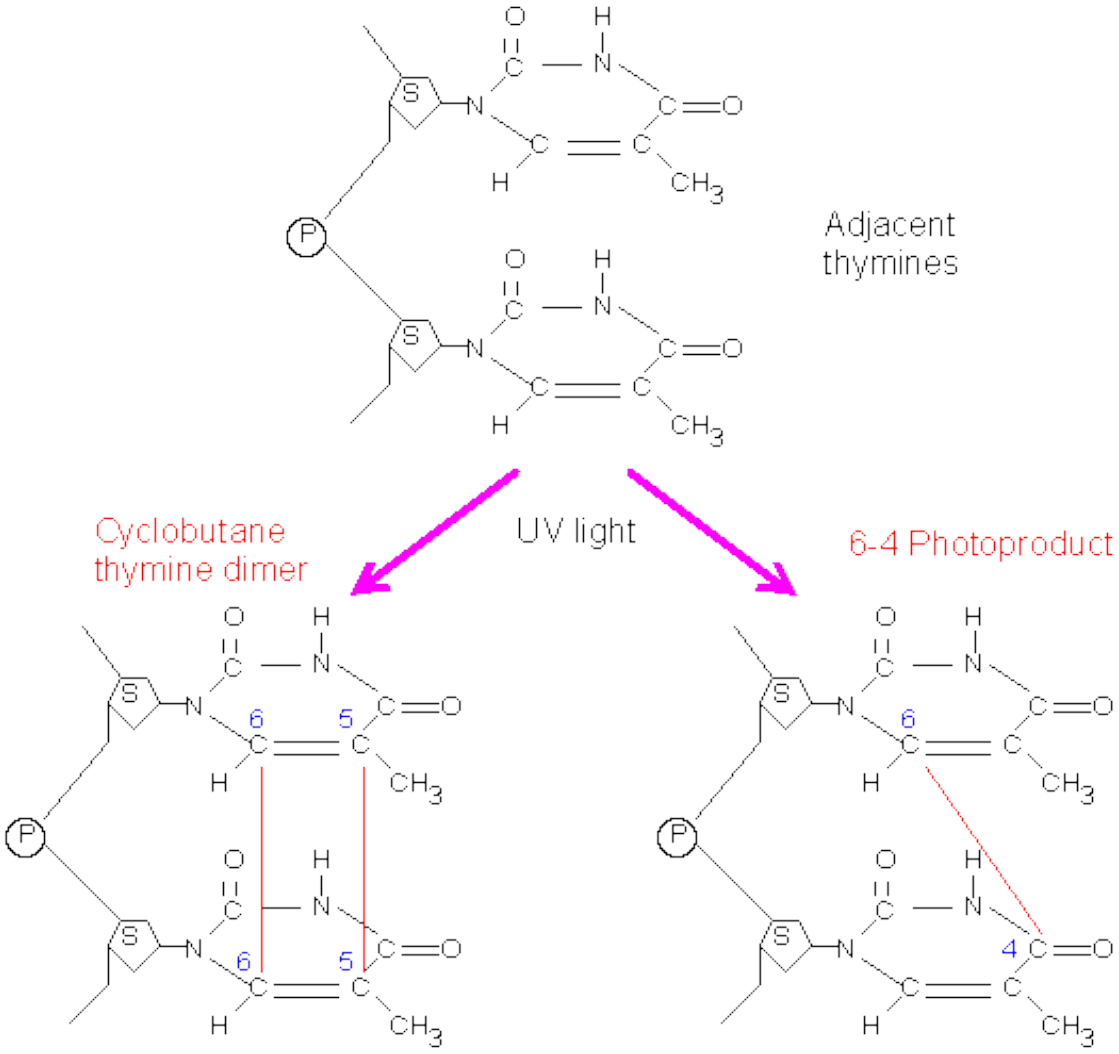

Figure 1.3. Pyrimidine dimer formation by UV light (13).

caused Bintsis (3) to note that there is little practical use in controlling microorganisms of this spectral range unless enhanced by some other means of control. It is worth noting that UVA and some visible light wavelengths (310 to 480nm) are involved in *photoenzymatic repair*. The repair occurs when the enzyme photolyase recognizes and binds to the pyrimidine dimers and then the absorption of light energy photolyzes the enzyme-dimer complex, releasing the enzyme and resulting in the conversion of the dimer into its repaired form (7) (Figure 1.4).

Research by Woodling and Moraru (28) demonstrated that the portions of the Pulsed Light spectrum responsible for bacterial inactivation were located in the spectral range $\lambda < 300$ nm (UVB and UVC), with some death taking place as a result to exposure to λ between 300 and 400 nm (UVA). No observable death was seen after treatment to $\lambda > 400$ nm (visible and NIR).

Repair of damaged sections of DNA can also occur without the requirement of light in a more complex process known as *dark repair*. This repair is also called *nucleotide excision repair* since the damaged portion of DNA is removed from the DNA and replaced. Nucleotide excision repair may occur in the presence or absence of light but the process is energy-dependent. In many prokaryotes, the complex UvrABC endonuclease is responsible for this repair. Two UvrA proteins bind with UvrB to form a trimer that detects DNA damage by identifying distortions and unzip the DNA (22). Cuts are made 4 nucleotides downstream and 7 nucleotides upstream of the DNA damage and the damaged oligonucleotides are removed by UvrD, a DNA helicase. The gap in the DNA is filled by DNA polymerase and sealed by DNA ligase. (Figure 1.5)

It has been previously hypothesized that the lethal action of Pulsed Light may also be due to photothermal effects, although not enough information exists to prove

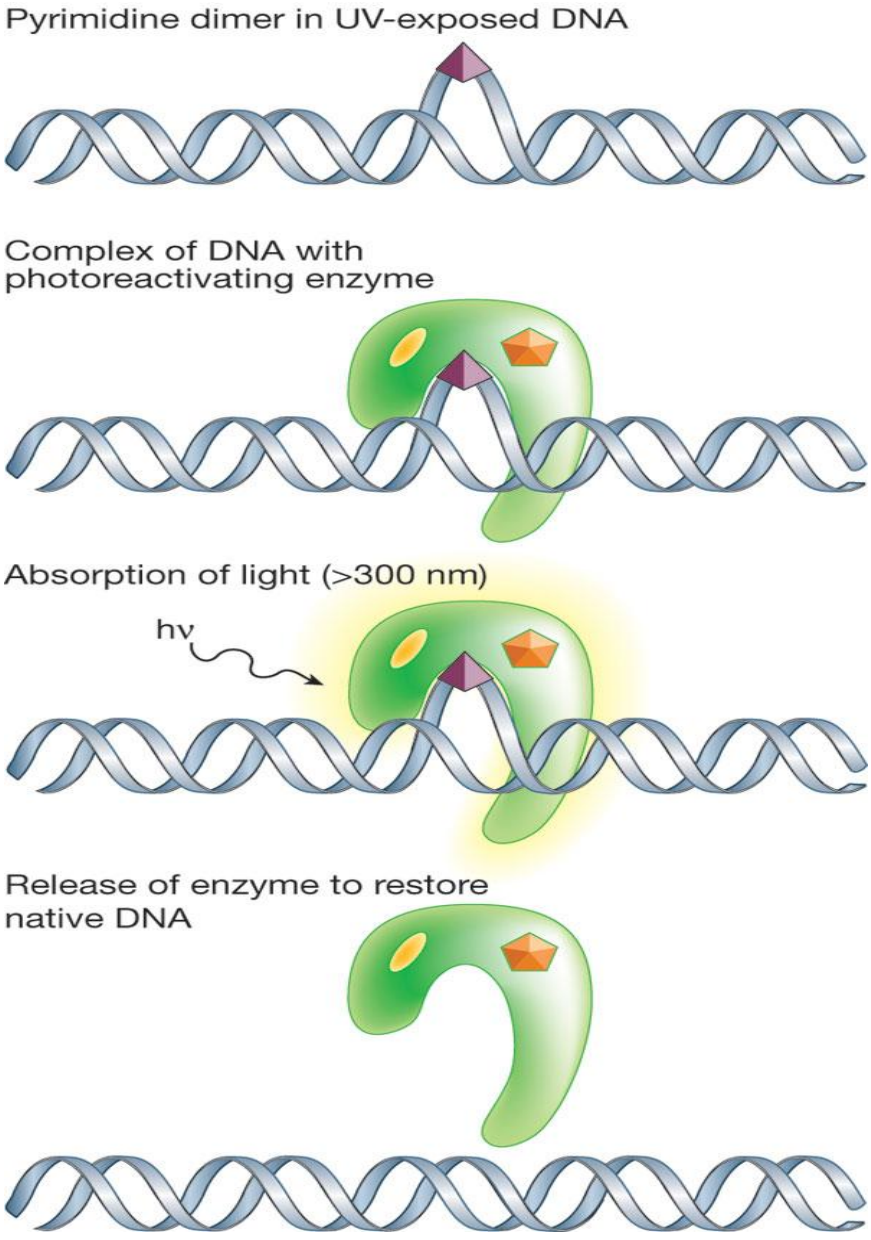

Figure 1.4. Photoreactivation of UV exposed DNA damage (5).

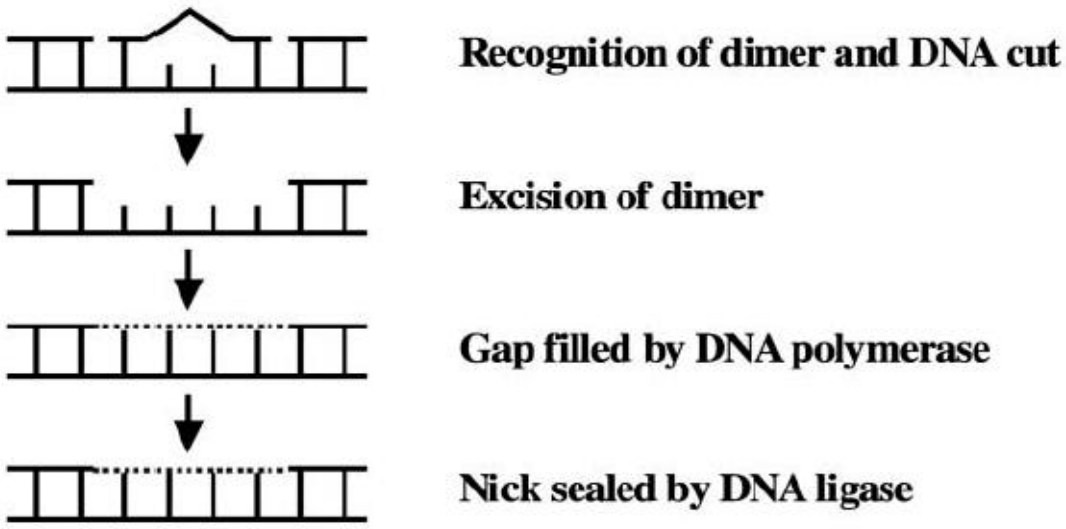

Figure 1.5. Nucleotide excision repair or "dark" repair (22)

this without a doubt. Several studies have observed effects on microbial cells after exposure to Pulsed Light. Wekhof (27) showed *Aspergillus niger* spores with ruptured tops, which were hypothesized to have resulted from the escaping of overheated spore contents. The ruptures left empty regions as a result of the "evacuation" of its contents. Wekhof based his theory on electron microscopy images of the treated spores, which brings the possibility of an artifact in sample preparation rather than an actual effect of Pulsed Light. The "overheating" effect reported by Wekhop has not been confirmed by other studies, but other physical effects on microbial cells have been reported elsewhere. Takeshita (25) studied the effect of Pulsed Light treatment on *Saccharomyces cerevisiae* and noted that in addition to single strand breaks in DNA and pyrimidine dimers, there was also increased protein elution and structural changes, enlarged vacuoles, cell membrane distortion, and change in circular shape of the cells. Anderson (1) found only minimal temperature increases, <1°C, when treating bacteria cells and fungal spores on agar plates. Pulsed Light thermal effects have seen an increase of 91°C on the muscle surface of salmon positioned 3 cm from the lamp and exposed for 60 s or 180 pulses (18). However, since the thermal effects were observed on the substrate level, they do not necessarily offer a prediction of what might be happening at a cellular level. Further investigations are therefore required in order to elucidate whether or not heating plays any role in microbial inactivation by Pulsed Light treatment.

Applications of Pulsed Light

Microbial inactivation in water and other liquids

The ability of Pulsed Light to reduce microbial counts in liquids depends on the distance of the source of light to the sample, the treatment dose, the turbidity and the thickness of the liquid layer. Huffman (9) examined the inactivation of bacteria,

viruses, and parasites suspended in water and treated with Pulsed Light at 0.25 J/cm^2 using a Pure Bright water treatment unit. For the bacteria *Klebsiella terrigena*, 2 pulses resulted in a reduction of >7.4 log CFU/ml. For viruses (Poliovirus type 1 and Rotavirus SA11), human viral surrogates (bacteriophages MS-2 and PRD-1) and a parasite (*Cryptosporidium parvum*), 2 pulses achieved an inactivation of >4 log reduction.

Bacteria and yeasts suspensions in buffers can also be greatly reduced by Pulsed Light. Takeshita (25) noted a decrease of 5.8 log CFU/ml of *Saccharomyces cerevisiae* when cells were suspended in potassium phosphate buffer in a 110 mm diameter watch glass after treatment with a total of 3.5 J/cm^2 using a flash lamp and reflector positioned above the sample. The SteriPulse-XL 3000 benchtop unit (Xenon Corporation) with a pulse width of 360 µs, 3,800V input power, and a pulse rate of 3 flashes per second was used in a large number of Pulsed Light inactivation studies. Krishnamurthy (12) suspended *Staphylococcus aureus* in phosphate buffer and treated the suspension with Pulsed Light for 5 s, at a distance of 80 mm from the lamp. They reported an inactivation level of 7.5 log CFU/ml. *E. coli* suspended in apple cider and apple juice was reduced by 5.5 and over 7 log CFU/ml, respectively, when samples were subjected to agitation and treated with Pulsed Light at fluence levels below 12 J/cm^2 (20). Milk samples treated with 25.1 J/cm^2 of pulsed UV light from a UV laser showed reductions of >2 log CFU/ml of *Serratia marcescens* (24). Further plating of treated milk samples did not show signs of growth after 21 days of storage.

Microbial inactivation on the surface of foods

A few studies have looked at the potential of Pulsed Light to inactivate microorganisms on the surface of meat products. Ozer and Demirci (19) examined the reduction of *L. monocytogenes* and *E. coli* O157:H7 on the muscle and skin side of raw salmon fillets using a SteriPulse-XL 3000. For *E. coli* O157:H7 a reduction of

0.30 and 1.09 log CFU/g was seen on the muscle and skin side, respectively when treated as a distance of 80 mm with 180 pulses. For *L. monocytogenes* on the salmon muscle and skin, reductions of 0.74 and 1.02 log CFU/g, respectively, were reached after 180 pulses at a distance of 80 mm. Surface temperatures increased after 60 s of Pulsed Light treatment from a distance of 80 mm by 28°C and 51 °C on the muscle and skin side, respectively. For treatments of less than 5 s, the muscle and skin surface temperatures did not increase. These differences in temperature changes are due to the differences in color between the darker skin side and the lighter muscle side.

A variety of minimally processed vegetables were examined to see the effect of Pulsed Light on mesophilic aerobic counts. Gomez-Lopez (6) examined celeriac, green bell peppers, iceberg lettuce, radicchio, soybean sprouts, spinach, and white cabbage treated with up to 2,700 pulses with a Xenon flash lamp with a pulse duration of 30 μs and an intensity of 7 J, at a distance of 128 mm from the lamp, with samples spread over a sterile 14×21 cm tray. Microbial reductions ranged from 0.56-log to 2.04-log for the variety of produce examined. The tested vegetables were naturally contaminated and not inoculated with a known microorganism, so the variability between the samples was hypothesized by the author to be due to different resistances of the natural microbial population, the location of the microorganisms, shadowing effects, or protective substances found in the vegetables. The processing (shredding, grating, chopped, or whole), shape or sample size did not produce any observable patterns in reduction.

Carrot slices inoculated with *S. cerevisiae* were exposed to Pulsed Light from two flash lamps located above and below the sample which emitted a total energy flux of 0.7 J/cm^2 (11). After the first 2 pulses, with each pulse delivering 0.7 J/cm^2, there was a reduction of 3.07 log CFU/g. After exposure to 24 pulses, a reduction on 4.93

log CFU/g was reached. The authors hypothesized that in combination with washing, Pulsed Light may reduce the level of yeast on sliced carrots by up to 6-log cycles. Strawberries inoculated with *Botrytis cinerea* and treated with Pulsed Light from a Xenon lamp with a pulse duration of 30 µs and an intensity of 7 J within a treatment chamber with reflective inner walls to enhance distribution of the light, showed no effect for surface decontamination to reduce storage rot (16). Additionally, induced resistances to fungal infections were not observed after Pulsed Light treatments. Previous studies had shown that similar treatment of *B. cinerea* conidia in phosphate buffer had resulted in a reduction of 3-4 log units (15), but the *in vitro* reductions did not occur on the strawberry fruit surface.

Alfalfa seeds inoculated with 5 log CFU/g of *E. coli* O157:H7 were reduced log 4.89 at a distance of 80 mm and 270 pulses from a SteriPulse-XL 3000 lamp (21). The same treatment performed at a distance of 130 mm resulted in a log reduction of 1.42. The distance from the lamp, 30 to 130 mm, did not significantly change the germination percentage of the seeds but did create an *E. coli* O157:H7 reduction range from 0.07-log to 4.89-log. Bialka and Demirci (2) noted a reduction of 1.1 and 4.3 log CFU/g of *E. coli* O157:H7 with 1.9 and 22.6 J/cm^2, respectively, on blueberries treated with a SteriPulse-XL 3000 lamp at a distance of 80 mm from the lamp. No significant differences were seen at other fluence doses between 1.9 and 22.6 J/cm^2. Treatments at a distance of 30 mm resulted in slightly greater reductions but resulted in damaged fruit. For treatments of *Salmonella* on blueberries, the differences in fluence did not create significantly greater reductions at 30 and 80 mm from the lamp. At 130 mm treatment distance however, the reduction of *Salmonella* increased significantly with fluence.

Fine and Gervais (4) achieved log reductions of 2.93 and 0.7 for Pulsed Light treated black pepper and wheat flour inoculated with *S. cerevisiae*. The difference in

inactivation between the two samples, both exposed to 31.12 J/cm^2 at a distance of 20 mm from the lamp, was assumed to be due to the difference in color between the black pepper and the wheat flour. A fluidized bed treatment unit was used to both mix samples and increase exposure. *Aspergillus niger* spores were greatly inactivated in corn meal (10). A log reduction of 4.95, when starting with an initial inoculum of 5 log CFU/g on the corn meal, was reached after 100 s of treatment. The reduction changed to 3.26 and 2.95 log CFU/g when the distance from the lamp was 30 and 130 mm, respectively. Clover honey was inoculated with *Clostridium sporogenes* spores to a level of 6.25 log/g (8). Spore reductions ranged from 0.0 - 5.65 log/g when treated with Pulsed Light from a SteriPulse-XL 3000 lamp at different honey depths, treatment times, or distances from the lamp. When the three parameters were modified, no combination could completely reduce all the spores present in the honey.

Pulsed Light treatment of packaging materials

Pulsed Light can be used to inactivate microorganisms on the surface of food packaging materials, and potentially on the surface of products packaged in "UV transparent" materials. The use of Pulsed Light could lead to a reduction in the need for preservatives or chemical sterilizing agents such as hydrogen peroxide, propylene oxide, or peracetic acid, which may leave a residue or require time to reduce to an acceptable level. When mold spores of *Cladosporium harbarum* were placed onto paper coated with polyethylene, a 2.7 log reduction was noted after 30 pulses at a distance of 70 mm from the lamp (26). One aspect that needs to be accounted for when using Pulsed Light to treat packaged products is that the treatment is limited to the surface of the product and is restricted by the ability of light to penetrate opaque and irregular surfaces. Additionally, undesirable chemical effects may be encountered. UV-treated low density polyethylene and polyethylene terephthalate have shown increased levels of surface oxidation products when compared to

untreated samples, which indicated that UV may accelerate the oxidation of the plastic film surfaces (18). Such phenomena could also occur in Pulsed Light treatments, and therefore any successful microbial inactivation study should be followed by an investigation of the potential side effects of the treatment.

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CHAPTER 2

RESEARCH OBJECTIVES

In recent years, several studies have examined the use of Pulsed Light to reduce the vegetative cells in various substrates. However, the kinetics of inactivation using Pulsed Light has not been quantitatively evaluated. Additionally, while Pulsed Light has been examined for its individual efficacy, the potential to increase its effectiveness when used in combination with antimicrobials has not been explored. There is also a lack of research data regarding the mechanisms of inactivation in Pulsed Light treatment. While the UV portion of Pulsed Light is believed to be primarily responsible for the inactivation of microorganisms, the effects of Pulsed Light on microbial cells and the cellular damage induced by Pulsed Light have not been systematically studied.

A better understanding of Pulsed Light and its strengths and weaknesses will help researchers and the food industry to evaluate future uses of this technology. In order to achieve an understanding of the aspects mentioned above, this thesis addresses the following research objectives

1. Evaluate the inactivation of *Listeria* in liquid and on the surface of solid substrates and develop models for the inactivation kinetics by Pulsed Light treatment.
2. Investigate the efficacy of using Pulsed Light treatment as part of a combination/hurdle treatment. Specifically, the combination of Pulsed Light with the antimicrobial nisin was studied.

3. Investigate the effects of Pulsed Light treatment on *L. monocytogenes* at the cellular level.

    3.1. Evaluate the potential increase in resistance to Pulsed Light of *L. monocytogenes* cells after repeated exposure to Pulsed Light.

    3.2. Examine the transcriptional gene response of *L. monocytogenes* to Pulsed Light exposure as compared to continuous UV treatments.

CHAPTER 3

INACTIVATION KINETICS AND FACTORS OF VARIABILITY IN THE PULSE LIGHT TREATMENT OF *LISTERIA INNOCUA* CELLS

ABSTRACT

Pulsed Light treatment can effectively reduce microbial populations in clear substrates and on surfaces, but its effectiveness varies as a function of substrate or treatment related factors. For Pulsed Light to be successfully adopted by the food industry, all factors of influence, as well as the inactivation kinetics for the microorganisms of concern must be elucidated. In this study, the inactivation kinetics of *Listeria innocua* and the effect of inoculum size on Pulsed Light inactivation were investigated. Stainless steel coupons (50.8 × 101.6 mm) of defined surface properties and transparent glass chamber slides (25.4 × 50.8 mm) were each inoculated with 1 ml of aqueous suspensions of *L. innocua* containing inoculum populations of up to 10^9 CFU. The thickness of the liquid layer in the glass slide was 1.16 mm. The inoculated substrates were exposed to Pulsed Light treatments of up to 17 J/cm^2 in a static Pulsed Light chamber equipped with a pulsed Xenon lamp. Survivors were recovered and enumerated by both standard plate counting and most-probable-number procedures. The data indicated that in clear liquids, Pulsed Light resulted in more than a 6-log reduction of *L. innocua* after a 12-J/cm^2 treatment, regardless of the initial inoculum size. For the stainless steel surfaces, less than a 4-log reduction after a 12-J/cm^2 treatment and a noticeable effect of substrate characteristics and inoculum size on inactivation were observed. The survivor curves showed pronounced tailing for all substrates used in the study. The Weibull model accurately predicted the survivor ratios for Pulsed Light treatment of *L. innocua* in clear liquids, with a shape and scale parameter of 0.33 and 3.01, respectively. The Weibull model resulted in significant

overestimation of Pulsed Light effectiveness for the stainless steel substrates, where the influence of various substrate properties and inoculum level on inactivation was significant.

INTRODUCTION

Pulsed Light is a U.S. Food and Drug Administration recognized technology capable of inactivating vegetative bacteria, spores, yeast, and molds on food surfaces, food contact surfaces, and medical devices (1–5, 8, 15, 16). In Pulsed Light treatments, a Xenon gas discharge lamp is used to generate light with wavelengths similar to sunlight, ranging from UV to near infrared, but of an intensity that is 20,000 to 100,000 times greater than the intensity of sunlight at sea level (20). The intensity of Pulsed Light treatment, called fluence, is measured in joules per square centimeter (J/cm^2).

It is generally accepted that the lethal effect of UV is the main cause responsible for the microbial inactivation effect (10, 19). The effectiveness of Pulsed Light is influenced by a range of substrate and treatment-related factors. Woodling and Moraru (19) showed that the levels of reduction achieved for *Listeria innocua* on stainless steel were affected by surface reflectivity, roughness, and hydrophobicity. The same study suggested that inoculum size also affects the level of Pulsed Light inactivation. This finding warranted a systematic investigation of the effect of inoculum size on inactivation, because this information is critical to the correct application of Pulsed Light technology for the inactivation of microorganisms in foods, which are characterized by variable levels of contamination.

Also important for designing Pulsed Light treatments of desired levels of microbial kill is the quantitative characterization of inactivation kinetics, which would allow correct process calculations to be made. First-order kinetics is widely accepted

for microbial inactivation processes, particularly heat treatment, and it has the advantage of defining and using simple kinetic parameters such as the *D*-values (decimal reduction times) and *z*-values (changes in temperature required to change the *D*-values) (13). However, such an approach is based on log-linear survivor curves, whereas in reality, concavity as well as shoulders and tails in survivor curves have often been reported. In Pulsed Light treatment, strong tailing effects have been previously reported by Woodling and Moraru (19) for the Pulsed Light inactivation of *L. innocua*, which deems the use of first-order kinetics approach unsuitable for this application.

Nonlinear kinetic models have been increasingly used in recent years to describe nonlinear microbial inactivation in food systems, both in heat treatments and non–heatbased treatments (9, 17). One of the most popular nonlinear models is the Weibull model, a nonmechanistic model that uses a power function to describe the variation of survivor ratio as a function of treatment intensity. For microbial inactivation, this function takes the following form:

$$\text{Log}(S) = \alpha\, t^{\beta}$$

where $S = (N/N_0)$ is the ratio of survivors after treatment over the initial number of organisms, α is the scale parameter, β is the shape factor that describes the shape of the survivor curve, and t is the treatment intensity (9, 17). When $\beta > 1$, a concave down curve is described, whereas a shape parameter $\beta < 1$ indicates a concave up curve. If $\beta = 1$, the survivor curve will assume a linear form. Because of its versatility, the Weibull model is potentially a useful tool for estimating the effectiveness of Pulsed Light treatment. The objectives of this study were to further elucidate the factors that affect Pulsed Light inactivation, specifically the inoculum size, and to explore the suitability of the Weibull model to quantitatively characterize Pulsed Light inactivation kinetics for a range of substrate characteristics and inoculum sizes.

This will facilitate a better understanding of Pulsed Light inactivation, which is expected to lead to a better appreciation of this technology by its potential users.

MATERIALS AND METHODS

Pulsed Light treatment

Pulsed Light treatments were performed by an RS-3000C SteriPulse System (Xenon Corporation, Woburn, Wash.). The system consists of a controller unit and a treatment chamber that houses a Xenon flash lamp. Each inoculated substrate (dried coupon or liquid-containing glass chamber) was centered individually on an adjustable stainless steel shelf in the Pulsed Light unit, at approximately 50.8 mm beneath the Xenon lamp, and treated with up to 15 pulses at a frequency of 3 pulses per s and a pulse width of 360 µs. All experiments were performed at least in triplicate.

Pulsed Light intensity measurements

Fluence measurements were taken by a pyroelectric head (PE25BBH) with a Nova II display (Ophir Optronics Inc., Wilmington, Mass.), with an aperture cover having a circular opening of 1 cm^2 and a pulse width setting of the meter of 1.0 ms. The pyroelectric head was placed approximately 50.8 mm from the lamp source. Pauses of at least 30 s between measurements were allowed in order to prevent overheating of the pyroelectric head. Fluence measurements were performed in triplicate.

Culture and inoculum preparation

A culture of *L. innocua* FSL C2-008 (environmental isolate from a smoked fish plant) was obtained from the frozen culture collection maintained by the Food Microbiology and Safety Laboratory in the Food Science Department at Cornell University (Ithaca, N.Y.). A fresh culture was obtained every 6 months. This culture was used to maintain a culture on tryptic soy agar slants (TSA; Difco, Becton

Dickinson, Sparks, Md.). Prior to the Pulsed Light treatments, the culture was streaked onto TSA and incubated for 24 ± 2 h at 35 ± 2°C. A single isolated colony was transferred into tryptic soy broth (TSB; Difco, Becton Dickinson) and incubated for 24 ± 2 h at 35 ± 2°C. A subsequent loop transfer into TSB and incubation for 24 ± 2 h at 35 ± 2°C was performed, typically resulting in populations in the range of 10^8 to 10^9 CFU/ml. If the culture grown from the slant appeared to deviate from previous streakings, a fresh culture was taken from the frozen stock. All Pulsed Light treatments were performed on *L. innocua* in stationary growth stage.

To study the effect of inoculum size on Pulsed Light effectiveness, a range of inoculum sizes spanning about 4 log cycles was used. Various inoculum populations were prepared by diluting the initial inoculum with Butterfield's phosphate buffer. For the solid substrates, maximum inoculum levels of about 9 log CFU/ml and minimum inoculum levels of about 5 log CFU/ml were used, while for the liquid substrates, maximum inoculum levels of about 7 log CFU/ml and minimum inoculum levels of about 3 log CFU/ ml were used. The maximum and minimum levels were dictated by the maximum concentration of cells that could be achieved during the need to dilute some of the inocula and the minimum level that would result in countable results after the Pulsed Light treatment. For the solid substrates, some of the inoculum levels were very close to 8 log CFU/ml, because the initial studies aimed at achieving a constant inoculum level of 8 log CFU/ml. Despite some of the inoculum levels being very close to each other, each data set was still presented separately because of the observed effect of inoculum level on Pulsed Light inactivation for the solid substrates.

Substrate preparation

The Pulsed Light treatments were performed on two types of model systems: stainless steel surfaces (solid substrate) and clear liquid broth (liquid substrate). To

simulate stainless steel surfaces that may be encountered in a food-processing environment, food-grade stainless steel coupons with different types of factory-controlled surface finishes were obtained from Pacific Sensor (Fountain Valley, Calif.). All coupons were rectangular, measuring 50.8 by 101.6 mm (2 by 4 in.). The following types of surfaces were used: electropolished (the smoothest), mill finish (control), and aluminum oxide treated (the roughest). Prior to the treatments, all coupons were washed for 30 min in an FS30H ultrasonic cleaner (Fisher Scientific, Pittsburgh, Pa.), which contained a 30:1 dilution of Fisherbrand Versa-Clean (Fisher) in water, individually rinsed three times in distilled water, air dried under a laminar hood, and then autoclaved at 121°C for 1h. For the liquid suspension experiments, sterile and transparent Lab-Tek II Chamber Slide 1 well glass slides (Nagle Nunc International, Naperville, Ill.) with chamber dimensions of 25.4 by 50.8 by 10 mm (width by length by height) were used to hold the liquid cell suspensions during the Pulsed Light treatment.

Substrate inoculation

The stainless steel coupons were placed under a laminar flow hood on ethanol-sanitized aluminum foil prior to being inoculated. Inoculation was done immediately by placing 1 ml of the appropriate inoculum in the center of the coupon. The inoculated coupons were then dried at room temperature (23 ± 2°C) under the laminar flow hood for about 90 min, which allowed the bacterial cells to adhere to the metal surfaces. The drying step was carried out to simulate a worst-case scenario of surface bacterial contamination, in which the cells are in direct contact with the solid surface and thus are more difficult to kill. The time required for the complete air drying of the aqueous inoculum was established experimentally. For the liquid suspension treatments, 1 ml of the inoculum was placed in the chamber of the Lab-Tek II Chamber Slide immediately before treatment. The height of the liquid inoculum in the

glass chambers was 1.16 mm.

Recovery and enumeration of survivors

It was previously determined that a special recovery and resuscitation procedure is necessary before determining the survivors of Pulsed Light treatment (18). The resuscitation procedure developed by Woodling and Moraru (18) was used, which consisted of placing each treated coupon in a 532-ml Whirl-Pak bag (Nasco, Inc., Modesto, Calif.) containing 100 ml of TSB and then hand massaging the coupon through the bag for 2 min. Massaging entailed rubbing the surface of the coupon for 45 s, shaking the bag vigorously for 15 s, and then repeating the procedure. The recovery broth was transferred to a new Whirl-Pak bag and incubated for 3 h at 30°C to allow any sublethally damaged cells to resuscitate. When developing the resuscitation procedure, cell growth was carefully monitored by plating and counting to make sure that resuscitation and not cell growth took place (18). Following the resuscitation step, the liquid was serially diluted in Butterfield phosphate buffer, after which 100 µl was spread plated onto TSA and incubated for 48 ± 2 h at 35 ± 2°C, and survivors were determined by standard plate counting (SPC).

For the Pulsed Light treated liquid suspensions, the treated inoculum (1 ml) was transferred to 7 ml of TSB, and the chamber was rinsed twice with 1 ml of TSB, adding the rinsed TSB to the treated inoculum. The recovery liquid (10 ml) was held for 3 h at 30°C to allow the resuscitation of sublethally injured cells, as discussed above. Following the resuscitation step, the liquid was serially diluted, plated, and incubated as stated above, and survivors were counted.

For those treatments characterized by low survivor counts, SPC and the most-probable-number (MPN) technique were used in parallel. When plate counts fell below the limit of detection by SPC, estimation of survivors was done by the MPN procedure. According to previous research by Woodling and Moraru (19),

SPC and MPN evaluations of Pulsed Light survivors yielded statistically similar results, which justify the use of both of these two evaluation techniques in the present study.

For the MPN method, the procedure reported by Swanson et al. (14) was used. Aliquots of the recovery broth were transferred into 100 ml of TSB in the following distribution: three with 10 ml, three with 1 ml, and three with 0.1 ml. The TSB was then incubated at 35 ± 2°C for 48 ± 2 h. Turbidity was used to presumptively identify positive samples, and any atypical positives were further confirmed by streaking on TSA, incubating at 37°C for 48 h, and identifying typical *L. innocua* colonies by their bluish gray color (11).

Evaluation of Pulsed Light efficiency

The initial counts (N_0) and survivor counts (N) were expressed in log CFU/ml. The level of microbial reduction was expressed as Log (N/N_0). An earlier study (18) indicated that in surface treatments, not all the cells that were inoculated on the metallic surfaces could be recovered in the TSB, presumably because of both a slight attachment of the cells onto the metallic surfaces and cell death during the drying step. These losses were found to be smaller than 1 log CFU, with standard deviations below 0.1 log CFU, as communicated by Woodling and Moraru (18). In the present study, the reported microbial reduction by Pulsed Light treatment did not take into account the recovery losses that occurred for the solid surfaces, which may have resulted in a slight overestimation of Pulsed Light effectiveness for those cases.

Survivor curves were built by creating log-linear plots of survivors counts (N) versus treatment intensity (fluence).

Statistical analysis

The parametric fit survival function by the Weibull distribution in the statistical package JMP 5.1 (SAS Institute, Cary, N.C.) was used to estimate the scale

(α) and shape (β) parameters. The parameters α and β were then submitted back into the Weibull function to generate predicted survivors as a function of Pulsed Light exposure. Analysis of variance and Tukey's honestly significant differences statistical tests were used to determine significant differences between treatments at $P < 0.05$.

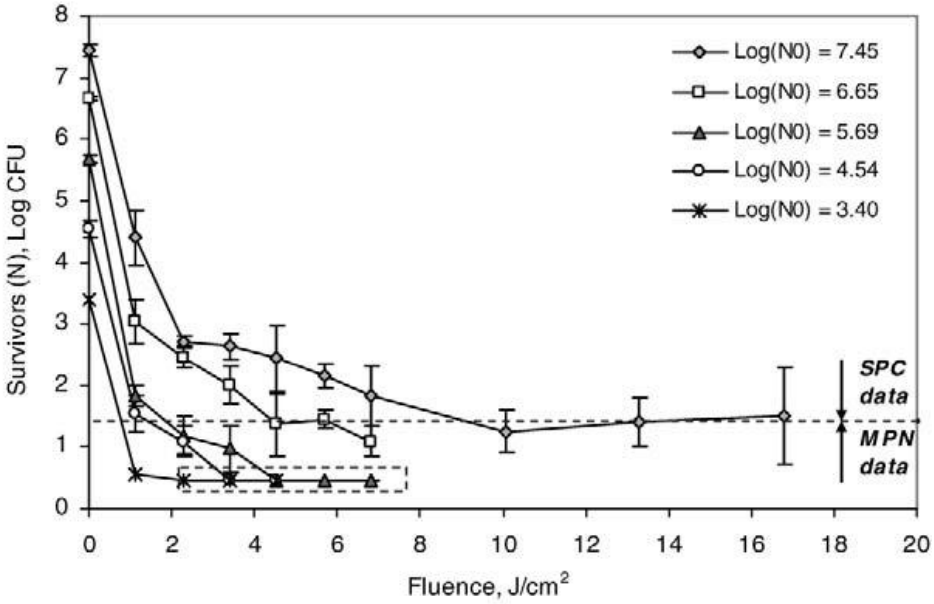
RESULTS AND DISCUSSION

Pulsed Light inactivation of *L. innocua* in a clear liquid substrate

Survivor and inactivation curves obtained by treating transparent liquid suspensions of *L. innocua* cells with inoculum levels (N_0) of 3.40 to 7.45 log CFU/ml with up to 12 pulses of light (13.30 J/cm^2) are presented in Figure 3.1A and 3.1B, respectively. Most data points in Figure 3.1A represent MPN estimates, because for many treatments, the SPC method yielded survivor counts (N) below 25 CFU per plate. For those treatments in which both SPC and MPN were used, survivor counts obtained with the two methods were practically identical, which supports the earlier observation that MPN and SPC methods can both be used for determining the survivors of Pulsed Light treatment (19). The data points inside the dotted rectangle in Figure 3.1A represent survivor levels that were at the sensitivity limit of the MPN method (survivor counts, <0.47 log CFU) and therefore were not used further for calculating inactivation levels.

Maximum inactivation levels of nearly 7 log were obtained when treating the clear suspension of cells with more than 9 J/cm^2, equivalent to a 3s treatment, which demonstrates the tremendous potential of Pulsed Light technology for microbial inactivation. All survivor curves in Figure 3.1A showed pronounced tailing, similar to the survivor curves obtained when treating *L. innocua* on solid substrates both in previous studies (18, 19) and in this study (see Figure 3.2A).

A)



B)

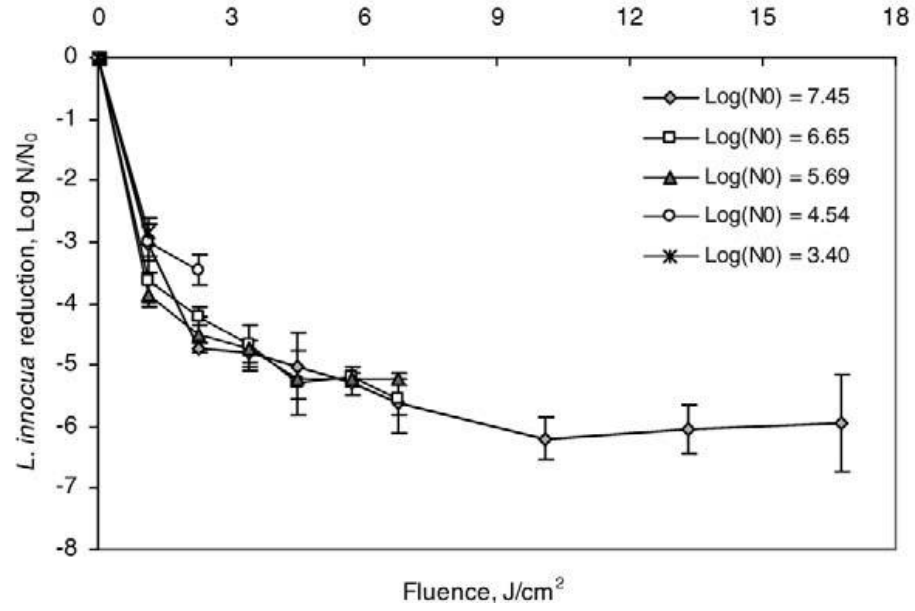
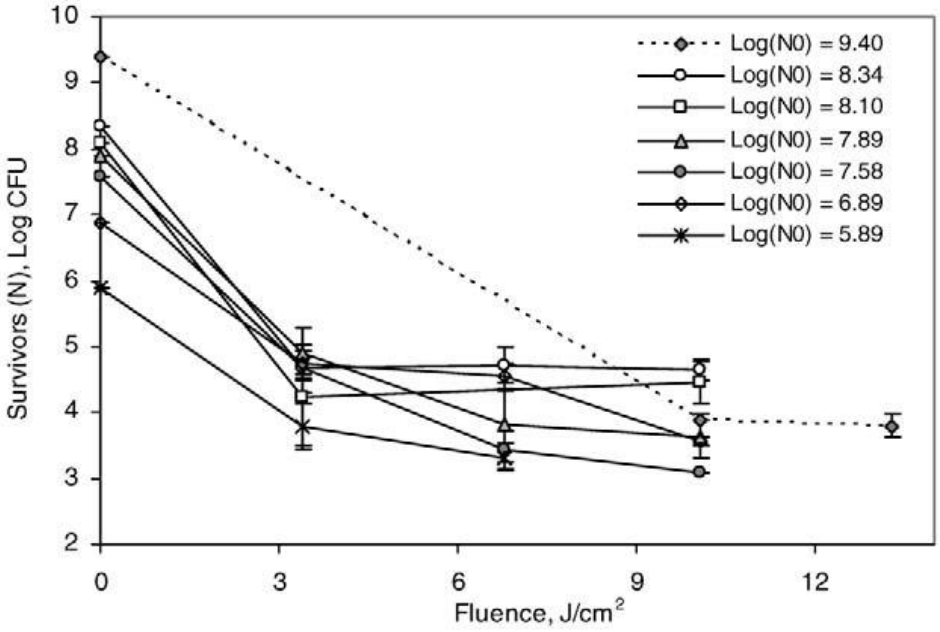


Figure 3.1. Survivor curves (A) and inactivation curves (B) for *L. innocua* treated with Pulsed Light in a clear buffer or broth solution at a distance of 50.8 mm from the Xenon lamp. Notes: (i) The dotted line (A) indicates the limit of the SPC data (N = 25 CFU) and (ii) the data points in the dotted rectangle (A) represent survivor counts < 0.47 log CFU (MPN estimates). The equivalent points are missing from (B), because accurate inactivation levels could not be calculated for these treatments.

A)



B)

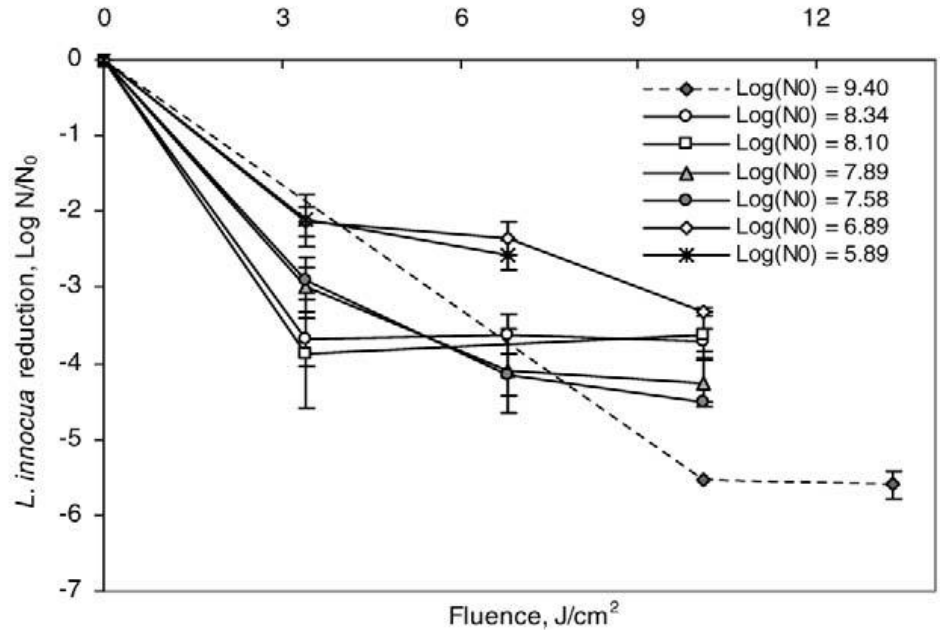


Figure 3.2. Survivor curves (A) and inactivation curves (B) for *L. innocua* treated with Pulsed Light on a mill finish stainless steel substrate at a distance of 50.8 mm from the Xenon lamp. Note: The dotted lines indicate incomplete survivor and inactivation curves for the highest inoculum level, for which only three data points were available.

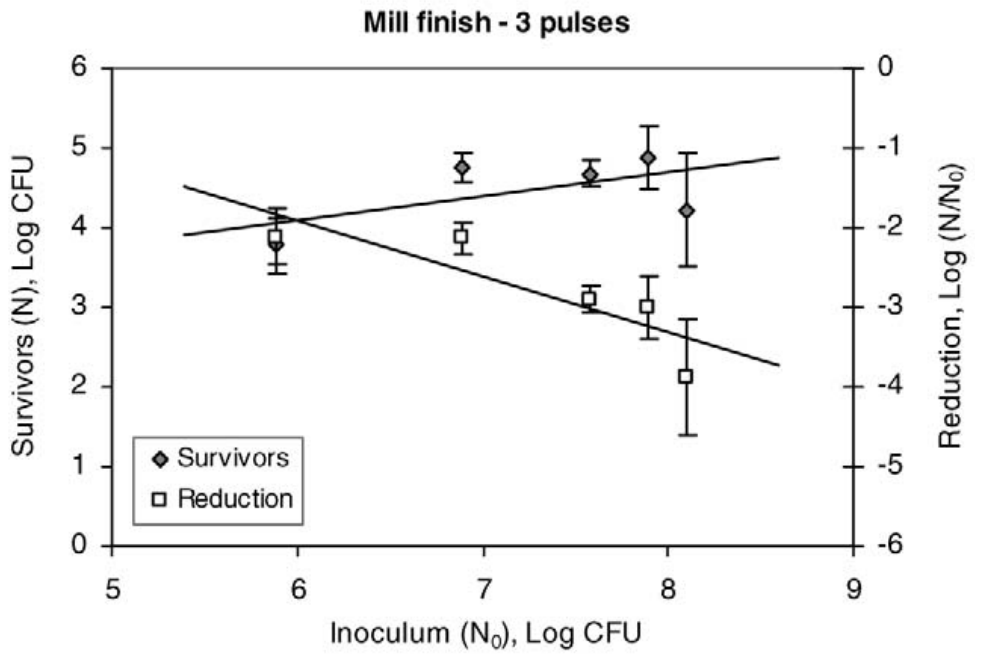
This shows that tailing is a characteristic of this microorganism's response to Pulsed Light treatment rather than a substrate- related effect.

As seen in Figure 3.1B, for the liquid substrate, the level of inactivation was not affected by the initial concentration of cells, with data points from all inoculum levels aligning closely along the same inactivation curve. This means that, for clear liquid substrates treated with a given treatment intensity (fluence), Pulsed Light is capable to deliver the same level of microbial reduction regardless of the level of contamination, which is extremely useful for the practical application of this technology.

For the Pulsed Light treatment performed on stainless steel coupons, survivor curves had profiles similar to those obtained for the liquid substrate, with the difference being that the plateau was established faster (at a lower fluence) and at higher survivor levels (Figure 3.2A). As seen in Figure 3.2B, inactivation levels for the mill finish did vary considerably between the data sets, with notably higher inactivation obtained when higher inoculum levels were used, which is significantly different from the liquid treatments.

To understand the reasons for this behavior, a detailed analysis of the inactivation data at the same fluence level was performed for the solid substrates. Figure 3.3 shows plots of survivors and inactivation levels as a function of initial inoculum size for the mill finish: Figure 3.3A at 3.40 J/cm^2 (3 pulses) and Figure 3.3B in the plateau region of the inactivation curve, which occurred at a fluence > 6.79 J/cm^2 (6 pulses). As observed in Figure 3.3, the level of microbial inactivation (reduction) was higher at higher inoculum sizes, whereas the survivor levels were relatively steady across the inoculum levels tested.

A)



B)

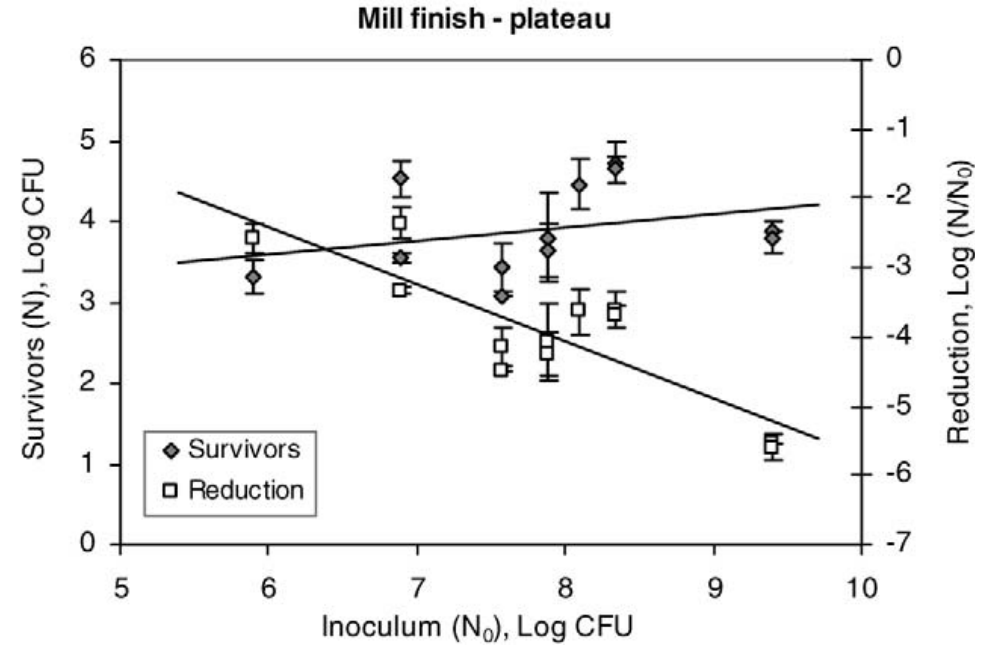


Figure 3.3. Survivors and reduction of *L. innocua* over a range of inoculum levels on the mill finish at the 3-pulse treatment (A) and in the plateau region of the inactivation curves (>6 pulses) (B).

The validity of the observed trends was verified with regression analyses, and correlations for which the coefficient and determination R^2 exceeded the critical value R_{cr}^2 were considered statistically significant. R_{cr}^2 was calculated based on the critical value of the correlation coefficient (R_{cr}), which was obtained from Sokal and Rohlf (12) as a function of the number of data points, at a probability level of 0.05. The correlation between inactivation (reduction) and inoculum was found statistically significant both for the 3-pulse treatment and for the treatments in the plateau region (Table 3.1). The statistical analyses also indicated that survivors were about constant throughout the range of inoculum levels used (near 0 slope of the regression equation and $R^2 \ll R_{cr}^2$).

This phenomenon was rather atypical, because for most microbial inactivation processes, the level of microbial reduction is constant, regardless of inoculum size. There are, however, several other reports in the literature that indicate an effect of inoculum size on inactivation. Molinari et al. (6) reported that in the pulsed electric field treatment of *Saccharomyces cerevisiae*, as the inoculum size increased, the levels of reduction achieved also increased. These authors reported a 2.2-log reduction when an initial inoculum of ~10^6 CFU/ml was used, while a 5.5-log reduction was achieved for an initial inoculum level of ~10^{11} CFU/ml (6). The authors hypothesized that this phenomenon was caused by changes in the conductivity of the cells, cell cluster formation due to the electric field, or distribution of the cells in the substrate.

For Pulsed Light treatment of microbial cells deposited on surfaces, it is very likely that the distribution of the cells on the surface is the main reason for varying reduction levels. If cells are hidden in the surface imperfections or layered beneath other cells, the concealed cells may survive the treatment, whereas the cells that are directly exposed to the Pulsed Light treatment are damaged and die.

Table 3.1. Analysis of the correlation between inoculum levels and the survivors and microbial reduction recorded after the Pulsed Light treatment for the solid substrates

Stainless steel finish	Pulsed Light fluence (J/cm^2)	No. of data sets	R_{cr}^2 [a]	Survivors: Survivors vs inoculum	Survivors: R^2	Reduction: Reduction vs inoculum	Reduction: R^2
Mill	3.40	6	0.658	y = 0.27x + 2.45	0.356	y = -0.73x + 2.45	0.795[b]
	> 6.79 (plateau)	12	0.331	y = 0127x + 2.58	0.095	y = -0.83x + 2.58	0.716[b]
Aluminum oxide	> 6.79 (plateau)	9	0.444	y = 0.09x + 4.50	0.013	y = -0.91x + 4.50	0.553[b]
Electropolished	> 6.79 (plateau)	8	0.499	y = 0437x + 1.95	0.289	y = -0.57x + 1.95	0.289

[a] Source: Sokal and Rohlf (12).

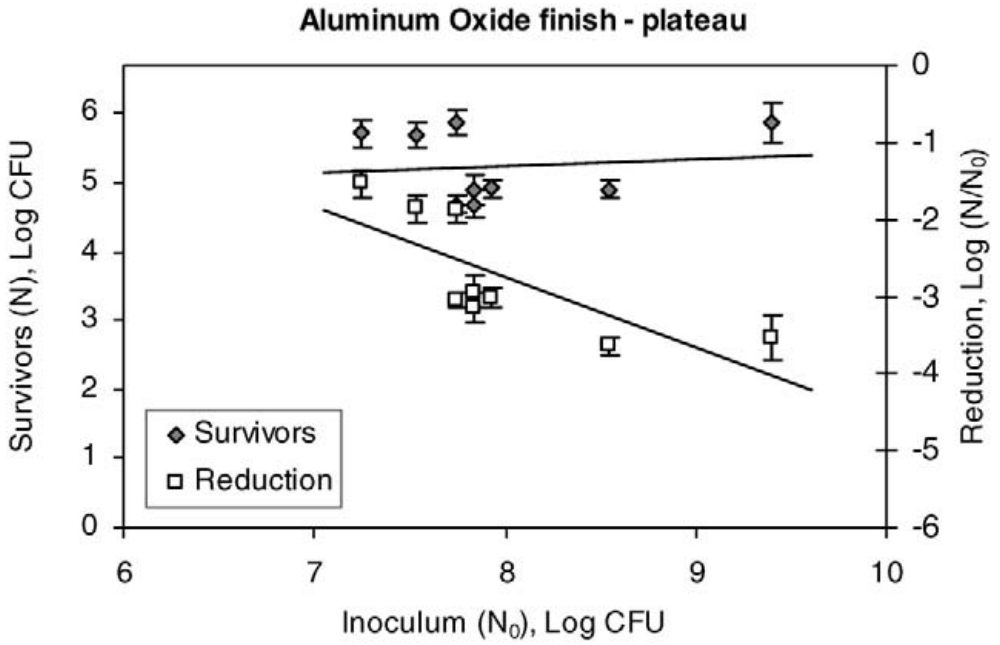
[b] Significant correlation between survivors and reduction and inoculum level ($R^2 > R_{cr}^2$).

The relatively constant number of survivors observed could possibly be explained by the ‘‘saturation’’ of the solid surface imperfections with cells that are shielded from the Pulsed Light treatment. As the inoculum level increases, the number of cells directly exposed to the Pulsed Light treatment also increases, which could explain the higher level of reduction achieved at high inoculum levels. This hypothesis is supported by the relatively constant amount of survivors of Pulsed Light treatment obtained for all three stainless steel finishes, particularly for the mill finish (Figure 3.3 and Table 3.1) and the aluminum oxide finish (Figure 3.4A and Table 3.1). The correlation between reduction and inoculum level was strongest for the aluminum oxide finish and insignificant for the electropolished surface (Figure 3.4B and Table 3.1), for which microbial hiding did not occur (18).

When comparing the survivor levels for the mill finish and aluminum oxide–treated finish, it was striking that the level of survivors was considerably higher for the latter, the rougher finish. The intercept of the survivor versus inoculum relationships shown in Table 3.1 could actually be used to estimate the level of survivors for each type of finish. Based on this analysis, the mill finish, which has a surface total roughness R_t = 2.88 µm (18), is able to ‘‘hide,’’ on average, about 2.58 log CFU of *L. innocua*, while the aluminum oxide–treated finish, with a total roughness R_t = 17.45 µm (18), is able to hide, on average, about 4.50 log CFU of *L. innocua*.

These results demonstrate once again that the efficiency of Pulsed Light depends on the substrate and that hiding effects can be significant on very irregular surfaces. The ability to estimate the microbial populations able to survive Pulsed Light treatment of a given substrate by an approach similar to the one presented above could be very useful in practical situations in which certain levels of microbial levels need to be met for either shelf life or safety reasons.

A)



B)

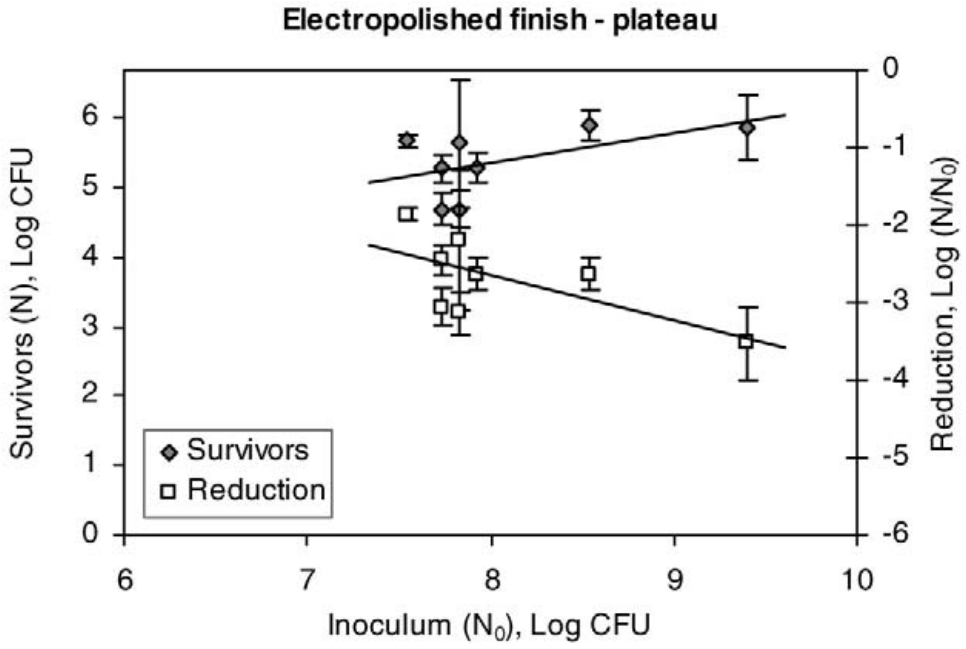


Figure 3.4. Survivors and reduction of *L. innocua* over a range of inoculum levels on aluminum oxide-treated (A) and electropolished (B) surface in the plateau region of the inactivation curves (>6 pulses).

Inactivation kinetics of *L. innocua* upon Pulsed Light treatment

A good understanding of inactivation by Pulsed Light treatment and a definition of specific kinetic parameters are necessary for both regulatory reasons and practical applications of this technology into the food industry.

The survivor curves clearly did not follow first-order kinetics, and the experimental data suggest that the tailing behavior was a result of a portion of the *L. innocua* cells being resistant to the Pulsed Light treatment, cell clumping effects, or both. Cell clumping effects may be substrate-specific, as observed in an earlier study performed on solid substrates (18), and could represent a strong reason for tailing. The exact reasons behind the tailing of survivor curves in Pulsed Light treatment require systematic investigation and will be addressed in a future study.

Otaki et al. (7), who studied the effects of low-pressure UV light and Pulsed Light on *E. coli* in turbid wastewater solutions, also observed tailing of the inactivation curves for the low pressure UV inactivation, with intensities of up to 600 $mW \cdot s/cm^2$ (0.6 J/cm^2), but reported first-order kinetics for Pulsed Light treatments, with intensities of up to 800 $mW \cdot s/cm^2$ (0.8 J/cm^2). Upon careful examination, even in the study by Otaki et al. (7), the inactivation curve for the Pulsed Light treatment of turbid water did show a hint of a tail. In terms of the level of inactivation, Otaki et al. (7) reported a 10- to 12- log CFU reduction of *E. coli* in turbid water treated with 0.6 to 0.8 J/cm^2 of Pulsed Light. A comparison of these results with the level of reduction of *L. innocua* achieved in the current study (about a 6-log CFU reduction after a 6 J/cm^2 treatment) suggests that *E. coli* is much more sensitive to the Pulsed Light treatment than *L. innocua.* This observation is also supported by the findings of Rowan et al. (10), who reported that *Listeria monocytogenes* averaged as much as 1.5 log CFU more survivors than *E. coli* after identical Pulsed Light treatments (200 pulses of high UV Pulsed Light), with both survivor curves showing strong tailing

effects (10).

Based on strong evidence that Pulsed Light inactivation does not generally follow a log-linear trend, the traditional first-order kinetics approach is not applicable for process calculations. Because of its ability to successfully model nonlinear inactivation, the Weibull model was used to describe the survival of *L. innocua* after exposure to Pulsed Light for the substrates used in this study.

The survivor curves for the liquid substrate showed an obvious non-log-linear decline, evidence of tailing, and a concave upward shape (Figure 3.1). The survivor ratios (N/N_0) for the Pulsed Light treatment with an inoculum size of 7.45 log CFU/ml were used to determine the Weibull parameters for the liquid substrate. For this data set, a shape parameter of $\beta = 0.33$ and a scale parameter of $\alpha = 3.01$ were used for the liquid suspension treated with Pulsed Light fluence of up to 13.30 J/cm^2. Similar shape parameter values have also been reported for various heat treatments, i.e., the heat treatment of *L. monocytogenes* in both milk and broth (17). Figure 3.5 shows both the experimental data and the survivor curve obtained with the calculated Weibull parameters.

Because the value of a microbial inactivation model resides in the possibility of using the model to predict inactivation levels in similar substrates without performing additional experiments, the validity of the Weibull model was tested for the data sets obtained for the other four levels of inoculum. As shown in Figure 3.6, which represents a plot of predicted survivor ratios versus measured survivor ratios, the Weibull model was able to predict very well the survivor ratios in Pulsed Light inactivation of *L. innocua* in clear liquids. The differences between the predicted data and the experimental data were within approximately ± 0.1 log (see Table 3.2), which is an extremely accurate prediction for microbiological data.

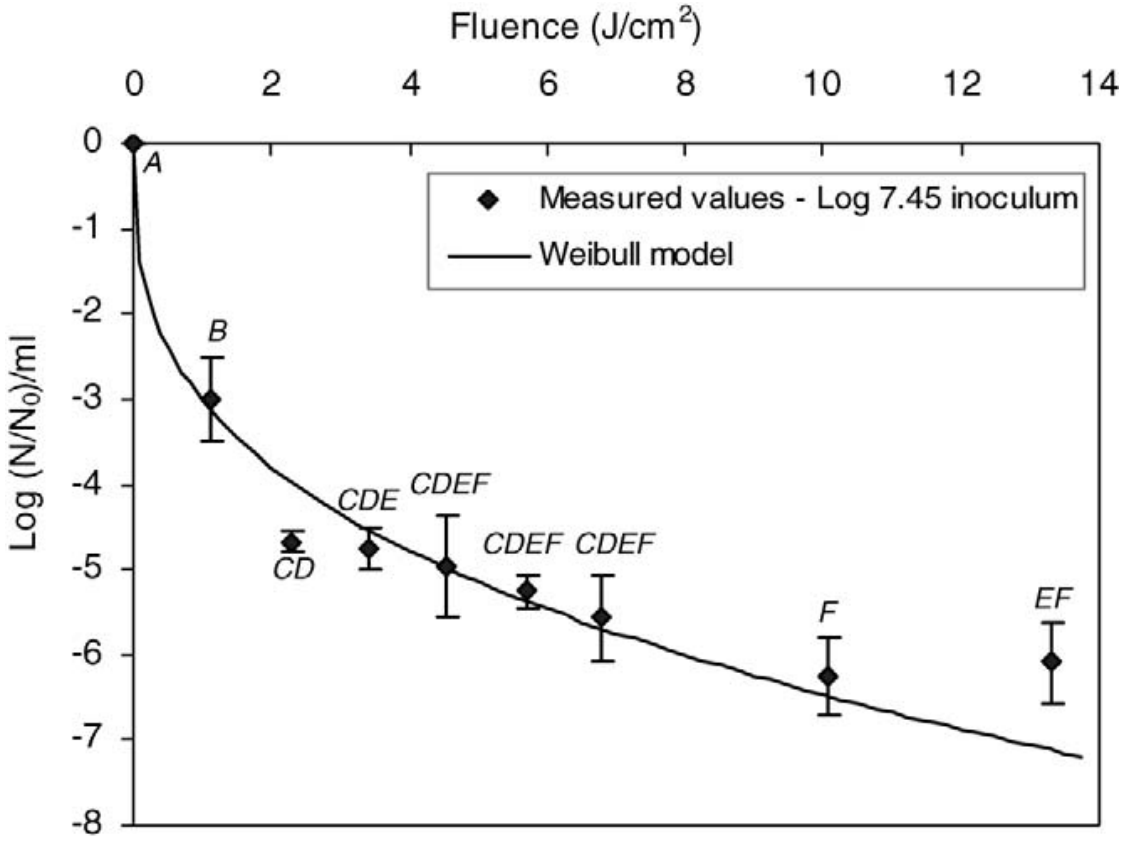


Figure 3.5. Experimental and Weibull-calculated survival ratios ($S = N/N_0$) for *L. innocua* treated with Pulsed Light in liquid suspensions with an initial inoculum of 7.45 log CFU/ml. Different letters indicate significant differences ($P < 0.05$).

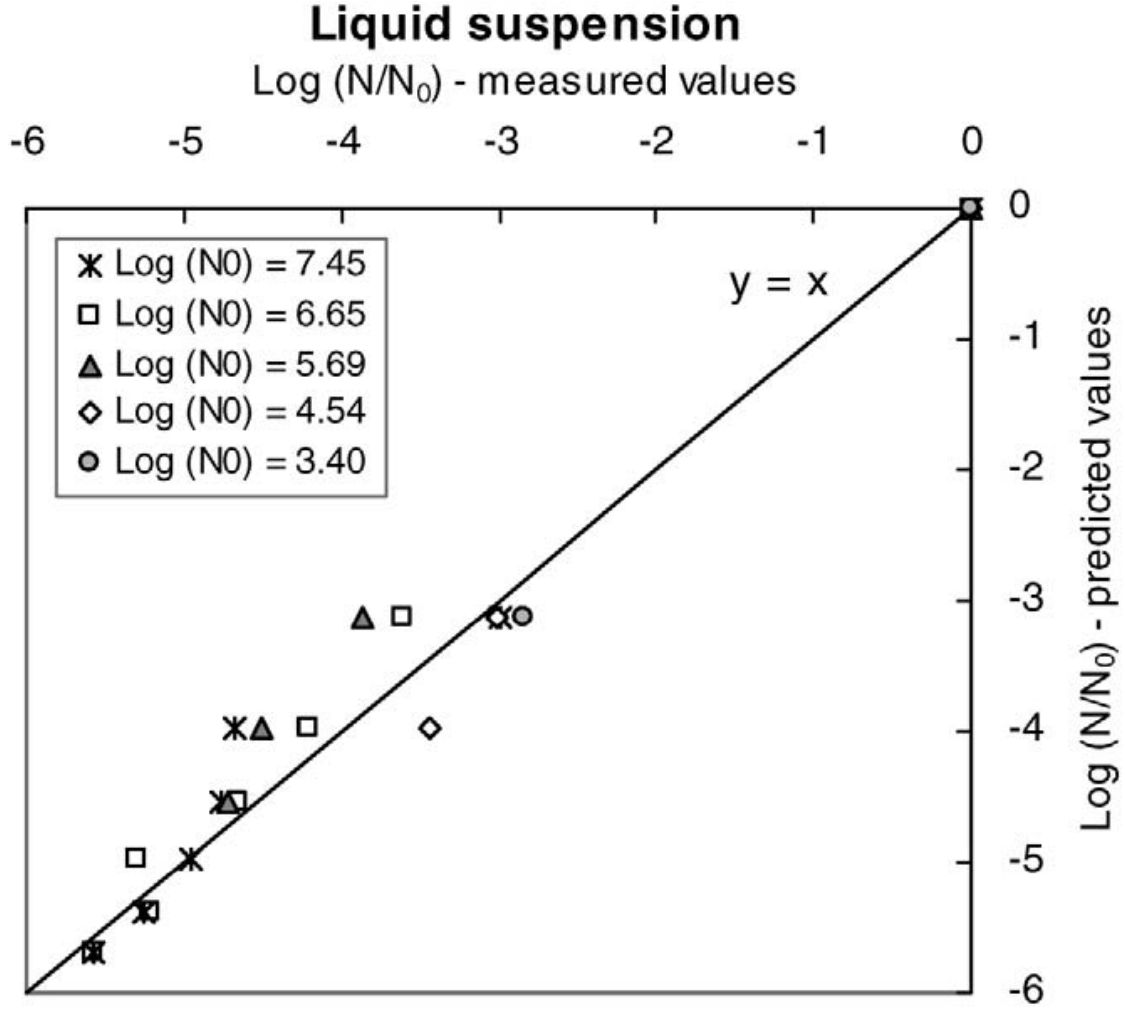


Figure 3.6. Measured versus Weibull predicted survival ratios ($S = N/N_0$) for *L. innocua* treated with Pulsed Light in liquid suspensions of various inoculum sizes. The Weibull prediction was based on the inactivation data obtained for a 7.45-log CFU/ml inoculum.

Table 3.2. Analysis of the Weibull prediction for Pulsed Light inactivation of *L. innocua* in a liquid substrate at various levels of inoculum [a]

Inoculum (Log CFU)	Model overestimation from exptl data (Log N/N_0)
6.65	0.02
5.69	0.11
4.54	0.10
3.40	0.10

[a] Prediction based on Pulsed Light inactivation data for an inoculum of 6.45 log CFU

It can therefore be concluded that survival ratios in the Pulsed Light treatment of clear suspensions of *L. innocua* cells can be accurately described by the following kinetic model:

$$\text{Log}(N/N_0) = -3.01\ F^{0.33}$$

where N is the number of survivors after Pulsed Light treatment (CFU), N_0 is the number of cells prior to the treatment (CFU), and F is fluence (joules per square centimeter).

On the basis of this model, it can be predicted that when treating a clear suspension of *L. innocua* cells with a fluence of 12 J/cm^2, which is the maximum level of Pulsed Light treatment currently permitted by the U.S. Food and Drug Administration regulations, a 6.87-log reduction can be obtained.

A similar approach was used to determine the Weibull parameters for the solid substrates. With the data set obtained from treating the mill finish coupons inoculated with 9.40 log CFU of *L. innocua*, the following Weibull parameters were obtained: $\alpha = 1.70$ and $\beta = 0.48$. When attempting to validate the model with data obtained at different inoculum levels, a clear departure of the model from the experimental data was observed (Figure 3.7). The difference between the model and the experimental data was more pronounced for the lower inactivation levels (Table 3.3), most likely because of the inoculation level effects discussed earlier in the article. When the mill finish model for the electropolished and aluminum oxide stainless steel finishes was used, the model significantly overestimated the level of Pulsed Light inactivation of *L. innocua* at levels that would be unacceptable for practical applications (Table 3.3) because of surface-related influences. It might be possible to attempt to incorporate the various substrate-related factors (e.g., optical properties, roughness) into the Weibull model, but that would require a systematic and laborious approach.

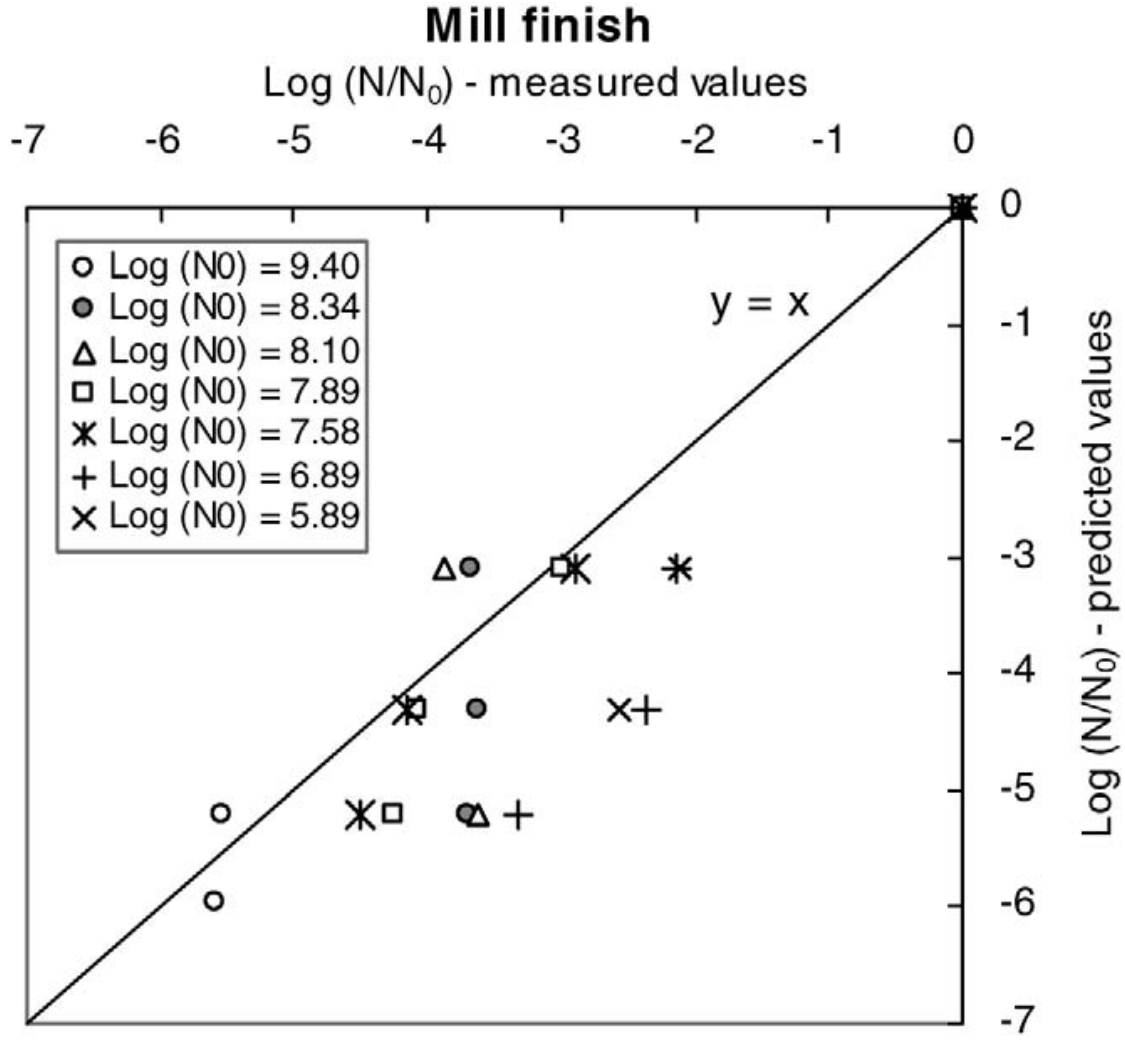


Figure 3.7. Measured and Weibull predicted survival ratios ($S = N/N_0$) for *L. innocua* treated with Pulsed Light on the mill-finished stainless steel coupons inoculated with various inoculum sizes. The Weibull prediction was based on the inactivation data obtained for a 9.40-log CFU inoculum.

Table 3.3. Analysis of the Weibull prediction for Pulse Light inactivation of *L. innocua* on stainless steel substrates at various levels of inoculum [a]

Mill finish		Electropolished finish		Aluminum oxide finish	
Inoculum (Log CFU)	Model overestimation (Log N/N_0)	Inoculum (Log CFU)	Model overestimation (Log N/N_0)	Inoculum (Log CFU)	Model overestimation (Log N/N_0)
8.34	0.15	9.40	0.48	9.40	0.48
8.10	0.09	8.79	0.60	8.79	0.20
7.89	0.12	8.54	0.64	8.54	0.18
7.58	0.10	7.93	0.64	7.93	0.43
6.89	0.61	7.83	0.66	7.83	0.47
5.89	0.59	7.74	0.61	7.74	0.84
		7.54	1.80	7.54	1.80
				7.25	2.39

[a] Prediction based on Pulsed Light inactivation data for the mill finish with an inoculum of 9.40 log CFU.

The results of this study demonstrate that microbial inactivation of *L. innocua* by Pulsed Light technology levels off at high treatment intensities, with the survivor curves showing strong tailing effects, both on rough and highly light-absorbing surfaces as well as in clear liquids. The complex influence of various factors on the effectiveness of Pulsed Light treatment was further substantiated by this study, because it was found that the level of inactivation was influenced by inoculum size when the treatment was applied to surfaces that allowed the hiding of microbial cells. The Weibull model allowed an excellent prediction of microbial inactivation in Pulsed Light treatment in clear liquids, because it accounted both for the concavity and tailing of the survivor curves. The model might also be useful for other relatively simple substrates, such as clear packaging or non-turbid juices, but it fails to give acceptable predictions for substrates when the influence of various substrate properties on inactivation is significant.

Pulsed Light treatment shows definite promise for use in the food industry, but one has to be aware that as substrate properties become more complex, the efficiency of Pulsed Light is diminished and less predictable. This is important to note, as foods and food contact surfaces are never simple, and many possess different attributes capable of affecting the efficiency of the treatment.

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CHAPTER 4

REDUCTION OF *LISTERIA* ON READY-TO-EAT SAUSAGES AFTER EXPOSURE TO A COMBINATION OF PULSED LIGHT AND NISIN

ABSTRACT

The risk of listeriosis associated with ready-to-eat foods is a major concern in the United States. Pulsed Light treatment has been effective for killing *Listeria.* The possibility of enhancing the antilisterial capability of Pulsed Light treatment by combining Pulsed Light with an additional hurdle, the natural antimicrobial nisin, was explored in this study. First, the ability of *Listeria innocua* to mimic the response of *Listeria monocytogenes* to Pulsed Light treatment was demonstrated. Subsequently, a series of inoculation studies was performed in which canned sausages were surface inoculated with *L. innocua* as a surrogate for *L. monocytogenes* and then treated with a commercial preparation of nisin (Nisaplin), Pulsed Light, or a combination of the two treatments. The application of a Nisaplin dip alone resulted in an immediate reduction of *L. innocua* by 2.35 ± 0.09 log CFU. Pulsed Light reduced *L. innocua* by 1.37 ± 0.30 log CFU after exposure to 9.4 J/cm^2. A total reduction of 4.03 ± 0.15 log CFU was recorded after the combined treatment of Nisaplin and PL for 48 h at 4°C. The long-term survival of *L. innocua* was evaluated on sausages stored at 4°C. Treatment with Nisaplin and Pulsed Light resulted in a 4- to 5-log reduction for two replicate studies. The combination treatment resulted in no significant microbial growth during 28 and 48 days of refrigerated storage in the first and second replicates, respectively. These results suggest that this combination treatment can be used as an effective antilisterial step in the production of ready-to-eat foods.

INTRODUCTION

Listeria monocytogenes in ready-to-eat foods

Listeria monocytogenes is a psychotropic Gram positive, non-spore forming, facultative anaerobic foodborne bacterial pathogen that targets high risk groups such as immune-compromised individuals, pregnant women, neonates and the elderly, as well as healthy people. *Listeria* is an unusual foodborne pathogen due to its ability to cause meningitis, septicemia, abortion, and a high mortality rate of 20-30%. The Centers for Disease Control and Prevention estimated that in the United States there are approximately 2,500 cases of listeriosis yearly, leading to about 500 deaths per year (38). The consequences of listeriosis reach beyond public safety; the estimated annual foodborne illness cost by the USDA from cases associated with *L. monocytogenes* is $2.3 billion (13). *L. monocytogenes* was first diagnosed in a human case in 1924 from a World War I soldier suffering from meningitis (45) but there is also speculation that listeriosis was the cause of Queen Anne's unsuccessful pregnancies in the 17th century (50). Pregnant women may have mild flu-like symptoms but the infection of the fetus may lead to abortion or stillbirth. In newborn children, symptoms can include sepsis, pneumonia, and meningitis (54). In nonpregnant adults of advanced age or in an immunosuppressed condition, listeriosis may result in sepsis, meningitis or focal infections. In noninvasive infections, gastrointestinal illness with fever and diarrhea are the most common symptoms.

L. monocytogenes is ubiquitous in the environment. This bacterium has the ability to grow in soil and water, often being found in decaying vegetation. Widespread *Listeria* in soil is believed to be aided by decaying material, plants and feces, which are providing nutrients while the soil provides a moist and cool environment (18). *L. monocytogenes* has been isolated in animal and bird feces, both showing signs of listeriosis and asymptomatic carriers. Human carries, both

symptomatic and asymptomatic, can also shed the bacteria in their feces.

There have been numerous major food outbreaks since the first documented outbreak associated with coleslaw in 1981 (40). Most recent cases of listeriosis have been traced back to ready-to-eat (RTE) foods such as frankfurters (8, 25) and deli turkey meat (9-11). Many cases of listeriosis seem to be the result of post-processing contamination, which is a serious concern with RTE foods. The ability of *L. monocytogenes* to survive and grow at refrigerated temperatures, (4) high salt concentrations and within acidified foods of pH $\geq$ 5.2 (30, 33, 55) represents a serious problem for many refrigerated RTE products with a long shelf life. Tompkin (57) reported that *Listeria* cannot be eliminated from the processing environment of RTE meat and poultry products but steps could be implemented to control *Listeria* which include environmental sampling programs to prevent the establishment of *Listeria*, preparing a rapid and effective response to positive detection, verification of detection and corrective action, and short and long term assessment to detect problems and trends.

The choices of postprocess bactericidal interventions for RTE products are very limited. The recent development of Pulsed Light technology, which can reduce the microbial load on foods and food contact surfaces (35-37, 58, 59, 61), may provide a viable solution for controlling postprocess contamination by *Listeria* in RTE products. Pulsed Light treatment consists of very short high-intensity pulses of broad spectrum light that is used to inactivate vegetative bacteria, spores, yeasts, and molds. The Pulsed Light treatment dose, called fluence, is expressed in joules per square centimeter. Pulsed Light treatment has been approved for use by the U.S. Food and Drug Administration for decontamination of food and food surfaces with the restriction that a xenon flash lamp be used as a light source, with pulse durations of < 2 ms and the cumulative treatment not exceeding 12 J/cm^2 (59). Although the exact

mechanisms responsible for cell death in Pulsed Light treatment are not yet fully elucidated, a majority of the Pulsed Light studies indicate a significant contribution of the UV portion of the spectrum, and the lower wave length UV radiation has a more pronounced role in inactivation (47, 60, 62). UV radiation, especially within 250 to 260 nm, is damaging to most microorganisms because it alters the microbial DNA through the formation of pyrimidine dimers, pyrimidine adducts, and DNA-protein cross-links (6).

In several studies, the ability of Pulsed Light to inactivate various microorganisms on food surfaces has been demonstrated: *L. monocytogenes* and *Escherichia coli* O157:H7 on raw salmon fillets (42), *E. coli* O157:H7 on alfalfa seeds (52), yeast on flour and black pepper (19), and fungi from fresh fruit (31), corn meal (28), and strawberries (36). Woodling and Moraru (61) reported a nearly 4-log reduction of *Listeria innocua* on the surface of stainless steel coupons of different surface roughness after a Pulsed Light dose less than 12 J/cm^2. Because of its effectiveness against *Listeria* and appropriateness for the inactivation of surface microflora, Pulsed Light could be applied as a postprocessing safety step to reduce surface contamination in RTE products. Particularly interesting is the potential to enhance the effectiveness of Pulsed Light treatment using a hurdle approach by combining Pulsed Light with another antilisterial treatment, such as an antimicrobial chemical compound.

The bacteriocin Nisin as a means to fight *Listeria monocytogenes*

Bacteriocins have become a popular microbial control method in recent years. They have been successfully shown to control bacteria in a wide range of foods including, cheese, vegetables, and meats. Nisin, in particular, has generated much interest dues to its GRAS status, antilisterial effects, and being a natural preservative.

Nisin, a low molecular weight antimicrobial protein produced by *Lactococcus*

lactis ssp. *lactis*, has a broad antimicrobial spectrum against Gram positive bacteria and spore-formers. Nisin is synthesized as a precursor peptide, modified post-translationally, and transported out of the cell. (2). These modifications include the dehydration of serine and threonine and the formation of lanthionine rings, making nisin classified as a lantibiotic. Two natural variants of Nisin exist, A and Z that are different at the 27^{th} amino acid: asparagine for A and histidine for Z (1).

When used against spores, nisin does not prevent spore germination but acts by not allowing outgrowth of the spore. In the case of vegetative cells nisin complexes with Lipid II, which is an important cell wall precursor which helps anchor the peptidoglycan during cell wall biosynthesis. The nisin-Lipid II complex inserts itself into the cytoplasmic membrane (7, 15, 26, 32) and this insertion leads to the formation of pores within the membrane, resulting in the leaking of cellular components and cell death.

Nisin is classified as Generally Recognized as Safe and is approved in over 80 countries including the European Union, the United States, India, China, and Australia (2). Commercially, nisin has been available since 1953 under the name Nisaplin®, which contains milk and milk solids and approximately 2.5% nisin (15). Pure nisin has an activity of 40×10^6 IU/g while Nisaplin® is standardized to 40×10^6 IU/g so that 1µg is equal to 1 IU. Nisin has been incorporated into a number of foods including many meat products (2, 15, 16, 34, 44). Applied as a spray, a dip, or incorporated in the product, nisin has been shown to control, with variable efficiency, post processing microbial growth in a number of meat products, including smoked rainbow trout (41), raw meat *(*3), fresh pork sausage (51), bologna (14, 49), hot dogs (21, 53, 56), and frankfurters (23, 24).

Applications often target *C. botulinum* spores or vegetative cells of *L. monocytogenes*. *C. botulinum* spores are less sensitive to nisin than *L.*

monocytogenes; where 10,000 IU/ml are needed to reduce 6 logs of spores while only 200 IU/ml are required for vegetative cells (39). Temperature also affects the efficacy of nisin, as nisin is more efficient at refrigeration temperatures than at elevated temperatures. *C. botulinum* growth has been shown to begin when nisin levels were below 154 IU/ml at 35°C while growth did not occur for nisin <12 IU/ml at 15°C (46). Chung (12) showed that *Listeria* was better inhibited at refrigerated temperatures than ambient temperatures. When used in food applications, nisin is primarily used in conjunction with another control step such as refrigeration or modified atmosphere.

The **objective** of this work was to develop a hurdle treatment for the control of *Listeria* on the surface of RTE foods by combining the bactericidal action of Pulsed Light treatment with the bactericidal and bacteriostatic effect of nisin. The appropriateness of using *L. innocua* as a surrogate for *L. monocytogenes* was tested, and subsequently *L. innocua* was used as a challenge organism. Commercial canned Vienna sausages were used as the food substrate. The survival of *L. innocua* was examined over prolonged storage at 4°C for four sausage treatments: (i) no treatment (control), (ii) nisin dip, (iii) Pulsed Light, and (iv) nisin dip plus Pulsed Light. The results of this work should help the food industry develop efficient hurdle treatments for control of postprocess *Listeria* surface contamination in RTE foods.

MATERIALS AND METHODS

Substrate

Armour brand Vienna sausages (Pinnacle Foods Corporation, Cherry Hill, NJ) were used as a model for RTE hot dogs and frankfurters. These sausages were made from mechanically separated chicken, water, beef, pork, salt, corn syrup, and less than 2% mustard, natural flavor, dried garlic, and sodium nitrite and were canned in a solution of chicken broth and caramel color. The pH of the sausages was 5.7. The

sausages were skinless and had a diameter of 20 mm and an average length of 53 mm.

Nisin

The commercial preparation Nisaplin (Danisco, New Century, KS) was used as a nisin source. A 0.5% Nisaplin solution was prepared by dissolving 1 g of Nisaplin into 200 ml of sterile, deionized water. Nisaplin contains a standardized nisin activity of 10^6 IU/g, resulting in a final nisin solution of about 5,000 IU/ml. This 0.5% Nisaplin solution is referred to as nisin throughout the article.

Pulsed light treatment

Pulsed Light treatments were performed with an RS-3000C SteriPulse System (Xenon Corporation, Woburn, MA). The system consists of a controller unit and a treatment chamber that houses a xenon flash lamp. Each sample was centered individually on an adjustable stainless steel shelf in the Pulsed Light unit 50.8 mm beneath the lamp and was treated with a variable number of pulses at a frequency of three pulses per second and a pulse width of 360 µs.

Fluence measurements

The Pulsed Light fluence was measured with a pyroelectric head (PE25BBH) and a Nova II display (Ophir Optronics Inc., Wilmington, MA) and was expressed in joules per square centimeter. To perform the fluence measurement, a stainless steel aperture cover was placed over the power meter head. The aperture covered the top surface of the detector head except for a 1-cm^2 circular opening that exposed the detector's surface. The pyroelectric head was placed 50.8 mm from the quartz face of the lamp. The settings on the Nova II display were a pulse width of 1.0 ms and a wavelength of < 0.3 µm. Pauses of at least 30 s between measurements were allowed to prevent possible overheating of the pyroelectric head.

Optical penetration depth measurements

To determine the optical penetration of Pulsed Light into the sausage, slices of

sausage of various thicknesses (0.58, 1.19, 2.46, and 3.76 mm) were placed over the 1-cm^2 opening of the pyrodetector head, which was located 50.8 mm from the lamp face. The detector was centered in the Pulsed Light chamber, and the fluence from three pulses was measured for each thickness. The resulting fluence values were plotted versus distance (thickness of the sausage slice), and the exponential decay of fluence with distance was used to calculate the optical penetration depth of the sausage. All fluence measurements were performed in triplicate.

Light absorption analysis

The absorption spectrum of the sausage was measured with a HR2000+CG-UV-NIR spectrometer (Ocean Optics Inc., Dunedin, FL). A 0.58-mm-thick slice of sausage was placed into a cuvette, which was then inserted into the spectrometer. The absorbance measurements were performed for the entire spectral range of the Pulsed Light treatment (180 to 1,100 nm). The absorption spectrum of the 0.5% Nisaplin in sterile deionized water also was determined.

Culture and inoculum preparation

L. innocua FSL C2-008 (environmental isolate from a smoked fish plant) and a five-strain *L. monocytogenes* human disease cocktail recommended by Fugett et al. (22) were obtained from the Food Microbiology and Safety Laboratory (Cornell University). The five-strain cocktail comprised the following *L. monocytogenes* isolates: FSL C1-056 (human isolate, sporadic infection), FSL J1-177 (human isolate, sporadic infection), FSL N1-227 (food-associated outbreak isolate, RTE meat product), FSL N3-013 (food-associated outbreak isolate, pate´), and FSL R2-499 (human isolate, outbreak associated with sliced turkey).

Before the experiment, the culture was streaked onto tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) and incubated for 24 ± 2 h at 35 ± 2°C. A single isolated colony was transferred into tryptic soy broth (TSB; Becton Dickinson) and

incubated for 24 ± 2 h at 35 ± 2°C. A loopful of this culture was transferred into TSB and incubated for 24 ± 2 h at 35 ± 2°C to produce an initial inoculum of 10^8 to 10^9 CFU/ml.

Building of growth curves for *L. innocua* and *L. monocytogenes*

A single colony of each isolate was selected from a TSA plate and inoculated into 5 ml of TSB and incubated for 12 to 18 h at 37 ± 2°C with shaking (225 rpm). After incubation, 50 µl of the culture was transferred to 5 ml of fresh TSB and incubated at 37 ± 2°C with shaking (225 rpm). When the optical density at 600 nm (OD_{600}) reached 0.4, 100 µl was transferred to 10 ml of TSB and incubated at 37 ± 2°C with shaking (225 rpm) for 24 h. For the *L. monocytogenes* cocktail, 2 ml of each strain culture at an OD_{600} of 0.4 were combined to create 10 ml of cocktail, which was incubated at 37 ± 2°C with shaking (225 rpm) for 24 h. At 0, 3, 4, 5, 6, 7, 9, 12, 18, and 24 h, appropriate dilutions were made into Butterfield's phosphate buffer (BPB), and 100 µl of *L. innocua* or *L. monocytogenes* cocktail was spread plated in duplicate onto TSA and incubated for 24 ± 2 h at 37 ± 2°C.

Pulsed Light treatment of *L. innocua* and *L. monocytogenes* in clear liquid suspensions

A 10-fold dilution of an inoculated 24h TSB culture was made with BPB for each *Listeria* isolate. For the *L. monocytogenes* cocktail, equal volumes of each strain were combined. Sterile transparent one-well glass Lab-Tek II Chamber Slides (Nagle Nunc International, Naperville, IL) with chamber dimensions of 25.4 by 50.8 mm were used to hold 1 ml of the liquid cell suspensions. The height of the liquid inoculum in the glass chamber was 1.16 mm. The chamber containing the suspension was centered individually and parallel to the lamp on an adjustable stainless steel shelf in the Pulsed Light unit approximately 50.8 mm beneath the xenon lamp and was treated with up to 12 pulses at a frequency of 3 pulses per s. The treated inoculum (1 ml) was transferred

to 7 ml of TSB, and the chamber was rinsed twice with 1 ml of TSB, adding the rinse TSB to the treated inoculum. The resulting 10 ml was serially diluted, plated, and incubated for 48 ± 2 h at 37 ± 2°C, and the survivors were enumerated. When plate counts fell below the limit of detection, the number of survivors was estimated with the most-probable-number technique. The recovery broth was diluted, 1 ml of each dilution was transferred into 10 ml of TSB (three tubes for each dilution), and the tubes were incubated at 37 ± 2°C for 48 ± 2 h. Turbidity was used to presumptively identify positive samples. Presumptive-positive samples were streaked onto modified Oxford medium (Becton Dickinson) and incubated at 37 ± 2°C for 24 ± 2 h. Tubes were considered positive when typical black esculin-positive colonies were observed. Survivor curves were built by plotting the log of the survivor ratios (N/N_0) versus fluence.

Pulsed Light treatment of *L. innocua* and *L. monocytogenes* on Vienna sausages

The broth was drained from the sausage cans, and the sausages were removed. Sausages were patted dry with sterile paper towels. For the nisin treatments, sausages were dipped into the 0.5% Nisaplin solution for 2 min, drained for 1 min, and allowed to dry for 15 min. After drying, nisin-treated and untreated (control) sausages were spot inoculated (20 µl) with *L. innocua* and *L. monocytogenes,* respectively, on the surface, and the inoculated sausages were allowed to dry for 30 to 45 min. Each inoculated sample was placed inoculated side up on a sterile petri plate, centered on an adjustable stainless steel shelf in the Pulsed Light unit 50.8 mm beneath the xenon lamp, and treated with up to nine pulses of light, corresponding to a maximum fluence of 10.1 J/cm^2. Treated sausages were then packaging into sterile 532 ml Whirl-Pak bags (Nasco, Modesto, CA) and were stored at 4 ± 2°C for up to 60 days.

Recovery and enumeration of survivors from treated sausages

At the appropriate time point for each sausage sample, 100 ml of TSB was

added to the Whirl-Pak bag, and samples were stomached for 2 min. The resulting sample was serially diluted in BPB, and 0.1 ml was spread plated onto TSA in duplicate. When low survivor counts were expected, 250 µl of the undiluted sample was spread plated four times onto TSA to improve the limit of detection. The total CFU count for the four plates provided the CFU per milliliter for that sample. When plate counts fell below 25 CFU/ml, survivor counts were determined by the most-probable-number technique. Aliquots of the inoculum in the recovery broth were transferred into TSB in the following distribution: three tubes with 10 ml, three with 1 ml, three with 0.1 ml, and three with 0.01 ml. The culture was then incubated at 35 ± 2°C for 48 ± 2 h. Turbidity was used to presumptively identify positive samples. Presumptive positive samples were streaked onto modified Oxford medium and incubated at 35 ± 2°C for 24 ± 2 h. Tubes were considered positive when typical black esculin positive colonies were observed.

Statistical analysis

An analysis of variance and Tukey's honestly significant differences test were used to determine whether differences between treatments were significant at $P < 0.05$ using the statistical package JMP 6.0.0 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Optical characteristics of the substrates

Light absorption by the sausage and nisin, particularly in the UV region, is very important for the outcome of the Pulsed Light treatment because absorption can affect the amount of fluence delivered to the microbial cells on the sausage surface.

The absorbance of the Vienna sausage was high across a broad spectrum, from 200 to 1,100 nm, but no particular spectral absorbance preference was observed,

which indicates that the sausage itself will attenuate the intensity of the treatment without modifying the spectral distribution of the incident light. The attenuation of light inside the sausage followed an exponential decay with sausage thickness (Figure 4.1). Based on this decay, the optical penetration depth of the sausage (defined as the depth at which the fluence decreases to 1/*e* of its value at the sausage surface) was calculated to be 2.3 mm. Thus, light treatments such as Pulsed Light will be effective only as a surface treatment for this type of substrate. Bacterial cells located beneath the sausage surface will not be effectively killed by the treatment because the sausage absorbs a significant amount of the incident light. Nisin had a lower absorbance value than the sausage, with near zero absorbance between 300 and 1,100 nm.

Nisin had preferential absorbance in the UV region, between 200 and 300 nm, with a sharp peak at approximately 225 nm. Consequently, nisin should diminish somewhat the amount of lethal UV delivered to the microbial cells, which might slightly reduce the effectiveness of Pulsed Light treatment when nisin is present on the sausage surface.

L. innocua* as a surrogate for *L. monocytogenes

Because of the pathogenic nature of *L. monocytogenes*, its use in the laboratory and particularly in pilot plant validation studies can pose significant risks. Thus, an indicator organism is needed in its place, in much the same way as *Clostridium sporogenes* is used as an indicator for the pathogenic *Clostridium botulinum* when evaluating process efficiency (29). *L. innocua* is often regarded as the nonpathogenic variant of *L. monocytogenes* (27) because it possesses many traits that are similar to those of its pathogenic relative; the greatest difference is the lack of hemolysin production (29).

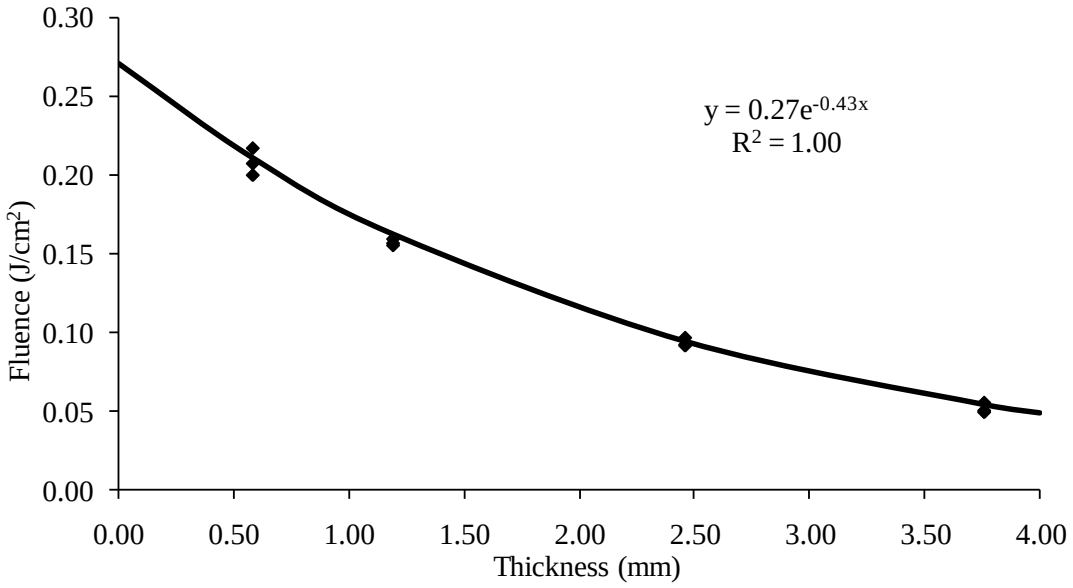

Figure 4.1. Optical penetration of Pulsed Light through sausage slices. Fluence at thickness 0 mm (no sausage) was 1.10 J/cm^2 (n = 3 at each thickness).

Kamat and Nair (29) concluded that *L. innocua* is a reasonable indicator organism for *L. monocytogenes* in a variety of treatments, including radiation, heat, lactic acid, NaCl, and nitrites. Because no data were available regarding the comparative response of *L. innocua* and *L. monocytogenes* to Pulsed Light treatment, a comparison between the growth characteristics and response to Pulsed Light for these two organisms was performed.

Numerous studies have been conducted to compare the growth of *L. innocua* and *L. monocytogenes* over a range of temperatures (0 to 36°C) and on a variety of substrates, including minced beef (17), lettuce (20), and crab meat (43). In these studies, no significant differences were noted between *Listeria s*pecies when grown at the same temperature. In this study, the growth kinetics of *L. innocua* and *L. monocytogenes* were compared at 37°C, which was the incubation temperature used for evaluating the survivors of the treatment. When comparing the growth curves at 37°C, no difference between the nonpathogenic *L. innocua* strain and the pathogenic *L. monocytogenes* cocktail were observed (Figure 4.2). Additionally, when comparing the survival of *L. innocua* and *L. monocytogenes* cocktail after treatment with Pulsed Light (Figure 4.3), similar reductions were observed at low fluence levels. As Pulsed Light dose increased, *L. innocua* appeared to have a slightly higher resistance to Pulsed Light than did *L. monocytogenes.* This finding suggests that Pulsed Light reductions that occur when utilizing *L. innocua* as the test organism may underestimate the Pulsed Light reduction of *L. monocytogenes* under similar conditions. Thus, reduction of *L. innocua* by Pulsed Light will give a conservative estimate of reduction of *L. monocytogenes* by Pulsed Light, which is a favorable result.

Overall, the physiological and metabolic similarity of these two organisms and the results in Figure 4.3 indicate that it is reasonable to use *L. innocua* as an indicator

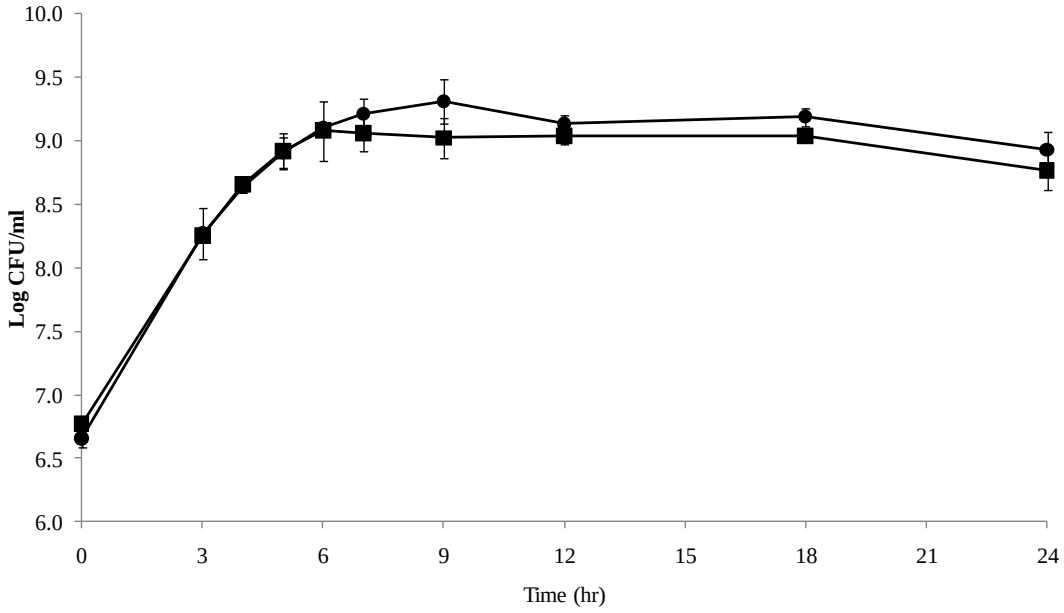

Figure 4.2. Growth of *L. innocua* (■) and *L. monocytogenes* cocktail (●) in TSB at 37°C with shaking at 225 rpm (n = 3).

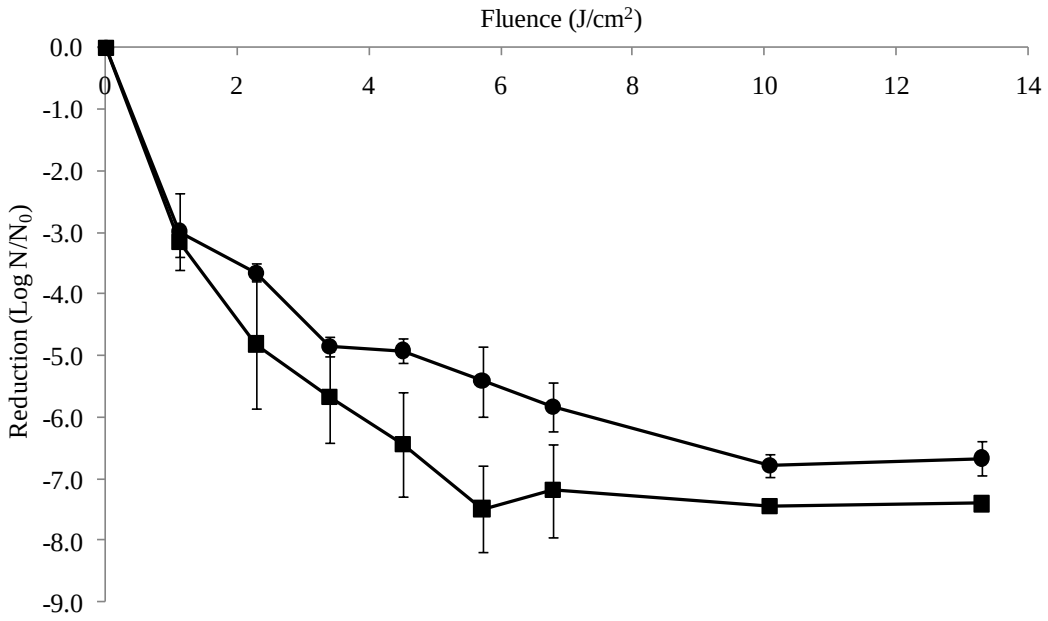

Figure 4.3. Reduction of *L. innocua* (●) and *L. monocytogenes* cocktail (■) after Pulsed Light treatment in BPB. Initial inoculum was 7.5 log CFU/ml for *L. innocua* and 7.9 log CFU/ml for *L. monocytogenes* cocktail (n = 3).

Overall, the physiological and metabolic similarity of these two organisms and the results in Figure 4.3 indicate that it is reasonable to use *L. innocua* as an indicator for *L. monocytogenes* in Pulsed Light treatment situations where the pathogen cannot be used. *L. innocua* also can be used as a general model for a food-associated microorganism for evaluating the influence of substrate related or treatment related factors on the effectiveness of Pulsed Light treatments. Because the present study involved prolonged refrigerated storage of the inoculated and treated sausage samples in facilities that did not allow the use of pathogens, *L. innocua* was used as an indicator for *L. monocytogenes.*

Reduction of *L. innocua* on sausages by exposure to Pulsed Light and nisin

As a first step, the necessary level of fluence and nisin exposure for each type of treatment was established. When subjecting the *L. innocua*–inoculated sausages to Pulsed Light alone, the inactivation curve had a clear plateau (Figure 4.4), with the highest reduction (1.37 log CFU per sausage) achieved after exposure to 9.4 J/cm^2 (nine pulses). The application of nisin alone resulted in an immediate reduction of 2.35 log CFU per sausage. This reduction was similar to that of *L. monocytogenes* reported by Geornaras et al. (23, 24) in both frankfurter and smoked sausages dipped in nisin, which had 2.4- and 2.1-log reductions, respectively. The combined Pulsed Light plus nisin treatment resulted in a significantly greater reduction compared with that achieved with the individual treatments, suggesting an additive effect of Pulsed Light and nisin (Figure 4.4). Additional reduction seemed to occur during refrigerated storage, although the reductions were not significantly different for the sausage samples held at 4°C for 0, 24, or 48 h. A total reduction of 4.03 log CFU per sausage was recorded for the samples subjected to nisin and Pulsed Light at 9.4 J/cm^2 after 48 h of storage at 4°C (Figure 4.4).

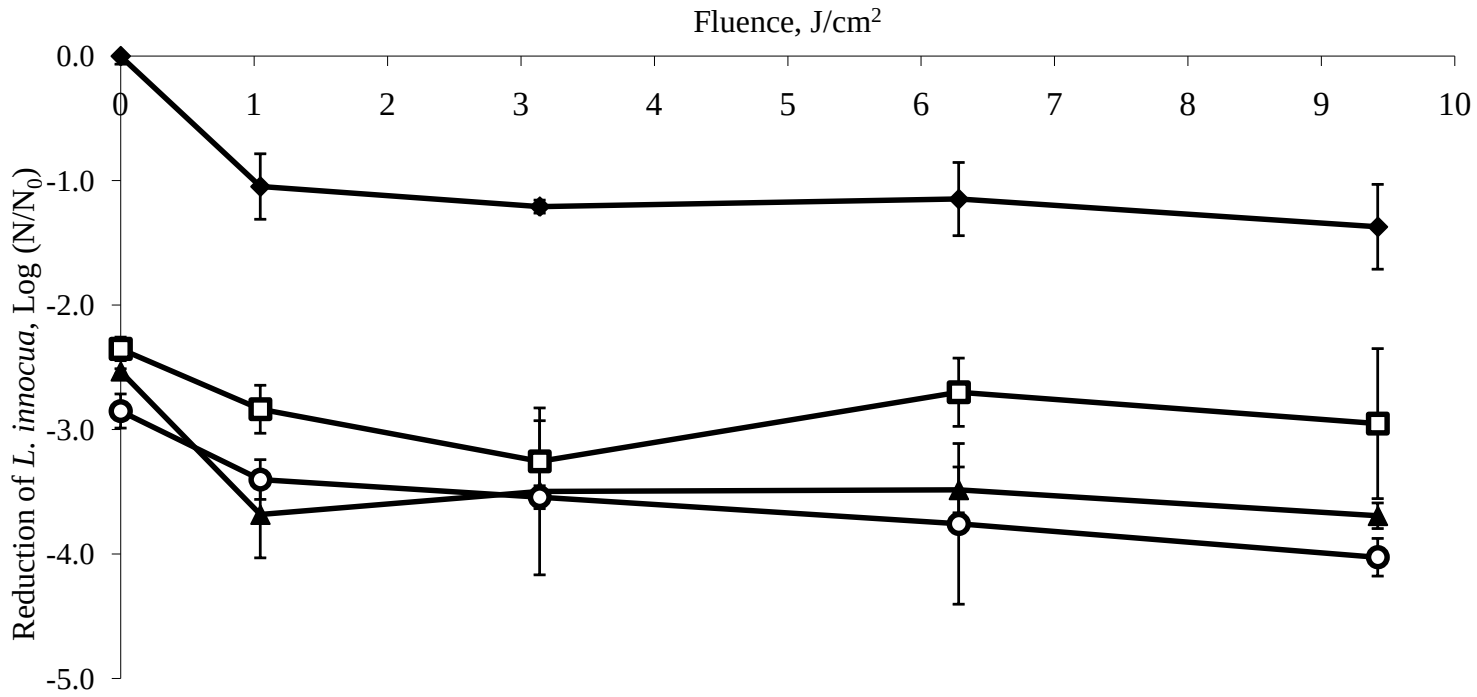

Figure 4.4. Reduction of *L. innocua* on Vienna sausages after treatment with nisin and various levels of Pulsed Light exposure: Pulsed Light only (♦), Pulsed Light plus nisin at 0 h (□), Pulsed Light plus nisin stored at 4 ± 2°C for 24 h (▲), and Pulsed Light plus nisin stored at 4 ± 2°C for 48 h (○). Reduction by nisin dipping alone was 2.35 log CFU per sausage. Starting inoculum was 6.5 log CFU per sausage (n = 3).

The combined treatment was able to repeatedly achieve a greater than 4-log reduction of *L. innocua,* with four replicates yielding very similar results. Although combination treatments involving Pulsed Light have not been reported previously, the ability to enhance the effectiveness of nisin by combining it with another antimicrobial treatment has been demonstrated. Combinations of nisin and organic acids (23, 24) or nisin and grape seed extracts in soy protein films (56) have also yielded better control of *Listeria* in frankfurters or sausages than did the individual treatments.

Under the conditions of this study, the sausages treated with Pulsed Light and/or nisin did not appear to undergo any noticeable changes in color or appearance compared with the untreated sausages. To accurately evaluate such aspects, a systematic sensory study is needed.

Long-term survival of *Listeria* on refrigerated sausages

The long-term survival of *L. innocua* was evaluated under refrigeration conditions on sausages subjected to the individual and combination treatments. A single level of Pulsed Light treatment was used at a fluence of 9.4 J/cm^2 (nine pulses). The results of two replicates are shown in Figure 4.5. Replicates are displayed separately because of quantitative differences observed between the two experiments.

In addition to the higher level of microbial inactivation achieved by the combination treatment compared with the individual treatments, the Pulsed Light plus nisin treatment also inhibited the growth of surviving *L. innocua* cells for a much longer period as compared with all other treatments. In the first replicate, the starting inoculum was 7.0 to 7.3 log CFU per sausage. After an initial drop of 4.61 log CFU per sausage immediately after the treatment (at day 0), the number of survivors for the combined treatment did not change over 28 days of refrigerated storage (Figure 4.5A). The greatest reduction, 5.10 log CFU per sausage, was observed for the combination treatment at day 4.

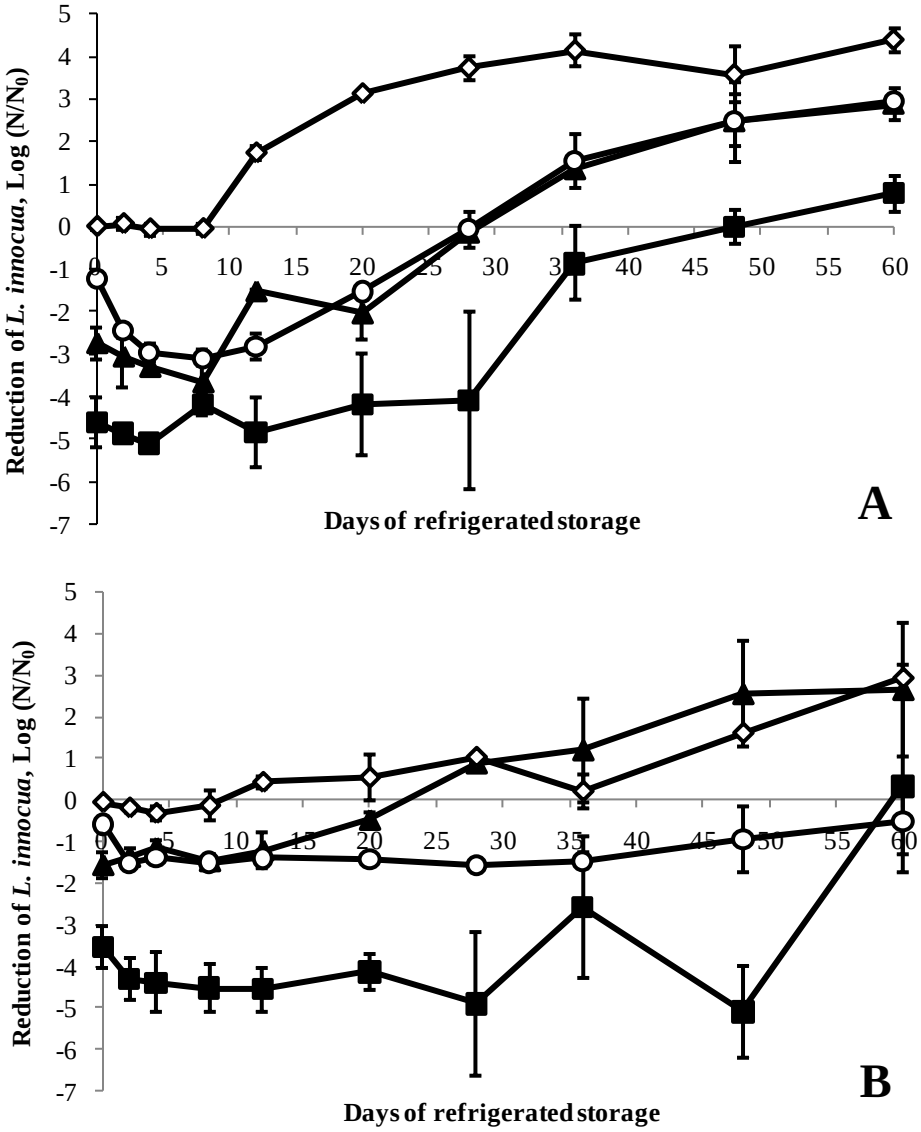

Figure 4.5. Reduction of *L. innocua* on Vienna sausages after the following treatments: Pulsed Light plus nisin (■), PL only (▲), nisin only (○), and untreated (◊). Samples were stored at 4 ± 2°C. Starting inoculum: (A) 7.0 to 7.3 log CFU per sausage (n = 4); (B) 6.5 to 6.6 log CFU per sausage (n = 4).

For the treatments with Pulsed Light only and nisin only, significant cell growth started to occur at the same time as for the untreated samples (after day 8) after an initial reduction of 2.74 and 1.24 log CFU per sausage, respectively. After 28 days of storage, the *L. innocua* counts increased to more than 3 log CFU above the initial inoculation level for the untreated samples. The number of survivors reached the initial inoculum level after 28 days in both the nisin- and Pulsed Light-treated samples and after approximately 40 days in samples from the combined treatment.

For the second replicate (Figure 4.5B), the starting inoculum was 6.6 log CFU per sausage. In the combined treatment, there was an initial reduction of 3.53 log CFU per sausage, with a reduction of 4 to 5 log CFU per sausage during the 28 days of storage. By day 60, the combined treatment counts had reached the initial inoculum level. Similar to the combined treatment, the number of survivors for the nisin treatment did not change over 28 days. The Pulsed Light treatment produced an initial reduction of 1.58 log CFU per sausage, but microbial counts became similar to those of the untreated samples after 12 days. After 60 days, the untreated (control) and Pulsed Light treatment samples had *L. innocua* counts about 3 log CFU per sausage higher than the initial inoculum level.

These results demonstrate that the combination treatment of nisin and Pulsed Light can significantly reduce *L. innocua* on RTE food surfaces and is more effective for delaying cell growth than is either treatment alone. Similar results were reported by Samelis (48, 49), who found that combinations of nisin and different organic acids used against *L. monocytogenes* extended the shelf life of frankfurters much longer than did the individual antimicrobials.

In this study, PL is believed to have caused damage to microbial DNA, whereas the bactericidal and bacteriostatic activity of nisin was probably the result of both membrane pore formation and the disturbance of cell wall biosynthesis. When

used together, the two treatments may cause major damage to bacterial cells, preventing the recovery of sublethally injured cells and significantly impeding the growth of the surviving cells. Like the use of organic acid or salt dips to control postprocessing *Listeria* contamination (5, 23, 24, 48, 49), the use of a nisin dip and Pulsed Light together provide another option for improving the safety and shelf life of RTE meat products.

Pulsed Light and nisin had additive antimicrobial effects when applied to the surface of Vienna sausages inoculated with *L. innocua*. The combination treatment also significantly delayed the growth of survivors as compared with the individual treatments. Because the results of this study indicated that *L. innocua* can be used as an indicator organism for the pathogenic *L. monocytogenes* in Pulsed Light treatment studies, the combination treatment should efficiently control *L. monocytogenes* on the surface of RTE meat products. The advantage of using Pulsed Light to reduce postprocess contamination is that this treatment would reduce the amount of chemical compounds need for such applications. Preliminary data also indicate that a unique attribute of Pulsed Light treatment is the possibility of applying it through UV-transparent packaging material. This could enable the development of an in-package terminal antimicrobial treatment, which would represent a quality step in ensuring the safety of RTE food products.

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CHAPTER 5

EFFECT OF PULSED LIGHT TREATMENTS ON THE GROWTH AND RESISTANCE BEHAVIOR OF *LISTERIA MONOCYTOGENES* 10403S, *LISTERIA INNOCUA*, AND *ESCHERICHIA COLI*[1]

ABSTRACT

Pulsed Light treatment has been shown to effectively inactivate a large proportion of contaminating bacteria on surfaces and in clear solutions. An important issue that needs to be investigated is whether repeated Pulsed Light treatment of causes any changes to the growth and resistance behavior of the surviving bacteria. To test this, three challenge microorganisms were used: *L. monocytogenes*, *L. innocua*, and *E. coli*. Cells of the challenge bacteria were suspended in Butterfields Phosphate Buffer (10^8 CFU/mm). 1 ml volumes were placed into glass chambers (2.54 x 5.08 cm) in a RS-3000C SteriPulse unit (Xenon Corp., MA), at 50 mm from the lamp, and exposed to low and high Pulsed Light doses (fluence levels of 1.1 and 10.1 J/cm^2, respectively). Survivors of the Pulsed Light treatment were enumerated, then isolated, re-grown and exposed again to Pulsed Light treatment. The cycle was repeated for a total of ten times. After each cycle of exposure, Pulsed Light survivor isolates and untreated cells were grown at 37°C with shaking for 24 hr in tryptic soy broth and their growth curves developed. Inactivation curves were also generated to examine possible differences between untreated, single treatment, and 10 cycles of repeatedly Pulsed Light treated cells. Growth curves of *L. monocytogenes*, *L. innocua* and *E. coli* isolates recovered from exposure to 1.1 to 10.1 J/cm^2 were not significantly different

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[1] *Experiments on E. coli were conducted by Lillian Hsu. Her assistance in this study is acknowledged and much appreciated.*

from the growth curves of untreated cells. Reduction levels of 3-4 and 5-6 log CFU were obtained after exposure to 1.1 and 10.1 J/cm^2, respectively, for the controls and the repeatedly treated and recovered isolates. These results show that Pulsed Light did not significantly change the growth kinetics or resistance to Pulsed Light of *L. monocytogenes, L. innocua,* or *E. coli* after multiple exposures. These findings have special significance for the use of Pulsed Light treatment in practical applications, as they demonstrate that this technology does not select for microorganisms with increased resistance.

INTRODUCTION

Pulsed Light utilizes high intensity broad spectrum light delivered in a short duration to inactivate a wide range of microorganisms including bacteria, viruses, and parasites. A growing concern for any antimicrobial treatment is the possibility that survivors could develop increased resistance to that particular treatment, as it has been observed for some bacterial strains to antibiotics. An important issue that needs to be investigated is whether repeated Pulsed Light treatment of causes any changes to the growth and resistance behavior of the surviving cells.

Throughout human history, there was a constant battle between infectious microorganisms and human beings including epidemics related to bubonic plague, malaria, and tuberculosis. With the discovery of penicillin in the 1940's, bacterial infections were dramatically reduced. Unfortunately, the widespread use of antimicrobials resulted in the development of resistance by bacteria. Resistant bacteria are a major concern to healthcare institutions and can lead to the failure of treatments of infected patients and problems with controlling infections that may lead to the spread of infection into the community. Examples of microorganisms that have developed resistance to antimicrobials include: methicillin-resistant *Staphylococcus*

aureus (3), vancomycin resistant *Enterococcus faecium* (8), and strains of *Listeria* (2), *Salmonella* (5), and *E. coli* (12) that have developed resistance to multiple antibiotics.

There are 4 major mechanisms of action by antibiotics: (1) inhibition or damage to the cell wall/cytoplasmic membrane, (2) inhibition of nucleic acid metabolism or synthesis, (3) protein biosynthesis, or (4) energy metabolism modification (9). While mechanisms of resistance to antibiotics by bacteria include: (i) mutations of the target protein; (ii) enzymatic inactivation of the antimicrobial; (iii) acquisition of genes for less susceptible target proteins from other species; (iv) preventing access to targets; and (v) bypassing the target (10). The acquisition of resistances may be acquired by mutations that can (i) alter the target protein; (ii) up-regulate enzymes that may inactivate the antimicrobial; (iii) up-regulate pumps that expel the antimicrobial; or (iv) down-regulate protein channels need for access (7). Additionally resistance may quickly develop through gene transfer between different bacteria via conjugation, transduction, and transformation that will provide the genes needed to resist particular antibiotics.

Pulsed Light inactivation is believed to be caused primarily by the absorbance of UV light. UV radiation induces detrimental effects to all living organisms from prokaryotes to humans. Repair systems have evolved as DNA damage repair has been necessary as life developed on earth, beginning with solar UV radiation induced damage, and is thus a highly conserved repair system with photolyase is among the oldest and simplest repair systems found in prokaryotes, archaebacteria, plants, and animals but not mammals (1). Resistance to UV radiation has been previously reported, but has not been correlated to the natural levels of solar radiation at the locations where isolates have been recovered. Moreover, it was observed that organisms that have resistance to UV radiation, such as *Deinococcus radiodurans*, are resistant due mechanisms including, rapid repair systems, multiple copies of their

genome, or they may contain UV absorbing pigments (4). In the study by Zenoff et al. (13), bacteria isolated from an extreme environment, a saline lake 4560 m above sea level in the northwestern Andes, showed resistance to UVB for prolonged periods of time, but this resistance could not be explained by pigmentation and mechanisms behind resistance were unknown.

To date, there is little understanding of the possible changes to the growth behavior and resistance to Pulsed Light treatment of the survivors of such treatments. This concern needs to be addressed before the extensive commercial adoption of any antimicrobial treatment or technology. Therefore, the main objective of this study was to conduct a systematic investigation of the effect of Pulsed Light treatment on the survival and growth characteristics of bacteria repeatedly exposed to both low and high treatment doses. Specifically, the objectives of this study were to: (i) expose isolates of *Listeria* and *E. coli* to low and high fluence levels, of up to ten repeated exposures (ii) examine and compare the growth behavior of unexposed cells and of survivors isolated after repeated exposure to Pulsed Light (iii) examine and compare any differences in Pulsed Light inactivation behavior of the challenge organisms after repeated exposure to low and high levels of Pulsed Light treatments.

MATERIALS AND METHODS

Cultures

L. monocytogenes serotype 1/2a strain 10403S and *L. innocua* FSL C2-008 (environmental isolate from a smoked fish plant) were obtained from the frozen culture collection of the Food Microbiology and Safety Laboratory at Cornell University. *E. coli* (ATCC25922) was obtained from the frozen culture collection of Dr. Randy Worobo at Cornell University. A fresh culture was obtained at least every 6 months and maintained on Trypticase Soy Agar (TSA, Difco, Becton Dickinson,

Sparks, Md.). Prior to experiments, the culture was streaked onto TSA and incubated for 24 ± 2 h at 37 ± 2°C.

Growth curve generation

A single colony was selected and inoculated into 5ml of tryptic soy broth (TBS, Difco, Becton Dickinson, Sparks, Md.) and incubated for 12-18 h at 37 ± 2°C, with shaking at 225 RPM. 50 µl was transferred to 5ml of fresh TSB and incubated at 37 ± 2°C, with shaking at 225 RPM, until an OD_{600} = 0.4 was reached. When an OD_{600} = 0.4 was reached, 50 µl was transferred to 5 ml of fresh TBS at desired temperature (37 ± 2°C), shaken at 225 RPM, and grown for 24 h. At various time points, 100 µl were taken from the growth tube and serially diluted in Butterfield's Phosphate Buffer (BPB); 100 µl were then plated in duplicate onto TSA plates and incubated for 24 ± 2 h at 37 ± 2°C.

Pulsed Light inactivation studies

Substrate preparation

For each culture, a single isolated colony was transferred into TSB and incubated for 24 ± 2 h at 37 ± 2°C with shaking at 225 RPM. A subsequent loop transfer into fresh TSB and incubation for 24 ± 2 h at 37 ± 2°C with shaking at 225 RPM was performed to produce an initial inoculum of about 10^9 CFU/ml. A 10-fold dilution was made in BPB to produce an initial inoculum of about 10^8 CFU/ml. One ml of the inoculum was placed in the chamber (25.4 × 50.8 mm) of a clear Lab-Tek I Chamber Slide well glass slide.

Pulsed Light treatments

The Pulsed Light treatments were performed using a RS-3000C SteriPulse System (Xenon Corp., MA), which consists of a controller unit and a treatment chamber that houses a Xenon flash lamp. Each inoculated sample was centered on an adjustable stainless steel shelf in the Pulsed Light chamber, at 50 mm beneath the

Xenon lamp, and exposed to Pulsed Light treatments of 1.1 and 10.1 J/cm^2 for low and high exposures, respectively, for the repeated treatments and up to 13.3J/cm^2 (12 pulses) for the development of inactivation curves. All Pulsed Light treatments were delivered at a frequency of 3 pulses per second.

Fluence measurements were taken using a pyroelectric head (PE25BBH) with a Nova II display (Ophir Optronics Inc., Wilmington, Mass.), with an aperture cover having a circular opening of 1 cm^2 and a pulse width setting of the meter of 1.0 ms. The pyroelectric head was placed at a known distance (50 mm) from the quartz face of the lamp. Pauses of at least 30 s between measurements were allowed in order to prevent overheating of the pyroelectric head.

Recovery and enumeration of survivors

Following the Pulsed Light treatment, the treated inoculum (1ml) was transferred to 7 ml of TSB and the glass chamber was rinsed twice with 1 ml of fresh TSB, adding the rinse TSB to the treated inoculum. The 10 ml liquid was serially diluted in BPB and 100 µl plated on TSA and incubated for 24 ± 2 h at 37 ± 2°C, after which survivors were counted. When counts below 25 CFU/ml were expected, 250 µl of the treated sample was plated onto each of four TSA plates and incubated for 24 ± 2 h at 37 ± 2°C to increase the sensitivity of plating.

Isolation and subsequent treatment of Pulsed Light survivors

Isolates recovered from the survivors of the initial Pulsed Light exposure were streaked for purity onto TSA and incubated for 24 ± 2 h at 37 ± 2°C. Isolates from the TSA plate were grown in TSB, placed in a liquid suspension, exposed again to Pulsed Light, then recovered and the survivors of the secondary Pulsed Light treatments enumerated as indicated above. A total of ten repeated exposures and recoveries were conducted, at both low (1.1 J/cm^2) and high (10.1 J/cm^2) fluence levels. The study was conducted in triplicate.

Statistical analysis

Analysis of variance (ANOVA) and the Tukey's honestly significant differences test were used to determine whether differences between treatments were significant at $P < 0.05$ using the statistical package JMP 7.0.0 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Inactivation curves for the Pulsed Light treatment of *L. innocua, L. monocytogenes,* and *E. coli* cells displayed an initial rapid reduction, followed by a plateau region (Figures 5.1, 5.2, and 5.3). A clear plateau was reached for *L. innocua* at fluence levels > 6 J/cm^2, with a reduction of slightly more than 5 log CFU/ml in the plateau region. Similarly for *L. monocytogenes,* the plateau was reached at a fluence level of about 6 J/cm^2, and a maximum reduction of 5.94 log CFU/ml was reached. For *E. coli* the plateau was reached at a higher dose, at a fluence of about 10 J/cm^2. A greater maximum reduction (6.26 log CFU/ml) was reached for *E. coli* than for either *Listeria* strain.

The reasons for the existence of a plateau could be either the presence of a microbial population with increased resistance to Pulsed Light, or the fact that a small proportion of the initial microflora has not been effectively exposed to Pulsed Light, due to shading effects, or both. In order to verify if the characteristics of the survivors from the plateau region are different from those of the initial population, growth curves and Pulsed Light inactivation curves were built in parallel for cells that have not been previously exposed to Pulsed Light treatment (control) and isolates recovered after exposure to various levels of Pulsed Light. Specifically, survivors from the initial portion of the inactivation curve (low fluence) and from the plateau region (high fluence) were isolated and repeatedly exposed to the same fluence level (either low or

high), for a maximum of ten times.

For all three microorganisms, cells unexposed to Pulsed Light (control) were compared to isolates recovered from the survivors of Pulsed Light treatment at low fluence (1.1 J/ cm^2) and high fluence (10.1 J/ cm^2) exposure, respectively (Figures 5.1, 5.2, and 5.3). Inactivation curves showed an initial rapid reduction after initial Pulsed Light exposure followed by plateaus reached at a treatment level of ~10 J/cm^2. The greatest reductions reached in the plateau region were of 5 to 6 log CFU/ml for both *L. innocua and L. monocytogenes* and while *E. coli* reached reductions of up to 7 log CFU/ml. As observed in Figures 5.7 and 5.8, repeated exposure and recovery of bacteria to low and high fluence levels of Pulsed Light did not induce any statistically significant differences in the inactivation curves as compared to the control (untreated cells).

The growth curves of *L. innocua, L. monocytogenes,* and *E. coli* are shown in Figures 5.4, 5.5, and 5.6 respectively. For *L. innocua* and *E. coli*, the growth curves for unexposed isolates were not statistically different from the growth curves of isolates that were exposed to 1.1 or 10.1 J/cm^2. For *L. monocytogenes*, slightly smaller populations, about 1 log less, were noticed for the time points in the stationary phases of the growth curves for the survivors exposed to 10 repeated treatments as compared to the untreated cells, but the cell counts after 24 h were not significantly different. Based on the experimental data, it can be concluded that, despite a somewhat slower growth of *L. monocytogenes* survivors, , the overall growth behavior of the three organisms evaluated in this study was not significantly affected by repeated exposure to Pulsed Light treatments.

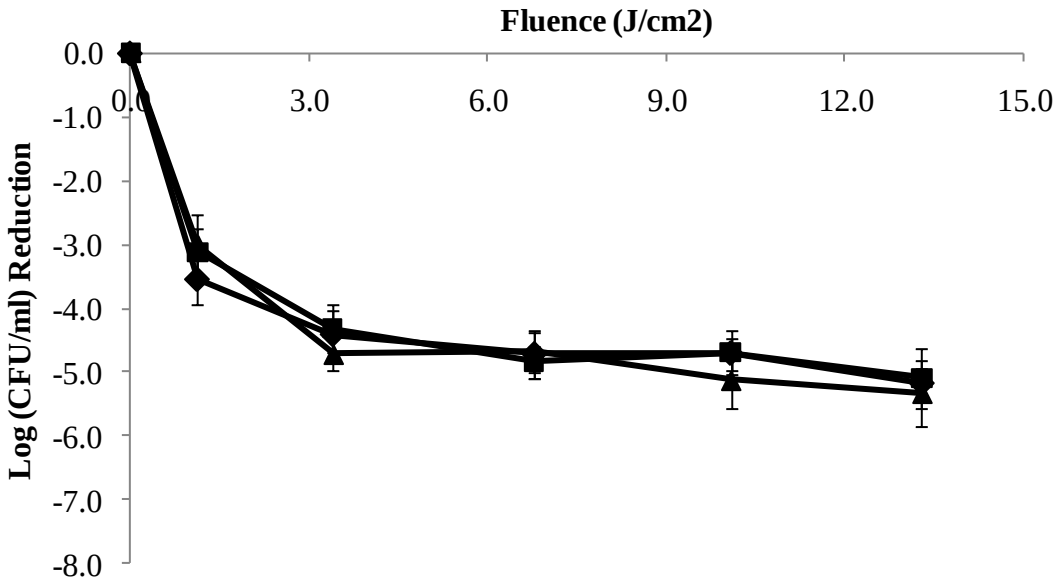

Figure 5.1 Reduction of *L. innocua* after exposure to Pulsed Light. Dosage of Pulsed Light exposure: 0 J/cm^2 (♦),10 x 1.1 J/cm^2 (■),and 10 x 10.1 J/cm^2 (▲) (n = 3).

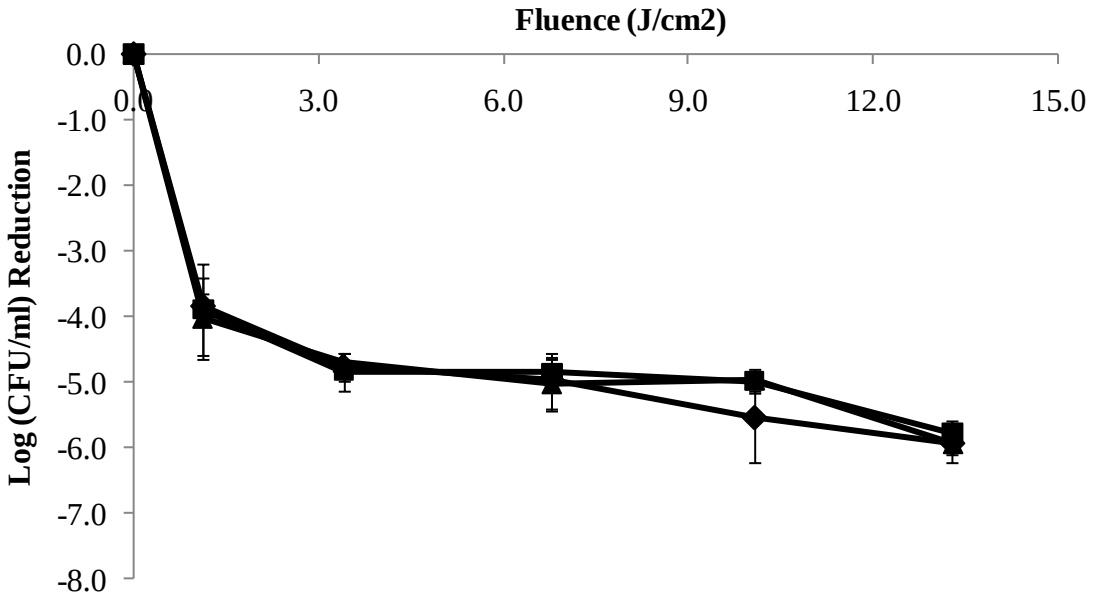

Figure 5.2 Reduction of *L. monocytogenes* after exposure to Pulsed Light. Dosage of Pulsed Light exposure: 0 J/cm^2 (♦),10 x 1.1 J/cm^2 (■),and 10 x 10.1 J/cm^2 (▲) (n = 3).

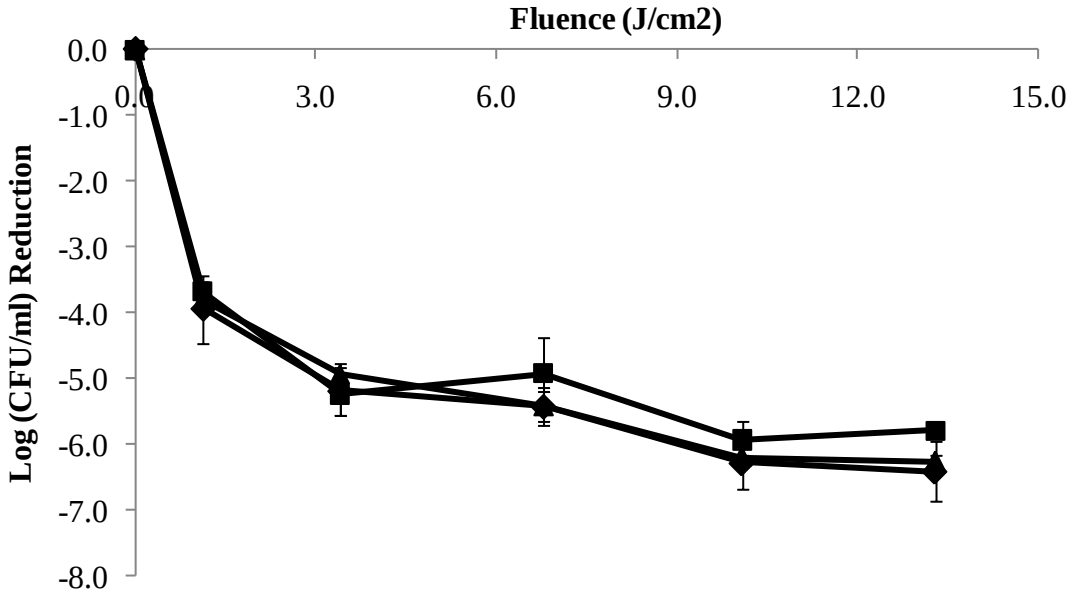

Figure 5.3 Reduction of *E. coli* after exposure to Pulsed Light. Dosage of Pulsed Light exposure: 0 J/cm^2 (♦),10 x 1.1 J/cm^2 (■),and 10 x 10.1 J/cm^2 (▲) (n = 3)

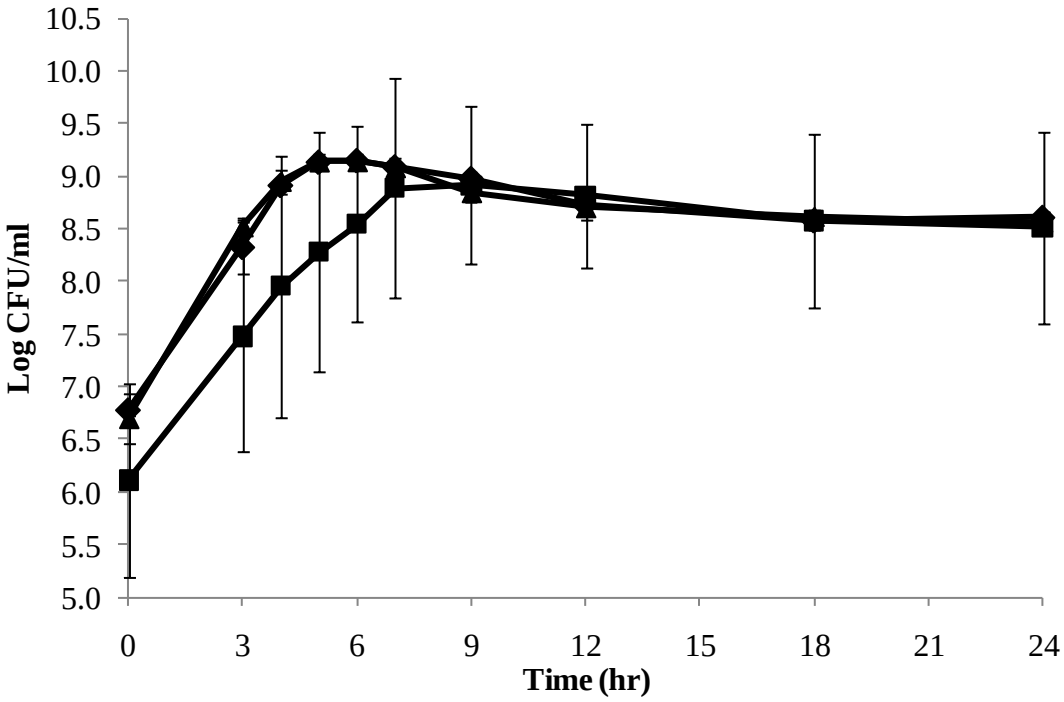

Figure 5.4 Growth of *L. innocua* in TSB at 37°C with shaking at 225 RPM. Dosage of Pulsed Light exposure: 0 J/cm^2 (no previous Pulsed Light exposure) (♦), 10 x 1.1 J/cm^2 (■) and 10 x 10.1 J/cm^2 (▲).

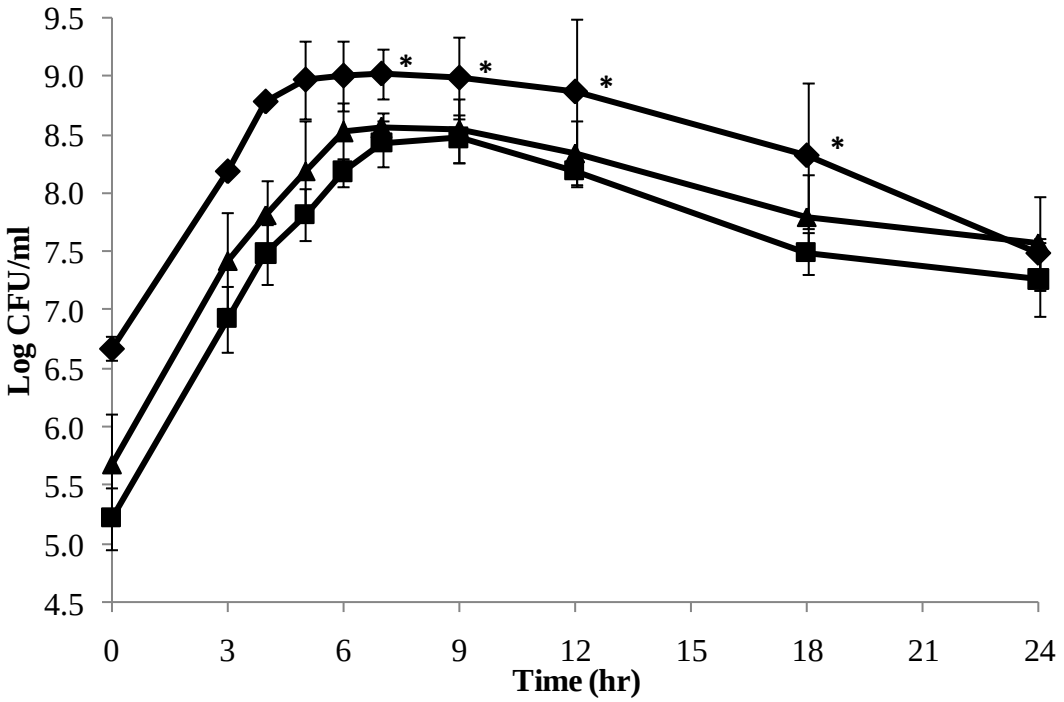

Figure 5.5 Growth of *L.monocytogenes*10403s in TSB at 37°C with shaking at 225 RPM. Dosage of Pulsed Light exposure: 0 J/cm^2 (no previous Pulsed Light exposure) (♦), 10 x 1.1 J/cm^2 (■) and 10 x 10.1 J/cm^2 (▲). ** Significant differences ($p<0.05$) at that time point between different Pulsed Light exposures* (n = 3).

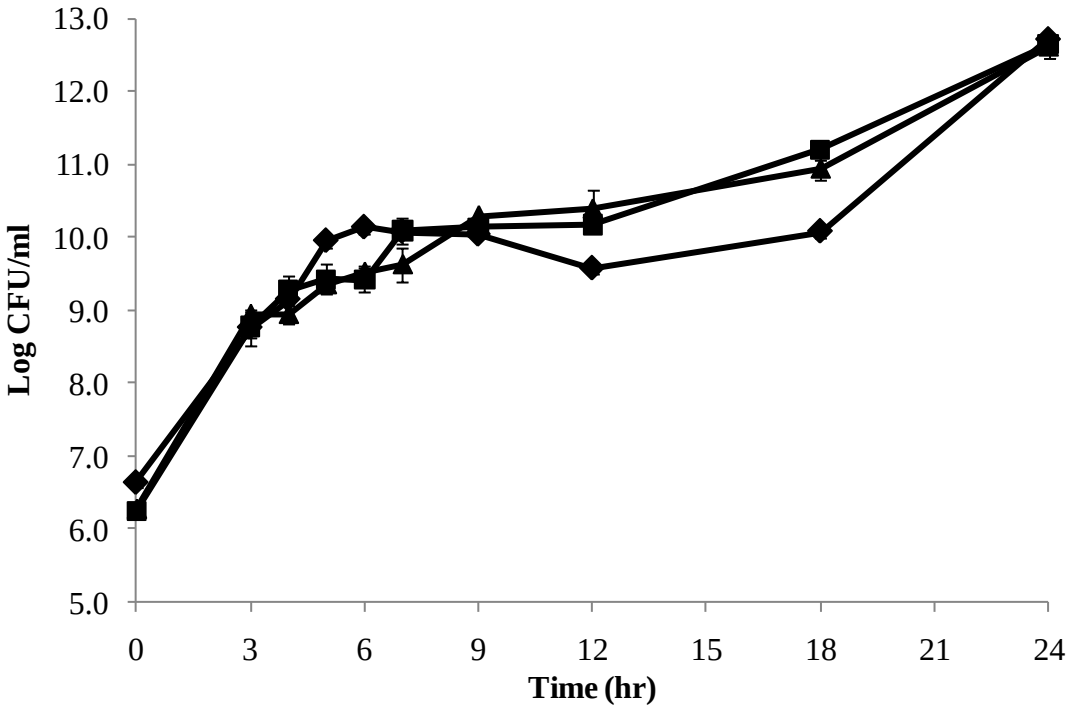

Figure 5.6 Growth of *E. coli* in TSB at 37°C with shaking at 225 RPM. Dosage of Pulsed Light exposure: 0 J/cm^2 (no previous Pulsed Light exposure) (♦), 10 x 1.1 J/cm^2 (■) and 10 x 10.1 J/cm^2 (▲).

Figures 5.7 and 5.8 show the growth reached after 24h and inactivation for *L. monocytogenes* and *L. innocua* for each of the ten exposures to low and high levels of Pulsed Light fluence. For the growth studies, there were no significant differences after each exposure and recovery, with total counts reaching between 7.5 to 8.0 CFU/ml. For the 1.1 J/cm^2 treatments, reductions were between 3 to 4 log CFU/ml for each of the repeated exposures, while treatment with 10.1 J/cm^2 resulted in reductions of 5 to 6 log CFU/ml. The results of the low and high fluence exposures were thus consistent and not significantly different between the repeated treatments.

Microbial inactivation by Pulsed Light treatment is believed to be due primarily to the absorption of UV light by nucleotides, which results in the formation of thymine-thymine, cytosine-thymine, and cytosine-cytosine pyrimidine dimers (6). These formations create deformations in the DNA and impair replication and transcription without proper repair. Repair occurs when the enzyme photolyase binds to the dimer and utilizes light energy to repair the dimer (6). Repair of damaged DNA may also be repaired by the nucleotide excision of the damaged portion of DNA, replacement of the nucleotides by DNA polymerase, and sealed by DNA ligase (11). Nucleotide excision repair protect cells from DNA damage induced by factors other than UV light and involved 3 proteins, UvrA, UvrB, and UvrC which identify and repair the damage.

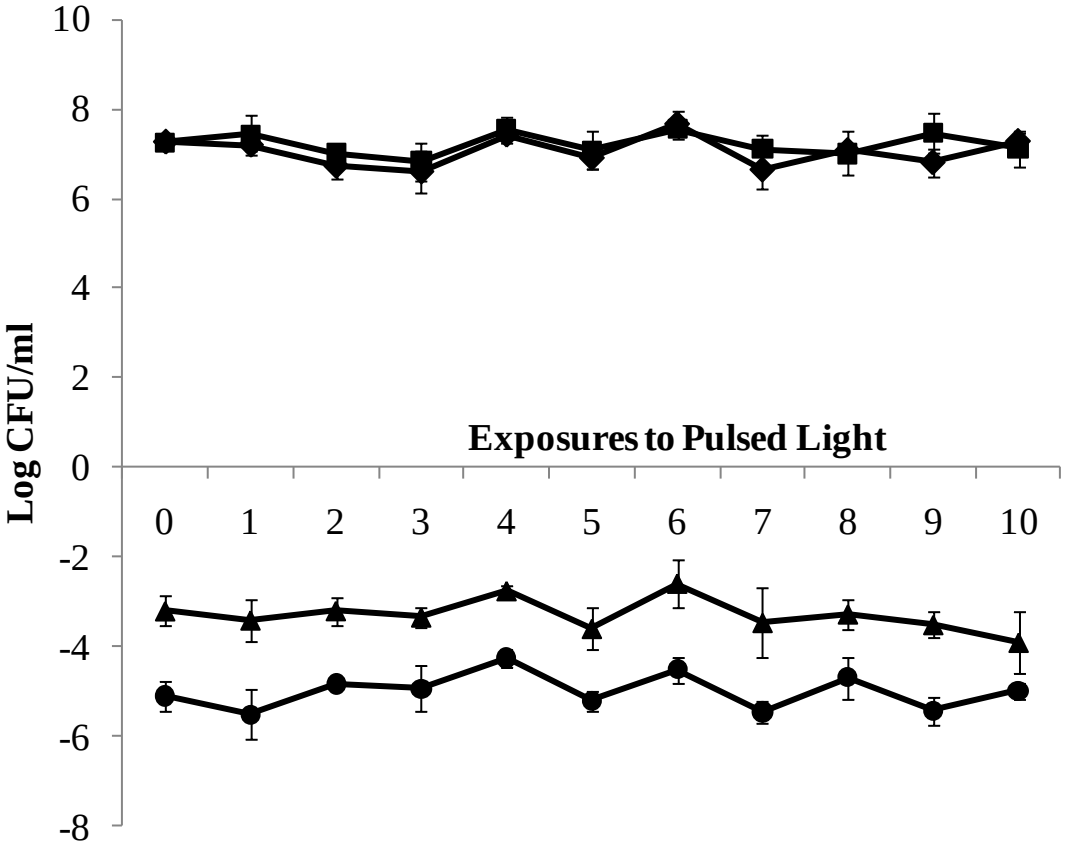

Figure 5.7 *L. innocua* 24 hr growth (upper) after Pulsed Light exposure to 1.1 J/cm^2 (♦) or 10.1 J/cm^2 (■) and reduction (lower) after Pulsed Light exposure to 1.1 J/cm^2 (▲) or 10.1 J/cm^2 (●) for each of the 10 exposure passages.

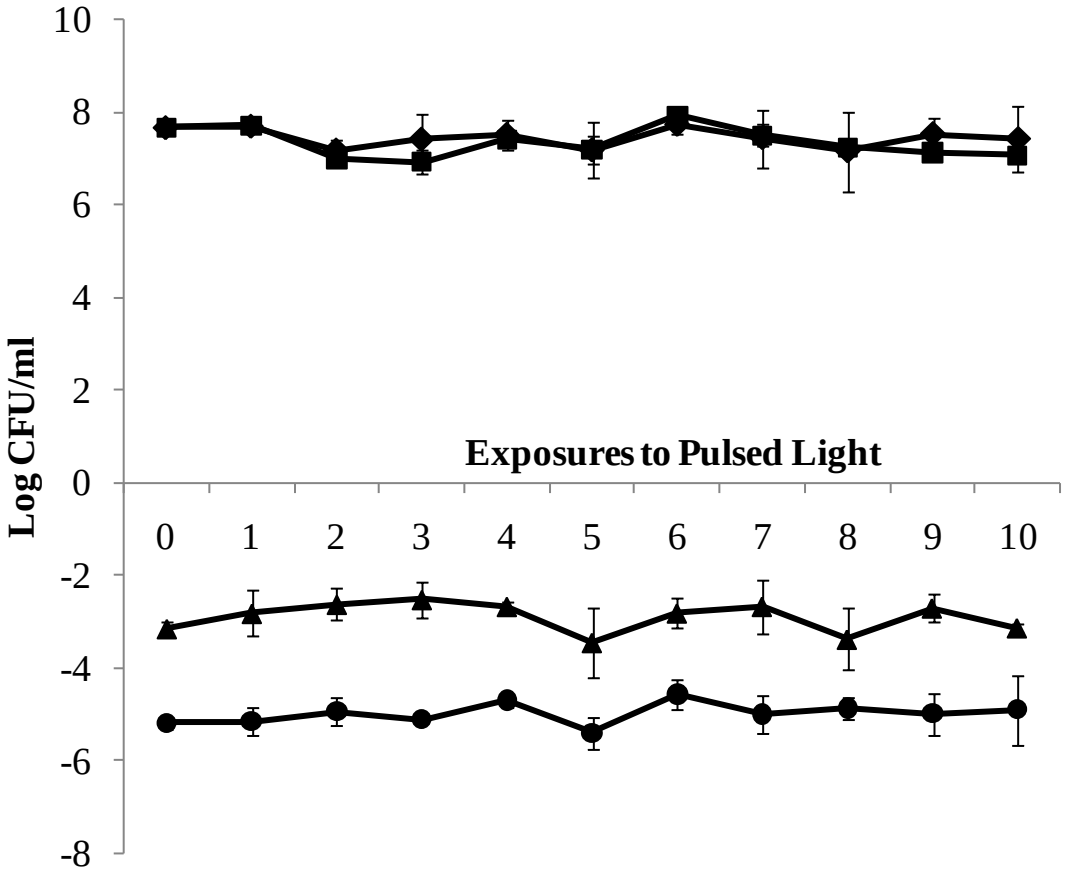

Figure 5.8 *L. monocytogenes* 24 hr growth (upper) after Pulsed Light exposure to 1.1 J/cm^2 (♦) or 10.1 J/cm^2 (■) and reduction (lower) after Pulsed Light exposure to 1.1 J/cm^2 (▲) or 10.1 J/cm^2 (●) for each of the 10 exposure passages.

These repair systems have developed alongside bacteria to protect the cell from UV inducedDNA damage, thus resist the effects of UV treatment – is different than the ability to resist an antibiotic compound, where the addition of a particular gene or mutation may grant resistance. It is possible that a rapid rise in resistance to UV light based treatments such as Pulsed Light was not seen, since UV resistance is not simply achieved via the acquisition of specific genes.

While the specific mechanisms responsible for this behavior are not yet fully understood, the results of this study demonstrate that Pulsed Light treatments can significantly reduce the populations of *L. monocytogenes*, *L. innocua*, and *E. coli* in clear liquids, while the cells that survive the initial treatment do not exhibit increased resistance or sensitivity. The surviving isolates for the bacterial strains investigated in the study did not show changes in their growth kinetics and did not select for increasing resistance when compared to unexposed, wildtype isolates, even after multiple Pulsed Light treatments. These findings suggest that Pulsed Light could be implemented in the food industry for a rapid reduction of bacteria in clear liquids without selecting for enhanced resistance.

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CHAPTER 6

COMPARATIVE ANAYLSIS OF GENE EXPRESSION PROFILES FROM *LISTERIA MONOCYTOGENES* FOLLOWING EXPOSURE TO PULSED LIGHT AND CONTINUOUS ULTRAVIOLET LIGHT

ABSTRACT

Pulsed Light treatment uses a broad spectrum of light that includes UV, visible, and NIR light to inactivate microorganisms. While it is generally believed that the UV portion of the spectrum is responsible for the microbicidal effects in Pulsed Light treatment, a direct comparison between the effects of Pulsed Light and continuous UV treatment at the cellular level has not yet been conducted. In this work, the cellular response of Pulsed Light treated and UV treated *Listeria monocytogenes* cells, at the same level of microbial inactivation, was investigated using whole genome DNA microarray analysis. In addition, the effect of the visible and NIR portions of the spectrum on the challenge organism was also evaluated by filtering out the UV component of the spectrum. First, suspensions of *Listeria monocytogenes* were treated with 3.20 J/cm^2 of Pulsed Light and 33mJ/cm^2 of UVC (254nm) from a germicidal lamp, respectively, which yielded a comparable level of inactivation In a separate experiment, cells were exposed to a similar dose (3.20 J/cm^2 and 3.25 J/cm^2) of full spectrum Pulsed Light and UV-blocked Pulsed Light ($\lambda > 400$ nm). Exposure to UV blocked Pulsed Light did not induce any inactivation; on the contrary, these treatments resulted in a slight increase in cell counts (0.14 log CFU/ml). Whole genome microarray experiments were performed to identify differential gene expression by *L. monocytogenes* after the three different treatments. Microarray analysis using a cutoff of $\geq$1.5 fold change and adjusted $P < 0.05$ revealed that 80 and 39 multiple stress related genes, motility genes, and transcriptional redulators showed

higher transcription levels for Pulsed Light and UV light treatments, respectively, as compared to the untreated samples. Blocked UV Pulsed Light resulted in 131motility related and cell membrane related genes that showed lower transcription levels, and no genes with higher transcription levels than the untreated cells. Both the inactivation data and the transcriptional gene response of *Listeria monocytogenes* as a result of exposure to continuous UV light and the UV and non-UV spectral ranges of Pulsed Light suggest that the microbial killing effects in Pulsed Light treatment are primarily caused by the UV portion of the spectrum, while the visible and NIR portion of the spectrum do not inflict any lethal effects on the exposed cells.

INTRODUCTION

The antimicrobial effects of ultraviolet (UV) treatment are known for years, and this technology has already commercial applications, both in the food industry as well as in water treatment. Pulsed Light technology emerged in recent years as another light based alternative to thermal treatment for killing undesirable microorganisms, and has been sometimes considered to be more potent than continuous UV treatment. UV uses germicidal mercury lamps that emit light at 254 nm to kill microorganisms, while Pulsed Light treatment uses Xenon lamps that emit short, intense pulses of light that includes UVC (200-280 nm), UVB (280 – 315nm), UVA (315 – 400nm), visible (400 – 700 nm), and near IR (700 - 1,100 nm). The mechanism of inactivation in UV treatment is believed to be the formation of pyrimidine dimers between adjacent bases (5). Without proper repair, the dimers interfere with DNA replication and transcription, and eventually lead to cell death. Due to the large amount of UV light generated in Pulsed Light treatment, it is generally believed that the absorbption of UV by nucleic acids and the resulting damage is the primary cellular target of Pulsed Light.

While there is evidence that the UV portion of Pulsed Light is responsible for the inactivation of microorganisms treated with Pulsed Light (73), additional cellular damage has also been reported (64, 71). Wekhof (28) showed *Aspergillus niger* spores with ruptured tops, which were hypothesized to have resulted from the escaping of overheated spore contents. The ruptures left empty regions as a result of the "evacuation" of its contents. This effect has not been confirmed by other studies. Takeshita (64) studied the effect of Pulsed Light treatment on *Saccharomyces cerevisiae* and noted that in addition to single strand breaks in DNA and pyrimidine dimers, there was also increased protein elution and structural changes, enlarged vacuoles, cell membrane distortion, and change in circular shape of the cells. Anderson (1) found only minimal temperature increases, <1°C, when treating bacteria cells and fungal spores on agar plates. Pulsed Light thermal effects have seen an increase of 91°C on the muscle surface of salmon positioned 3 cm from the lamp and exposed for 60 s or 180 pulses (46). However, since the thermal effects were observed on the substrate level, they do not necessarily offer a prediction of what might be happening at a cellular level.

Pulsed Light studies have focused on its ability to inactivate various microorganisms in different substrates (4, 19, 22, 26, 29, 31, 35, 39, 40, 46, 51, 57, 59, 66, 67, 68) but studies have not examined cellular responses following treatment. Therefore, the main objective of this work is to investigate the transcriptional response of bacterial cells exposed to UV and Pulsed Light, and to evaluate the similarities and/or differences between the mechanisms of inactivation between the two treatments. For this purpose, *L. monocytogenes* has been chosen as a challenge organism.

L. monocytogenes is a psychotropic Gram positive, non-spore forming, facultative anaerobic foodborne bacterial pathogen that targets high risk groups such

as immunocompromised individuals, pregnant women, neonates and the elderly, as well as healthy people. *L. monocytogenes* has the ability to cause meningitis, septicemia, abortion, and a high mortality rate, of 20-30%. The Centers for Disease Control and Prevention estimated that in the United States there are approximately 2,500 cases of listeriosis yearly, leading to about 500 deaths per year (43). *L. monocytogenes* can tolerate salt concentrations up to 10%, pH range 5-9, and temperatures ranging from 1 to 44°C (38). Survival in this broad range of environmental conditions can lead to DNA damage that requires repair. The SOS response in cells is regulated by LexA and Rec A proteins. Typically, the LexA protein suppresses the SOS genes but RecA is activated by the detection of DNA damage. The SOS response is induced to repair DNA damage as well as repair replication forks that have been stalled by DNA damage (14). *L. monocytogenes* exposed to UV light can create dimer formations that can be repaired by reversing the damage using DNA photolyase in the presence of visible light (24) or removing the damaged portion of DNA with nucleotide excision repair (58). In *L. monocytogenes* and many other prokaryotes, the complex UvrABC endonuclease is responsible for this repair. Two UvrA proteins bind with UvrB to form a trimer that detects DNA damage by identifying distortions and unzips the DNA. Cuts are made 4 nucleotides downstream and 7 nucleotides upstream of the DNA damage and the damaged oligonucleotides are removed by UvrD, a DNA helicase. The gap in the DNA is filled by DNA polymerase and sealed by DNA ligase.

Whole genome microarray technology was selected as the means to investigate the genomic response of the bacterial cells to UV and Pulsed Light, respectively. DNA microarray technology was developed by Schena (52) as a method to monitor the expression of many genes densely positioned in parallel on a small glass chip. This has become a powerful tool in genetic analysis by allowing the quantitative

examination of thousands of genes at a time. Microarrays have a broad application to many areas including genomic, biomedical, pharmaceutical, and food safety research.

Microarrays utilize a number of techniques that have been developed over time. These include DNA complementary base pairing identified by Watson and Crick, DNA binding to complementary RNA or DNA, immobilization of DNA to surfaces, determining nucleic acid sequences, and DNA blotting hybridization or Southern blotting. At its most basic, microarrays take advantage of base pairing of complementary sequences through hybridization. Probes are created with known DNA sequences of interest that are made up of oligonucleotides that are specific to each gene. These probes are orderly arranged and a few nanoliters are robotically deposited on the surface of a chip or slide made of glass or silicon and immobilized to the slide surface. Densities of spots can reach into the thousands per square centimeter. From the target microorganism of interest, total RNA is collected and reverse transcribed to produce single strand cDNA. The cDNA is either labeled directly, by incorporating labeled nucleotides into the cDNA, or indirectly, in which case, modified nucleotides are utilized to generate cDNA and then following synthesis; the cDNA is labeled with a fluorescent dye or marker. In a two channel microarray, the target cDNA is labeled with one dye and the control or reference cDNA is labeled with a different dye to allow comparisons between treatments. The dyes most commonly used fluoresce green or red when excited by their respective wavelength. The cDNA is denatured and the slide is blocked to prevent non-specific cDNA binding to non-target DNA spot sites, and hybridization with the target DNA or RNA is allowed to occur. Following hybridization, unbound cDNA is removed and the slide is imaged. Two lasers, one for each of the samples and dye, are used to excite the fluorescently-labeled DNA on each spot and a scanner captures the resulting image. Software analyzes the intensity of each spot and the relative cDNA that was affixed to

each spot will be shown as a ratio of red to green, the color of the dyes for labeling. The ratios allow a relative expression of each gene between the control and experimental sample. The experiments are typically repeated with the difference being the alteration of the dye association, from target to control, to correct for any differences influenced by the dye itself (7).

Microarrays have the ability to be very powerful tools in understanding bacteria under a wide range of conditions. Microarray technology has been successful in analyzing global gene expression of pure cultures under numerous conditions. These include but are not limited to anaerobic conditions for fermentation versus nitrate/nitrite electron acceptors (75), resistance to ionizing radiation (37), response to drugs and drug discovery (15), pathogen detection in environmental samples (10) as well as environments such as limited iron, acidic, or cell densities (53, 54). Additionally, microarrays have also been developed to detect bacteria and examine microbial community structure, function, and microbial ecology dynamics (76). Microarrays have been specifically created to study the genomic response of *Listeria* for a variety of purposes, including divergences of genes between different lineages, strain differentiation between closely related isolates, and finding genetic markers for epidemiological studies (8, 11). The role that stress-responsive alternative sigma factor σ^B plays as well as the cellular response during invasion of host cells has been examined with microarrays to help identify the mechanisms that allow *L. monocytogenes* to survive a variety of environments and persist in them (13, 33, 42, 27). Specific responses to salt, heat, cold, or osmotic stresses have been closely examined using whole genome microarray experiments to identify numerous previously known and unknown genes that respond when the cells are exposed to different conditions and stresses (50, 12, 28). The technology has allowed previously unknown genes and their functions to be identified.

The aim of this study was to examine the global transcriptional response of *L. monocytogenes* to conventional germicidal UV light, and Pulsed Light.. This study also attempts to identify any gene expression effects caused by the visible and NIR spectrum of Pulsed Light to determine if the inactivation is solely UV related or if there are contributions by the other spectral components of Pulsed Light. This will help elucidate the mechanisms of inactivation for the two treatments.

MATERIALS AND METHODS

Bacterial strain and growth conditions

L. monocytogenes serotype 1/2a strain 10493S (6) from the culture collection of the Food Microbiology and Safety Laboratory at Cornell University was used in this study. For each experiment, *L. monocytogenes* was streaked onto Brain Heart Infusion (BHI; Difco, Sparks MD) from glycerol stock cultures stored at -80°C and incubated at 37°C for 24h. Cultures were maintained on slants of Tryptic Soy Agar (TSA; Difco, Sparks MD) for 3 months at 4°C. For experiments, stock culture was streaked to TSA plates for isolation and incubated at 37°C for 24h. A single colony was subsequently inoculated into 5 ml of Defined Medium (DM) for *L. monocytogenes* (48) and incubated for 12 – 18h at 37°C with shaking (225 RPM). Following overnight incubation, 50 µl of the culture was transferred to 5 ml of fresh pre-warmed (37°C) DM and incubated at 37°C with shaking (225 RPM) until an optical density at 600 nm (OD_{600}) of 0.4 (early log phase) was reached. When the culture reached OD_{600} = 0.4, 1.0 ml was transferred to 100 ml of pre-warmed (37°C) DM and incubated at 37°C with shaking (225 RPM) until an OD_{600} = 1.0 plus 3h was reached (early stationary phase).

Pulsed Light and UV light treatments

Once cells reached early stationary phase, 15ml of the inoculum was transfered into a petri plate of 100 mm diameter, which was then placed onto an MS 3 basic orbital shaker (IKA Works, Wilmington, NC) and set to 500 RPM to generate turbulence and ensure uniform treatment for both the UV light and Pulsed Light treatments (51). Bacterial cells were collected for RNA isolation stress conditions as follows: (i) Pulsed Light vs. UV light treatments and (ii) full spectrum Pulsed Light vs. UV light blocked Pulsed Light.

For UV light treatments, the inoculum was exposed to a germicidal lamp (254nm) located inside a biological safety cabinet (NuAire 425, NuAire Laboratory Equipment Supply; Plymouth, MN). Treatments lasted 120s and cells were exposed to 33 mJ/cm^2 of light. UV fluence measurements were made using a UVX radiometer (Ultra-Violet Products; Upland, CA), in triplicate. For the control treatments, the inoculum was placed on the shaker and shaken for 120s while the lamps in the cabinet were turned off.

For Pulsed Light treatments, the petri plate containing the inoculum and the shaker were placed into a RS-3000C SteriPulse System (Xenon Corporation, Woburn, MA). To avoid both the change in the spectral composition and the pathway of the light caused by the presence of the shaker inside the chamber, as well as any damage to the shaker, all the elements of the shaker were wrapped in Aluminum foil. The Pulsed Light system consists of a treatment chamber that houses a Xenon flash lamp and a control unit that delivers Pulsed Light at a frequency of 3 pulses per second with a pulse width of 360 μs. For Pulsed Light treatments, cells were exposed to a fluence of 3.20 J/cm^2 (6 pulses). To evaluate the effect of removing the UV spectrum from Pulsed Light, a UV-blocking filter, which only allowed the transmission of $\lambda >$ 400nm was placed in the Pulsed Light unit at 5 mm from the lamp housing on a solid

metal shelf that was designed to limit the passage of light around the filter or secondary reflections. For UV blocked treatments, the cells were exposed to the same fluence as in the case of the full spectrum Pulsed Light (actual fluence was 3.25 J/cm^2). Due to the removal of the high energy UV portion of the spectrum, a number of12 pulses were necessary to reach the same fluence (as compared to 6 pulses in the full spectrum Pulsed Light (73). The fluence of the Pulsed Light treatments were measured using a pyroelectric head (PE25BBH) with a Nova II display (Ophir Optronics Inc. Wilmington, MA). The pyroelectric head was placed in the center of the treatment chamber and adjusted to the same distance as the cells during treatment. A stainless steel aperture cover was placed over the head and only allowed a 1 cm^2 circular opening of exposure on the detector's surface. For the Nova II display, the unit was set to a pulse width of 1.0 ms and the wavelength settings was < 0.3. Fluence measurements were performed in triplicate with pauses of at least 60 s between each measurement to prevent possible overheating of the sensor.

Following each treatment, a 1 ml sample was taken for cell enumeration and the remaining cell culture was transferred to a foil wrapped centrifuge tube and placed in the incubator at 37°C with shaking (225RPM) for 5 min. Following the 5 min incubation, cells were harvested and used for RNA isolation. For cell culture enumeration, dilutions were performed in Butterfield's Phosphate Buffer and 100 µl was spread plated in duplicate onto TSA plates and incubated for up to 48hr at 37°C. Three biological replicates were performed for each experimental condition.

Total RNA isolation

Following 5 min of shaking, 1.5 ml of 10% phenol: 90% ethanol was added to the culture and centrifuged for 10 min at 13,000 RPM and 4°C. After centrifugation, the supernatant was removed and the cells resuspended in 5ml of Ambion Tri Reagent (Applied Biosystems; Foster City, CA) with 0.1 mm zirconium beads, in an 8 ml tube.

The sample was homogenized in for 5 min in a Mini-Beadbeater-8 (BioSpec Products, Inc. Bartlesville, OK). Following homogenization, RNA isolation was carried out according to the manufactures directions, with the following changes: (1) Following addition of isopropanol, centrifugation at 12,000*g* was for 20 min at 4°C. (2) After washing with 75% ethanol, a second wash with 100% ethanol was conducted. (3) The RNA pellet was resuspended in nuclease free water. The RNA was checked for quality by performing UV spectrophotometer readings at 260 and 280 nm with the NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE). 100 µl of total RNA was then combined with 10 µl RNasin (Promega, Madision, WI), 20 µl 10X DNase buffer (Promega), 70 µl RQ1 DNase (Promega) and 2 µl 0.1M dithiothreitol (DTT) (Invitrogen Inc., Carlsbad, CA) and incubated at 37°C for 1h. Following incubation, the RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA), as directed by the manufacturer. A third wash with 500 µl of 80% EtOH was added to the protocol prior to elution of the RNA. Following RNA cleanup, the RNA integrity was checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a RNA 6000 Nano kit (Agilent Technologies). If RNA was not used immediately, purified and unpurified RNA was stored at -80°C.

Microarray construction

Whole-genome microarrays were designed to include all 70-mer oligonucleotides representing 2,857 *L. monocytogenes* EGD-e ORFs identified in the genome sequence of *L. monocytogenes* EGD-e (21). In addition, an *ilnD* probe was designed using Array OligoSelector (http://arrayoligosel.sourceforge.net/), which was based on the *inlD* sequence for *L. monocytogenes* 10403S, since *inlD* is not present in strain EGD-e (21). Probes targeting five *Saccharomyces cerevisiae* genes were used as non-hybridizing controls as previously described (42, 74). Salmon sperm DNA and

serial dilutions of chromosomal *L. monocytogenes* 10403S DNA were also spotted on the glass array for quality control and signal normalization purposes, respectively. The Array-Ready Oligo sets for 2,857 ORFs from *L. monocytogenes* EGD-e as well as other 70-mer oligonucleotides were purchased from Operon Technologies (Huntsville, AL). *L. monocytogenes* strains 10403S and EGD-e both represent the same *L. monocytogenes* lineage (II), serotype (1/2a), and ribotype (DUP-1039C) (72); therefore, probes designed using the EGD-e genome were expected to hybridize well with 10403S genes. Mismatches of the selected targets are unlikely to generate false positives (differentially regulated genes even though they are not significantly different) since the mismatches would occur in both of the compared RNA samples.

The controls and 70mer-oligonucleotides were spotted in duplicate on Corning UltraGAPS slides (Corning, NY) using a custom built XYZ arrayer located at the Microarray Core Facility at Cornell University (Ithaca, NY). After the oligonucleotides were spotted onto the slides, the slides were UV cross-linked (300mJ for 1 min) to immobilize the oligonucleotides to the slide surface. Slides were stored in a desiccator protected from light at room temperature until ready for use.

cDNA labeling and competitive microarray hybridization

For each RNA sample, 6 µg of total RNA was reversed transcribed into cDNA using Superscript III RT (Invitrogen). 6 µg of total RNA, 1 µl of random hexamer primers (Invitrogen) and nuclease free water up to 17.4 µl total volume. The sample was incubated at 70°C for 10 min and then placed on ice for 10 min. After chilling on ice, 6 µl of 5x First-Strand Buffer (Invitrogen), 3 µl 0.1M DTT, 1 µlRNaseOUT (Invitrogen), 2 µl Superscript III RT (Invitrogen), and 0.6 µl of 25 mM aa-dUTP/dNTP in a 2:3 mix were added to the sample. The sample was incubated at 42°C for 16-18h in a water bath.

Following incubation, the reaction was stopped by adding 10 µl of 1 N NaOH, 10 µl of 0.5M EDTA was added and the sample incubated at 65°C for 15 min. The sample was neutralized with 10 µl of 1 N HCl and the sample was purified with a QIAquick PCR purification kit (Qiagen). Manufacturer's directions were followed but the wash was conducted with phosphate wash buffer (5 mM K_2HPO_4, pH 8.0, 80% EtOH) instead of Buffer PE, and elution was done with 4 mM K_2HPO_4, pH 8.5 instead of Buffer EB.

cDNA was dried completely and 4.5 µl of 0.1M sodium carbonate pH 9.3was added to each sample followed by 4.5 µl of the appropriate Cy dye. Cy 3 or 5 mono-reactive dye packs (GE Healthcare, United Kingdom) were resuspended in 72 µl of DMSO and 4.5 µl of the appropriate dye was added to the sample. Samples were incubated for 2 – 18 h, which do not produce significantly different results between incubation times (data not shown). After incubation, 35 µl 0.1M sodium acetate of pH 5.2 was added and the labeled cDNA was purified with a QIAquick PCR purification kit, as instructed by the manufacturer. Elution was done with nuclease free water. The two target cDNAs were combined, dried, and resuspended in 55 µl 1x hybridization buffer (5X SSC [1XSSX is 0.15M NaCl and 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate (SDS), 0.1mM DTT, 0.5x formamide, 600 µg/ml salmon sperm DNA), denatured for 5 min at 95°C, and applied to the microarray slide by the use of mSeries LifterSlips (Erie Scientific, Portsmouth, NH). Immediately prior to hybridization, microarray slides were blocked by incubating slides in a solution of 1% bovine serum albumin, 5X SSC, and 0.1% SDS for 1 h at 42°C. Following incubation, slides were washed twice by dipping slides in 0.1X SSC and rocking for 5 min on The Belly Dancer (Stovall Life Science, Inc. Greensboro, NC), using a fresh solution of 0.1X SSC. This was followed by two washes with H_2O and 30 s rocking with a fresh wash of H_2O. Slides were dried by centrifugation at 1800 RPM for 3 min.

Lifter slips were prepared prior to hybridization by washing for 30s in the following: 1% SDS, H_2O, 100% EtOH, H_2O, and H_2O. Slips were dried by filtered air and stored in clean 50 ml tubes until needed. After hybridization, slides were washed to remove unbound labeled cDNA by washing slides in 2X SSC and 0.1% SDS at 42°C for 5 min of rocking. This was followed by two washes in 2X SSC with 5 min of rocking, two washes in 0.2X SSC with 5 min of rocking and a final shake in H_2O. Slides were centrifuged to dry for 3 min at 1800 RPM. Within 1h, slides were scanned at the Microarray Core Facility at Cornell University with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) with the following parameters: pixel size, 10; focus position, 20, laser power 100%. Microarray experiments were performed using RNA from three biological replicates.

Microarray data analysis

Raw TIFF images were gridded and analyzed using GenePix Pro 6.0 software. Spots flagged from the data and removed from the analysis included poor morphology spots, oversaturated spots in both channels, and empty spots. Microarray data was analyzed using LIMMA (linear models for microarray data) software (60) in R from the Bioconductor project (20), including the performance of background correction and the normalization and assessment of differential expression. Background corrections were performed for each microarray slide by using the "normexp" method (with offset=50) (60), resulting in reduced variability in the log ratios for genes with low transcript levels. The "print-tip loess" method (62) was used to correct for spatial variation and intensity-dependent bias within-array normalization. Between-array normalization was performed to scale the normalization of log ratios to the same median absolute deviation across arrays in a given data set. Correlation between duplicate spots on each array was calculated using the "duplicateCorrelation" function (61). A linear model was fitted to the normalized log ratios for each gene, followed by

empirical Bayes smoothing to calculated moderated *t* statistics and *P* values were generated to identify genes with differential expression. Statistical significance of differential expression results was assessed based on adjusted *P* values (*P* values were adjusted for multiple comparisons by controlling for the false discovery rate). Genes with an adjusted P value of < 0.05 were considered statistically significant and a fold change of ≥ 1.5 was used as a minimum for the identification of differentially expressed genes.

RESULTS AND DISCUSSION

Inactivation of *L. monocytogenes* by Pulsed Light and UV light treatments

Inactivation curves for Pulsed Light and UV light treatments are shown in Figure 6.1A and Figure 6.1B, respectively. Pulsed Light resulted in a fairly linear reduction over the fluence range used in this study. Previous studies have shown tailing in reduction curves at higher fluence levels than those used in this study (up to 12 J/cm^2) (67). The UV light treatment showed a shoulder at the beginning of the curve, followed by a relatively linear inactivation curve after the initial UV exposure.

In order to evaluate the transcriptional response of cells exposed to the two treatments, it was important that treatment dosese that induce the same level of microbial kill were selected. Therefore, for subsequent microarray studies, a fluence of 3.20 J/cm^2 (6 pulses) for Pulsed Light and 33 mJ/cm^2 (120s) for UV light, which resulted in reductions of 3.10 log CFU/ml for Pulsed Light and 3.14 log CFU/ml for UV light. When treatments were performed at the selected fluence levels prior to the microarray study, slightly different inactivation levels of *L. monocytogenes* were obtained. Following exposure to 3.20 J/cm^2 of Pulsed Light, *L. monocytogenes* was reduced by 2.33± 0.36 log CFU/ml (Table 6.1). Germicidal UV light reduced *L. monocytogenes* by 3.06 ± 0.60 log CFU/ml, which was not statistically different than the reduction by Pulsed Light.

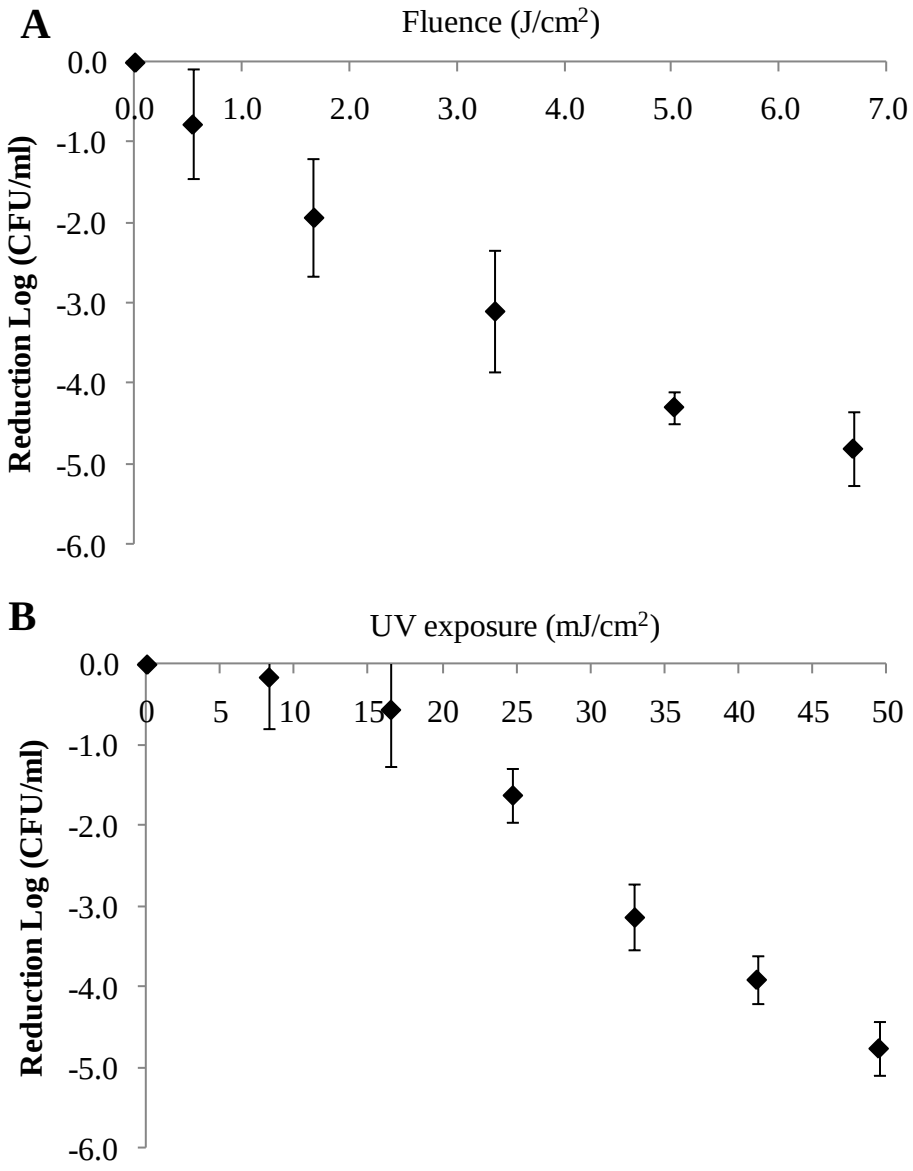

Figure 6.1 Reduction of *L. monocytogenes* after exposure to Pulsed Light (A) and UV light (B).

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Table 6.1. *L. monocytogenes* after exposure to Pulsed Light, blocked UV Pulsed Light and germicidal UV light

Untreated Control	Pulsed Light	Blocked UV Pulsed Light	Germicidal UV Light
9.47 ± 0.54[a]	-2.33 ± 0.36[b]	0.14 ± 0.20[c]	-3.06 ± 0.60[d]

[a] Log CFU/ml

[b] Reduction following exposure to fluence of 3.20 J/cm^2

[c] Reduction following exposure to fluence of 3.25 J/cm^2

[d] Reduction following exposure to fluence of 33mJ/cm^2

As previously mentioned, one of the objectives of this study was also to evaluate the effect of the visible and NIR portions of the spectrum on the *Listeria* cells. In order to study this, the UV portion of Pulsed Light was removed by using a filter that only allowed transmission of light of $\lambda > 400$ nm (Figure 6.2) so the cells were only exposed to the visible and near IR spectrum. The same fluence as for the full spectrum Pulsed Light treatment was used in this case (3.25 J/cm^2). As a result of the UV blocked Pulsed Light, a slight microbial increase of 0.14 log CFU/ml, was noticed instead of any microbial inactivation (Table 6.1), although the increase was not significant. Based on the cell counts, the cells did not appear to even receive sublethal injurywithout the UV portion of the spectrum,. This phenomenon was first noted by Woodling and Moraru (73). They also noted that when using a UV-transmitting filter, which allowed a partial transmission of light between 300 to 400 nm, UV-A, reductions were much less than reductions that allowed UV-B and UV-C exposures, showing the importance of the $\lambda < 315$ to microbial inactivation.

Global gene expression analysis

Whole genome expression profiles of the survivors of the three treatments (Pulsed Light, UV light, and UV blocked Pulsed Light), after growth at 37°C, were compared to those of cells unexposed to any of the light treatments. A total of 80 genes from *Listeria* cells exposed to Pulsed Light and 39 genes from *Listeria* cells exposed to UV light, representing 2.8 and 1.4% of the2,857 ORF's in the array, respectively, showed higher transcription levels ($\geq$ 1.5 fold change, adjusted P value < 0.05) in early stationary phase cells grown at 37°C (Table 6.2). No increase in transcription was observed after treating *L. monocytogenes* with UV blocked Pulsed Light, although 131 (4.6%) genes showed lower transcription levels after this treatment as compared to untreated cells (Table 6.3).

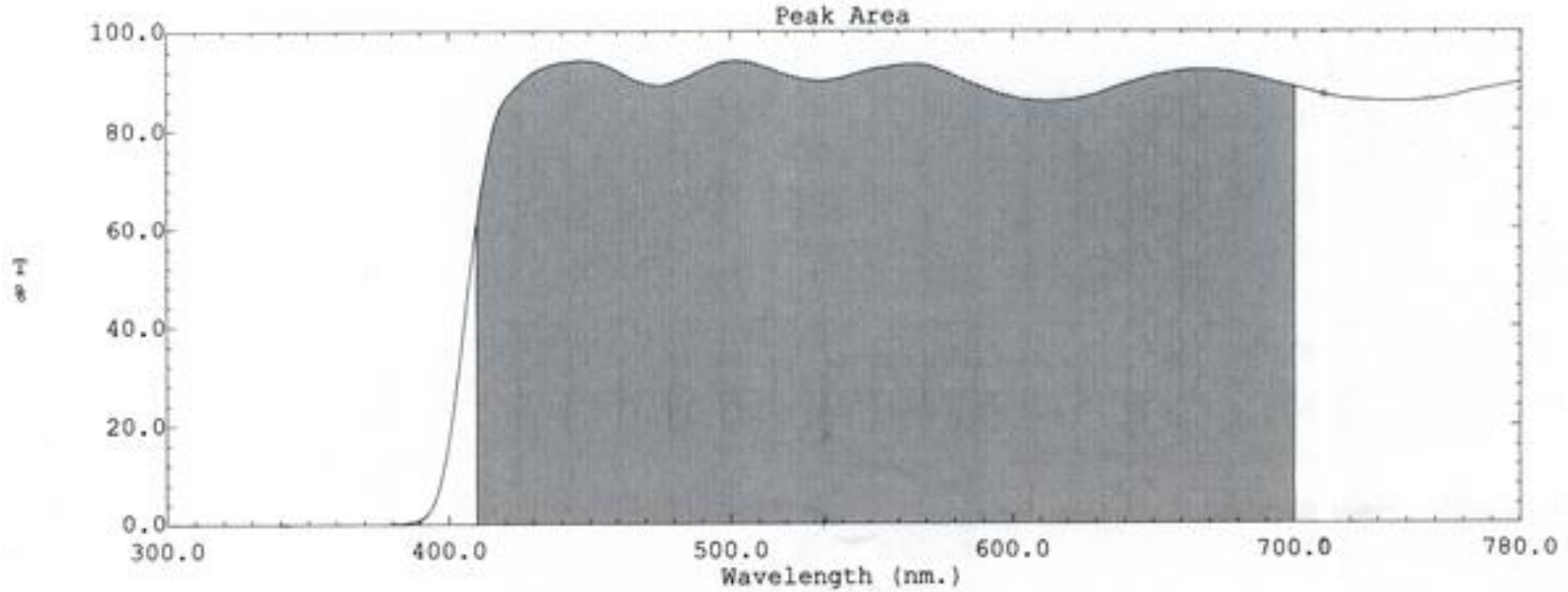

Figure 6.2 Transmission spectrum of UV blocking filter.

Table 6.2. Genes identified by microarray analysis to be up-regulated following exposure to Pulsed Light, UV Light, or UV Blocked Pulsed Light[a]

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Stress				
lmo0609	Similar to *E. coli* phage shock protein E		1.6	
lmo1138	Similar to ATP-dependent Clp protease proteolytic component	1.6		
lmo2461 (*sigL*)	RNA polymerase sigma-54 factor (sigma-L)		1.7	
lmo2485	Similar to *B. subtilis* yvlC protein	1.6		
lmo2748	Similar to *B. subtilis* stress protein YdaG	1.7		
Transcription or Translation				
lmo0266	Similar to transcriptional regulators	1.8	2.3	
lmo0376	Similar to putative transcription regulator	1.6		
lmo0382	Similar to *B. subtilis* transcription repressor of myo-inositol catabolism operon IolR		1.7	
lmo0575	Similar to transcription regulator GntR family		1.6	
lmo0602	Weakly similar to transcription regulator	1.5		
lmo0770	Similar to transcriptional regulator (LacI family)	1.5	1.6	
lmo0815	Similar to transcription regulators		1.5	
lmo0822	Similar to transcriptional regulators	1.7		
lmo0873	Similar to transcriptional regulator (antiterminator)		1.6	

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Transcription or Translation (continued)				
lmo1263	Similar to transcriptional regulator	1.7	1.5	
lmo1716	Similar to putative transcription regulators		1.6	
Cell membrane function				
lmo0263 (*inlH*)	Internalin H	1.6		
lmo0593	Similar to transport proteins (formate?)	1.7		
lmo0721	Putative fibronectin-binding protein	1.5		
lmo0767	Similar to ABC transporter, permease protein	3.0		
lmo1697	Similar to putative transmembrane proteins	1.5		
lmo1870	Similar to alkaline phosphatase	1.5	1.5	
lmo2157 (*sepA*)	sepA	1.7		
lmo2575	Similar to cation transport protein (efflux)		1.8	
Phage related				
lmo0113	Similar to protein gp35 from Bacteriophage A118	1.6		
lmo2276	Similar to an unknown bacteriophage protein	1.5		
Motility				
lmo0690 (*flaA)*	Flagellin protein		1.8	

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Phosphotransferase systems				
lmo0369	Conserved hypothetical protein, highly similar to *B. subtilis* YeeI protein		1.8	
lmo0631	Similar to PTS system, fructose-specific IIA component		1.5	
lmo0875	Similar to PTS system, beta-glucoside enzyme IIB component	1.5		
lmo1972	Similar to pentitol PTS system enzyme II B component		1.6	
lmo2780	Similar to cellobiose PTS enzyme IIA	1.5		
DNA/RNA metabolism				
lmo0213 (*pth*)	Similar to peptidyl-tRNA hydrolase		1.7	
lmo1096 (*guaA*)	Highly similar to GMP synthetase		1.5	
lmo1691	Similar to deoxyuridine triphosphate nucleotidohydrolases		1.5	
lmo1698	Similar to ribosomal-protein-alanine N-acetyltransferase	1.7		
lmo1880	Similar to similar to RNase HI	1.7		
lmo1881	Similar to 5-3 exonuclease		1.7	

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Metabolism				
lmo0266	Similar to succinyldiaminopimelate desuccinylase	1.8		
lmo0359	Similar to D-fructose-1,6-biphosphate aldolase	1.5		
lmo0539	Similar to tagatose-1,6-diphosphate aldolase	1.6		
lmo0580	Weakly similar to carboxylesterase	1.6		
lmo0857	Similar to carboxylesterase	1.7		
lmo1051	Similar to formylmethionine deformylase and to *B. subtilis* YkrB protein	1.9		
lmo1091	Similar to glysosyltransferases	1.7	1.8	
lmo1180	Similar to putative carboxysome structural protein	1.6		
lmo1244	Weakly similar to phosphoglycerate mutase 1	1.5		
lmo1285	Conserved hypothetical protein, similar to *B. subtilis* YneT protein	1.5		
lmo1647	Similar to 1-acylglycerol-3-phosphate O-acyltransferases	1.7		
lmo1883	Similar to chitinases	1.7		
lmo2385	Similar to B. subtilis YuxO protein	1.5		
lmo2433	Similar to acetylesterase	1.5		
lmo2542	Similar to protoporphyrinogen oxidase	1.6		
lmo2674	Similar to ribose 5-phosphate epimerase	1.6		
lmo2830	Similar to thioredoxin	1.7		

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins				
lmo0019	Unknown	1.6		
lmo0216	Highly similar to *B. subtilis* YabO protein		1.7	
lmo0254	Unknown	1.5		
lmo0267	Similar to other proteins	1.5	1.9	
lmo0267	Similar to other proteins			
lmo0310	Unknown	1.6	1.5	
lmo0310	Unknown			
lmo0321	Similar to unknown proteins	1.7		
lmo0377	Unknown		1.5	
lmo0397	Similar to unknown proteins		1.6	
lmo0515	Conserved hypothetical protein	1.6		
lmo0579	Similar to unknown protein	1.5		
lmo0628	Unknown	1.6		
lmo0629	Unknown		1.5	
lmo0654	Unknown	2.2	1.6	
lmo0661	Similar to unknown proteins		1.5	
lmo0758	Unknown	1.7	1.6	
lmo0771	Unknown	1.6	1.7	
lmo0800	Similar to *B. subtilis* YqkB protein	1.6		
lmo0903	Conserved hypothetical protein	1.8		

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins (continued)				
lmo0911	Unknown	1.6		
lmo0953	Unknown	2.0		
lmo1069	Similar to *B. subtilis* YlaI protein	1.5		
lmo1140	Unknown	1.7		
lmo1236	Similar to *B. subtilis* YslB protein	1.5		
lmo1245	Unknown	1.6		
lmo1312	Unknown	4.5		
lmo1468	Similar to unknown proteins	1.6		
lmo1515	Similar to unknown protein	1.5	1.6	
lmo1526	Similar to unknown proteins	1.8		
lmo1580	Similar to unknown protein	1.7		
lmo1612	Similar to unknown proteins		1.6	
lmo1670	Similar to conserved hypothetical proteins	1.7		
lmo1790	Similar to unknown proteins	1.6		
lmo1830	Similar to conserved hypothetical proteins	1.7		
lmo1888	Similar to hypothetical proteins	1.7		
lmo1919	Similar to unknown proteins	1.5		
lmo2158	Similar to *B. subtilis* YwmG protein	1.6		
lmo2177	Similar to unknown protein		1.8	
lmo2210	Unknown		1.6	

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins (continued)				
lmo2213	Similar to unknown protein	1.7		
lmo2255	Unknown	2.1		
lmo2263	Similar to unknown proteins	1.5		
lmo2269	Unknown	1.7		
lmo2311	Unknown	1.8	1.7	
lmo2391	Conserved hypothetical protein similar to *B. subtilis* YhfK protein	1.7		
lmo2432	Unknown	1.9	1.8	
lmo2454	Unknown	1.9	1.7	
lmo2574	Unknown		1.7	

Table 6.2 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins (continued)				
lmo2670	Conserved hypothetical protein	1.6		
lmo2673	Conserved hypothetical protein	1.5		
lmo2723	Similar to unknown proteins		1.6	
lmo2724	Similar to unknown proteins	1.7		

[a] Genes that met the criteria (a ≥ 1.5 fold change and an adjusted P value of < 0.05) for upregulation after exposure to Pulsed Light, UV light, or UV blocked Pulsed Light.

[b] Protein functions are based on annotations provided by ListiList (http://genolist.pasteur.fr/ListiList/), TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi), and the KEGG Sequence Similarity Database (http://www.genome.jp/kegg/ssdb/).

[c] Change(n-fold) indicates the transcriptional level ratio between *L. monocytogenes* 10403S cells exposed to a light source compared to untreated cells (determined by microarray analysis). Values indicate that transcript levels are higher for light exposed than untreated cells (e.g. a vaule of 2.0 indicates a 2.0-fold higher transcript level after light exposure than untreated).

Table 6.3. Genes identified by microarray analysis to be down-regulated following exposure to Pulsed Light, UV Light, or UV Blocked Pulsed Light[a]

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Transcription or Translation				
lmo2173	Similar to sigma-54-dependent transcriptional activator			-1.7
Cell membrane function				
lmo0186	Similar to *B. subtilis* YabE protein			-1.6
lmo0641	Similar to heavy metal-transporting ATPase			-1.5
lmo0683	Similar to chemotactic methyltransferase CheR			-2.0
lmo0689	Similar to CheA activity-modulating chemotaxis protein CheV			-1.6
lmo0843	Similar to *B. subtilis* protein YsdA			-1.6
lmo0912	Similar to transporters (formate)			-1.8
lmo1636	Similar to similar to ABC transporter (ATP-binding protein)			-2.9
lmo1634	Similar to Alcohol-acetaldehyde dehydrogenase			-17.5
lmo1637	Similar to membrane proteins			-3.3
lmo1699	Some similarities to methyl-accepting chemotaxis proteins			-1.5
lmo1852	Similar to putative mercuric ion binding proteins			-2.1
lmo1853	Similar to heavy metal-transporting ATPases			-2.0
lmo1884	Similar to xanthine permeases			-1.9
lmo2035 (*murG*)	Similar to peptidoglycan synthesis enzymes, putative phospho-N-acetylmuramoyl-pentapeptide-transferase			-2.0
lmo2036 (*murD*)	Similar to UDP-N-acetylmuramoylalanine D-glutamate ligase			-1.6

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Cell membrane function (continued)				
lmo2037 (*mraY*)	Similar to phospho-N-acetylmuramoyl-pentapeptide transferase			-1.7
lmo2504	Similar to cell wall binding proteins			-2.0
lmo2528 (*atpC*)	Highly similar to H+-transporting ATP synthase chain epsilon			-1.5
lmo2529 (*atpD*)	Highly similar to H+-transporting ATP synthase chain beta			-1.6
lmo2530 (*atpG*)	Highly similar to H+-transporting ATP synthase chain gamma			-1.7
lmo2531 (*atpA*)	Highly similar to H+-transporting ATP synthase chain alpha			-1.7
lmo2532 (*atpH*)	Highly similar to H+-transporting ATP synthase chain delta			-2.1
lmo2533 (*atpF*)	Highly similar to H+-transporting ATP synthase chain b			-1.7
lmo2534 (*atpE*)	Highly similar to H+-transporting ATP synthase chain c			-2.0
lmo2535 (*atpB*)	Highly similar to H+-transporting ATP synthase chain a			-1.6
lmo2536 (*atpI*)	Highly similar to ATP synthase subunit i			-1.8
lmo2634	Similar to *B. subtilis* YbaF protein			-1.6
lmo2715 (*cydD*)	Highly similar to ABC transporter (ATP-binding protein) required for expression of cytochrome BD			-1.7
lmo2716 (*cydC*)	Highly similar to ABC transporter required for expression of cytochrome BD			-1.7
lmo2717 (*cydB*)	Highly similar to cytochrome D ubiquinol oxidase subunit II			-1.5
lmo2530 (*atpG*)	Highly similar to H+-transporting ATP synthase chain gamma			-1.7

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Motility				
lmo0681	Similar to flagellar biosynthesis protein FlhF			-2.1
lmo0682	Similar to flagellar hook-basal body protein FlgG			-2.2
lmo0685	Similar to motility protein (flagellar motor rotation) MotA			-2.6
lmo0686 (*motB*)	Similar to motility protein (flagellar motor rotation) MotB			-2.2
lmo0696	Similar to flagellar hook assembly protein			-1.5
lmo0697	Similar to flagellar hook protein FlgE			-1.9
lmo0698	Weakly similar to flagellar switch protein			-2.6
lmo0699	Similar to flagellar switch protein FliM			-2.5
lmo0700	Similar to flagellar motor switch protein fliY			-2.1
lmo0705	Similar to flagellar hook-associated protein FlgK			-1.6
lmo0707	Similar to flagellar hook-associated protein 2 FliD			-2.0
lmo0710	Similar to flagellar basal-body rod protein flgB			-1.7
lmo0712	Similar to flagellar hook-basal body complex protein FliE			-1.7
lmo0713	Similar to flagellar basal-body M-ring protein fliF			-1.8
lmo0714	Similar to flagellar motor switch protein fliG			-2.0
lmo0716	Similar to H+-transporting ATP synthase alpha chain FliI, flagellar-specific			-2.0

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Phosphotransferase systems				
lmo0096	Similar to PTS system mannose-specific, factor IIAB			-1.5
lmo0097	Similar to PTS system mannose-specific, factor IIC			-1.6
lmo0098	Similar to PTS system mannose-specific, factor IID			-1.5
lmo0875	Similar to PTS system, beta-glucoside enzyme IIB component			-1.5
lmo1972	Similar to pentitol PTS system enzyme II B component			-1.6
DNA/RNA metabolism				
lmo0280	Highly similar to anaerobic ribonucleotide reductase activator protein			-1.7
lmo1322 (nusA)	Highly similar to N utilization substance protein A (NusA protein)			-1.5
lmo1324	Conserved hypothetical protein, similar to B. subtilis YlxQ protein			-1.7
lmo1325 (*infB*)	Highly similar to translation initiation factor IF-2			-1.6
lmo1327 (*rbfA*)	Highly similar to ribosome-binding factor A			-1.8
lmo1596 (*rpsD*)	Ribosomal protein S4			-1.9
lmo1754 (*gatB*)	Glutamyl-tRNA(Gln) amidotransferase (subunit B)			-1.5
lmo1755 (*gatA*)	Glutamyl-tRNA(Gln) amidotransferase (subunit A)			-1.6
lmo1756 (*gatC*)	Glutamyl-tRNA(Gln) amidotransferase (subunit C)			-1.7

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
DNA/RNA metabolism (continued)				
lmo2460	Similar to *B. subtilis* CggR hypothetical transcriptional regulator			-2.7
lmo2559 (*pyrG*)	Highly similar to CTP synthases			-1.9
lmo2611 (*adk*)	Highly similar to adenylate kinases			-1.8
lmo2612 (*secY*)	Highly similar to preprotein translocase subunit			-1.5
lmo2613 (*rplO*)	Ribosomal protein L15			-1.6
lmo2614 (*rpmD*)	Ribosomal protein L30			-1.9
lmo2615 (*rpsE*)	Ribosomal protein S5			-1.7
lmo2616 (*rplR*)	Ribosomal protein L18			-2.0
lmo2617 (*rplF*)	Ribosomal protein L6			-1.9
lmo2618 (*rpsH*)	Ribosomal protein S8			-1.9
lmo2619 (*rpsN*)	Ribosomal protein S14			-1.8
lmo2620 (*rplE*)	Ribosomal protein L5			-2.2
lmo2621 (*rplX*)	Ribosomal protein L24			-1.8
lmo2622 (*rplN*)	Ribosomal protein L14			-2.4
lmo2623 (*rpsQ*)	Ribosomal protein S17			-2.0
lmo2624 (*rpmC*)	Ribosomal protein L29			-1.8
lmo2625 (*rplP*)	Ribosomal protein L16			-1.7
lmo2626 (*rpsC*)	Ribosomal protein S3			-1.9

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Metabolism				
lmo0355	Similar to Flavocytochrome C Fumarate Reductase chain A			-3.0
lmo0717	Similar to transglycosylase			-2.0
lmo0961	Similar to proteases			-1.7
lmo1091	Similar to glysosyltransferases			-1.6
lmo1151	Similar to *Salmonella typhimurium* PduA protein			-1.6
lmo1200	Similar to cobalamin biosynthesis J protein CbiJ			-1.6
lmo1672 (*menE*)	Similar to O-succinylbenzoic acid-CoA ligase			-1.6
lmo1803	Similar to FtsY of and SRP receptor alpha-subunit			-1.6
lmo1817	Weakly similar to thiamin *E. coli* pyrophosphokinase			-1.6
lmo1917 (*pflA*)	Similar to pyruvate formate-lyase			-1.6
lmo1989 (*leuC*)	Similar to 3-isopropylmalate dehydratase (large subunit)			-1.5
lmo2034 (*divIB*)	Similar to cell-division initiation protein divIB			-1.8
lmo2455 (*eno*)	Highly similar to enolase			-2.5
lmo2456 (*pgm*)	Highly similar to phosphoglycerate mutase			-3.1
lmo2457 (*tpi*)	Highly similar to triose phosphate isomerase			-3.3
lmo2458 (*pgk*)	Highly similar to phosphoglycerate kinase			-3.5
lmo2459 (*gap*)	Highly similar to glyceraldehyde 3-phosphate dehydrogenase			-1.8
lmo2635	Weakly similar to *E. coli* MenA protein			-1.9
lmo2636	Conserved hypothetical lipoprotein			-1.8

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins				
lmo0047	Unknown			-1.7
lmo0350	Unknown			-1.5
lmo0573	Conserved hypothetical protein			-1.5
lmo0684	Unknown			-1.8
lmo0687	Unknown			-2.1
lmo0688	Similar to unknown protein			-2.0
lmo0701	Unknown			-2.9
lmo0702	Unknown			-2.9
lmo0703	Unknown			-1.6
lmo0704	Unknown			-1.6
lmo0715	Unknown			-2.0
lmo0718	Unknown			-1.8
lmo0731	Unknown			-1.6
lmo0788	Unknown			-2.7
lmo0954	Unknown			-2.2
lmo1257	Unknown			-3.5
lmo1326	Conserved hypothetical protein similar to *B. subtilis* YlxP protein			-1.7
lmo1700	Unknown			-1.7
lmo1796	Similar to unknown protein			-1.6
lmo1854	Similar to conserved hypothetical proteins			-2.9

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins (continued)				
lmo1966	Similar to unknown proteins			-1.7
lmo2029	Similar to unknown proteins			-1.5
lmo2116	Unknown			-1.6
lmo2151	Similar to unknown proteins			-1.5
lmo2258	Unknown			-1.8
lmo2410	Unknown			-1.8
lmo2486	Unknown			-3.2
lmo2487	Similar to *B. subtilis* YvlB protein			-2.2
lmo2567	Unknown			-2.7
lmo2568	Unknown			-3.3
lmo2594	Unknown			-1.6

Table 6.3 (Continued)

Protein category and gene	Protein function[b]	Change (n-fold) in cells exposed to[c]: Pulsed Light	UV Light (254 nm)	UV Blocked Pulsed Light
Other or hypothetical proteins (continued)				
lmo2669	unknown			-1.5
lmo2711	Similar to hypothetical proteins			-1.7

[a] Genes that met the criteria (a ≥ 1.5 fold change and an adjusted P value of < 0.05) for upregulation after exposure to Pulsed Light, UV light, or UV blocked Pulsed Light.

[b] Protein functions are based on annotations provided by ListiList (http://genolist.pasteur.fr/ListiList/), TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi), and the KEGG Sequence Similarity Database (http://www.genome.jp/kegg/ssdb/).

[c] Change(n-fold) indicates the transcriptional level ratio between *L. monocytogenes* 10403S cells exposed to a light source compared to untreated cells (determined by microarray analysis). Values indicate that transcript levels are higher for light exposed than untreated cells (e.g. a vaule of 2.0 indicates a 2.0-fold higher transcript level after light exposure than untreated).

No genes showed decreased transcription levels following either Pulsed Light or UV light. Overall, the results show that: (i) a number of *L. monocytogenes* genes were differentially expressed following exposure to with Pulsed Light or UV light; and (ii) a number of *L. monocytogenes* genes showed lower transcription levels after exposure to UV blocked Pulsed Light.

L. monocytogenes genomic expression following exposure to Pulsed Light, UV light or UV Blocked Pulsed Light

Stress response genes

The *L. monocytogenes* genome contains a number of stress-response genes to allow for the survival of *L. monocytogenes* in a number of environmental conditions, including temperature (25, 12), pH (18), and osmolarity (56). For the Pulsed Light treatments, lmo1138, lmo2485, and lmo2748 and for the UV treatments, lmo0609 and *sigL* (lmo2461) showed increases in transcription. Both lmo2485 and lmo2748 are grouped as class II stress response genes, representing a general stress response that is regulated by the alternative sigma factor SigB (69); however, the SigB encoding gene did not show increased differential expression in any of the treatments. lmo2485 was previously identified as being SigB regulated in *L. monocytogenes* (33). lmo1138, similar to Clp, is an ATP dependent protease that is a heat stress response gene involved in the degradation of proteins that are misfolded and is important for tolerating many stresses (36). For the UV light only treatment, lmo0609, a gene whose protein function is similar to an *E. coli* phage shock protein, showed increased transcription after treatment. The phage shock protein operon is usually induced by phage infection, but also induced by other stress conditions such as heat, osmotic stress, and ethanol exposure (45); therefore its up-regulation would be expected after exposure to UV light. The gene *sigL* (lmo2461), which showed raised levels after UV light exposure, has been shown to be induced under a variety of stresses that include

low temperatures (4°C), organic acid, and increased osmolarity (49, 12).

It was surprising that the "dark repair" genes, *uvrA* (lmo2488), *uvrB* (lmo2489),and *uvrC* (lmo1234),did not show an increase in transcription; additionally there was no increase in *recA* (lmo1398), the major regulator of the SOS response involved in DNA repair and the resuming of replication that have stalled (41). Since photolyase (lmo0588), an indicator for light repair of damaged DNA (63), was not expected to increase, since cells were kept in aluminum foil wrapped dark tubes following treatment this may also be a reason there was no increase in *uvrABC.* For cells exposed to UV blocked Pulsed Light, there were no stress response genes that exhibited transcriptional changes compared to the untreated cells. This agrees with the findings of the inactivation study (see Table 6.1), which illustrate that cells exposed to UV Blocked Pulsed Light showed a slight but not significantly different growth as compared to the untreated cells, indicating that no stresses were placed on those cells.

Motility genes

The flagellin gene *flaA* (lmo0690) showed up-regulation following exposure to UV light. The flagella provide motility that allows *L. monocytogenes* cells to move away from unfavorable conditions such as a UV light rich environment. Flagella production is usually regulated by temperature, and at 37°C there is little motility and flagella due to bacterial flagellum being recognized and initiating an immune response in host cells (70). *L. monocytogenes* has maximum transcription of *flaA* when grown at 22°C (47, 16). The increased transcription in *flaA* after UV light exposure of 120s took place in response to adverse environmental conditions for *L. monocytogenes*. Differences in treatment exposure time, 2 s for Pulsed Light and 120 s for UV light, may be the reason why flagellin increased in UV but not Pulsed Light, despite the same treatment dose. With the Pulsed Light's short treatment time, the cells may not recognize a prolonged negative environment, thus flagellin expression was not

increased. While the total duration of the Pulsed Light treatment was 6 pulses or 2 seconds since each pulse has a pulse width of 360 μs, the actual cumulative exposure time of the cells to Pulsed Light lasted only a few milliseconds. In case of the 120 s of continuous UV light exposure, *L. monocytogenes* tended to move away from the stress/UV source, as indicated by the up-regulation of flagellin. This is an interesting difference observed between the two treatments.

For cells exposed to UV blocked Pulsed Light, there was a down regulation in 16 genes related to flagella production. As stated previously, flagella production in *L. monocytogenes* is temperature regulated, with minimal production at 37°C or higher temperatures, while the cells are highly flagellated and motile at low temperatures (23). These results indicate that during exposure to the visible and near IR spectrum there was little need for the cells to be motile, which suggests that these conditions do not impose lethal or growth impeding stresses on the *L. monocytogenes* cells.

Transcription and translation genes

The Pulsed Light and UV treated cells expressed a total of 12 genes with increased regulation, 6 genes from Pulsed Light, 9 from UV and 3 common for the two treatments. The cells treated with UV blocked Pulse Light displayed down-regulation of lmo2173, a protein similar to the sigma-54-dependent transcriptional activator. σ^{54} is primarily involved in the control of carbon metabolism by regulation of the phosphotransferase system (PTS) (2). The expression of *mptACD* operon, as well as of several others PTS operons, appears to be in direct control of σ^{54}. The *mptACD* operon encodes subunits of the PTS permease of the mannose family. This mannose regulation coincides with the additional down-regulation of lmo0096, lmo0097, and lmo0098, which are similar to the PTS mannose specific system.

The gene with increased transcription in both the Pulsed Light and UV treated cells, lmo0770, is similar to transcriptional regulators in the *lac*I family. *lac*I acts as

an inducer for the *lac* operon. The defined growth medium used for *L. monocytogenes* in this study contained glucose as the carbohydrate source, so regulation of the *lac* operon would be predicted as being tightly controlled. There was also an increase in the repressor of myo-inositol operon *iolR* (lmo0382). Glucose was shown to repress the expression of the transcripts of the myo-Inositol operon in *Clostridium perfringens* (32) so increases of the repressor protein are expected. A similar scenario is seen with lmo0557, a regulator of the GntR family, which is involved in gluconate repression (65). Additionally, numerous undefined transcriptional regulators showed increased expression levels (lmo0266, lmo0376, lmo0602, lmo0815, lmo0822, lmo0873, lmo1263, and lmo1716). *L. monocytogenes* genes that were positively regulated included several related to a variety of PTS genes including YeeI (lmo0369) for glucose involved regulation, fructose (lmo0631), beta-glucoside (lmo0875), pentitol (lmo1972), and cellobiose (lmo2780). Not including lmo0369, the other PTS related genes involve either enzyme IIA or IIB of the PTS system. This variety of PTS systems for multiple types of sugars may be an indication that the cell is under stress and up-taking as many sugars as possible for use as catabolites for possible cell repair. PTS operons have been suggested to play a role in carbon assimilation at low temperatures or exposure to stresses such as energy (12, 50).

Cell membrane associated genes

The cell wall of bacteria is a complex structure and provides the first defense of the cell to the environment and potential stresses as well as chemotactic transmembrane receptors that allow the cell to move toward or away from favorable or unfavorable environments. For cells treated with UV blocked Pulsed Light there were several genes associated with cell growth that were down-regulated, these include those associated with peptidoglycan synthesis *mugG* (lmo2035) and *murD* (lmo2036) (44) and cell wall synthesis *mraY* (lmo2037) (9). These changes in expression

regarding cell growth and division were possibly reduced due to the cells in early stationary phase having left the growth phase prior to exposure to the UV blocked light. In addition to the down-regulation in cell wall synthesis, three genes (lmo0683, lmo0689, lmo1699) involved with chemotaxis also showed lower transcription levels. The light treatment did not induce additional stress response genes and these down-regulations may be involved in the transition into stationary phase.

The Pulsed Light appears to induce several important virulence genes, *inlH* (lmo0263), *sepA* (lmo2157), and lmo0721. These proteins appear to be associated with *L. monocytogenes* pathogenesis and internalization. InlH is a cell surface protein that is in the family of internalin related proteins that help promote invasion of host cells (55). lmo0721 is a putative fibronectin-binding protein that helps cells bind to the surface of host cells. SepA is a major extracellular protein of *Shigella* and involved in tissue invasion (3). Possibly the damage caused by the UV induced the *Listeria* to seek a more favorable environment within a host cell away from the Pulsed Light. Interestingly, after exposure to Blocked UV Pulsed Light, lmo1634, alcohol-acetaldehyde dehydrogenase, was down regulated by the largest fold change, 17.5, of all the genes. Lmo1634 was recently determined to be *Listeria* adhesion protein (LAP) (34) and LAP was found to be an important adhesion factor and interacts with mammalian heat shock protein to help initiate infection. The reason for up and down regulations of proteins with similar properties after Pulsed Light and UV Pulsed Light is not currently understood. The Pulsed Light and UV light also increased other cell membrane proteins such as alkaline phosphatase (lmo1870) and a cation efflux transport protein (lmo2575). The commonality of these surface induced proteins is their involvement in signaling and interacting with the external environment. Efflux pumps often remove toxins from the cell and the other proteins can provide feedback to external factors that *L. monocytogenes* may encounter.

Metabolic and unknown genes

Genes were up-regulated following exposure to Pulsed Light and UV light with regard to carbohydrate, amino acid, and nucleic acid metabolic pathways and downregulated following treatment with UV blocked Pulsed Light for the same categories of metabolic pathways. For the UV light and the Pulsed Light, there is an exonuclease (lmo1881) and an RNase (lmo1880), respectively, that were up-regulated. This may indicate the need to catabolize RNA that was generated in response a stress response system or that single stranded DNA that was damaged will be broken down to its nucleotides.

CONCLUSIONS

This work represents the first genome-wide analysis of Pulsed Light and its effects on the gene expression of *L. monocytogenes*. When compared to the traditional germicidal UV, there were little differences in the gene types that were up-regulated between the two treatments. The results of the genomic analysis show that numerous stress response, transcription/translation, motility, and cell membrane genes were up-regulated following exposure to Pulsed Light or UV light. The UV Blocked Pulse Light treatment did not induce any stress response genes and showed down-regulated transcription/translation, motility, and cell membrane associated genes. The cell counts following treatments showed significant differences when the UV portion of Pulsed Light was filtered. The Pulsed Light and UV light showed reductions of 3 log CFU/ml of *L. monocytogenes* while the removal of the UV spectrum showed slight growth when cells were only exposed to the visible and NIR spectrum. Pulsed Light achieved reductions in 2 s of treatment time compared to the 120 s for similar reduction by UV. These results provide evidence that the UV portion of the Pulsed Light spectrum is responsible for the inactivation of microorganisms and that the

visible and NIR spectrum do not adversely affect the treated cells. The rapid treatment time of Pulsed Light also makes the technology a viable option to traditional germicidal UV light to processing applications.

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CHAPTER 7

CONCLUSIONS

Previous research utilizing Pulsed Light had shown the technology was capable of reducing vegetative cells in a variety of substrates. However, the objective of this work was to examine the kinetic inactivation following Pulsed Light treatment as well as examine the potential of combining Pulsed Light with an antimicrobial to create a potential multiple hurdle treatment. Additionally, there was little understanding of the effects at the cellular level when microorganisms were exposed to Pulsed Light and whether cells may potentially develop resistance to Pulsed Light or if the mechanism of inactivation was something beyond UV induced damage. Through a combination of kinetic modeling, applications of the antimicrobial nisin, repetitive exposure and recovery to Pulsed Light, and whole genome microarrays, the major conclusions were determined below.

- Application of the Weibull model to predict Pulsed Light inactivation and account for non-linear reductions in clear liquids was accurately determined but as the substrate properties became more complex, the model overestimated the inactivation on stainless steel surfaces.
- Combining Pulsed Light with the antimicrobial nisin significantly reduced surface *Listeria* and effectively delayed cell growth at refrigerated temperatures then either treatment alone, from 8 days to 28-48 days.
- Repeated exposure to either low or high fluence levels of Pulsed Light did not result in changes in growth kinetics, inactivation kinetics, or increase resistance to *L. monocytogenes, L. innocua,* or *E. coli.*

- Inactivation curves of *L. monocytogenes* treated with Pulsed Light or UV light showed initial shoulders after UV treatment and linear reductions at low level so Pulsed Light treatment. A similar 3 log CFU/ml reduction was reached after 120s of UV light (33mJ/cm^2) or 2s of Pulsed Light (3.25 J/cm^2), while when UV light was blocked during Pulsed Light treatment (3.25 J/cm^2) cell counts showed no significant difference between untreated cells. These results demonstrated the speed advantages Pulsed Light provides compared to UV light.
- Following Pulsed Light or UV light exposure showed higher transcription levels of stress related, motility, and transcriptional regulators genes. Reduced transcriptional levels of genes related to motility and cell membrane were seen following blocked UV Pulsed Light treatments. There were limited differences between the genes up-regulated following Pulsed Light and UV treatments suggesting that the method of inactivation is not different than germicidal UV.

Suggestions for future work

Based on the results and conclusions of this work, the following suggestions regarding future work are as follows:

- Additional parameters incorporating the substrate properties could be incorporated to increase the accuracy of the inactivation predictors. In order to utilize Pulsed Light treatments through packaging or for surface decontamination of food contact material, incorporating the packaging film's properties into the model would be beneficial
- For food surface decontaminations, the sensory effects have not been examined for either immediate or extended shelf life studies. This information would be necessary for potential food industry applications.

- The role of the different UV spectrums, A, B, and C, generated by Pulsed Light have not been fully examined regarding their individual contributions to the inactivation of microorganisms due to filter limitations. Additionally, it is possible to alter the spectral output of the lamp an optimal inactivation setting may be identified and changing the intensity of the UV, vis, and NIR ranges may allow better understanding of potential cellular effects of each spectrum.