PULSED LIGHT BASED TREATMENTS AS A NON-THERMAL STRATEGY FOR MICROBIAL CONTROL ON CHEESE SURFACE

A Thesis

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In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Food Science & Technology

by

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ABSTRACT

Cheese products made from pasteurized milk often undergo post-pasteurization contamination when subjected to cutting, slicing, or packaging, either at the processing plant or in retail environments. Post-processing cross-contamination has significant health and economic consequences when leading to either hospitalizations or losses due to microbial spoilage.

Pulsed Light (PL) treatment, consisting of short, high-energy light pulses, known to effectively inactivate microorganisms on surfaces, was evaluated as a solution to address surface decontamination of cheeses, either as a stand-alone treatment, or in combination with the antimicrobials nisin and natamycin. The effect of PL on color change, oxidative stability and onset of mold growth was also investigated.

Slices of white cheddar and processed cheese were spot inoculated with *Pseudomonas fluorescens* 1150, *Escherichia coli* ATCC 25922 and *Listeria innocua* FSL C2-008, at a concentration of either 5 or 7 log CFU/slice. The inoculated samples were exposed to PL doses of 1.2 to 13.4 J/cm^2, directly or through UV-transparent packaging. For combination treatments, cheese slices were dipped into a 2.5% Nisaplin (nisin) or a 50 ppm Natamax (natamycin) solution prior to inoculation. The antimicrobial treatments were tested both before and after PL application. The survivors were recovered and enumerated by standard plate counting (SPC). When survivor counts fell below the SPC detection limit, the most probable number (MPN) technique was used. Experiments were performed in triplicate and data was analyzed using a general linear model. Color change, oxidative stability, and onset of molding

were monitored periodically on non-inoculated cheddar cheese samples stored at 6 ºC for one month. Color measurements were taken before and after PL treatment, and expressed as CIELAB values. Development of lipid peroxides was monitored colorimetricaly as a measure of oxidative stability, and the onset of molding was assessed visually on a daily basis.

PL treatment alone was most effective against *E. coli*, achieving a maximum reduction of 5.4 ± 0.1 log CFU, at a dose of 13.2 J/cm^2. For *P. fluorescens*, a maximum reduction of 3.7 ± 0.8 log CFU was obtained while a 3.4 ± 0.2 log CFU maximum reduction was achieved for *L. innocua*. The packaging, inoculum level and cheese type had no effect on *L. innocua* and *P. fluorescens* inactivation levels, while *E. coli*'s response was more variable, depending on treatment conditions. PL combination treatments with antimicrobials showed that the presence of natamycin in cheese may interfere with PL, while a synergistic effect between PL and nisin was observed against *Listeria*, and only when nisin was applied after the PL treatment.

PL was also found to be effective in extending the shelf life of cheese. Treatment of cheddar cheese slices with 9 PL pulses, or 10.1 J/cm^2, delayed the onset of molding by a week, and slowed down the rate of molding after that. In terms of the effect of PL on cheese quality, no significant color change or increase in lipid peroxides was observed at a dose of 10.1 J/cm^2 after one month of refrigerated storage.

Overall, this work suggests that PL has strong potential for decontamination of cheese surface, and thus for improving the safety and extending the shelf life of cheese.

BIOGRAPHICAL SKETCH

Jade Proulx was born on October 18, 1989 in Montreal, Quebec, Canada. She is the youngest daughter of Donald Proulx and Manon Daoust, with two older siblings, Chloé Proulx and Donald Proulx, Jr.

Jade completed her education in French until the end of high school, after which she enrolled in Champlain College to get a *Diplôme d'Études Collégiales* in Health Sciences. She graduated in 2009, which would mark her first degree obtained from an English educational institution.

After developing an interest for organic chemistry while at Champlain College, Jade went on to complete a Bachelor of Science in Food Chemistry at McGill University in 2012. Her undergraduate career was punctuated by internships at two different flavor companies, Kerry Ingredients & Flavors Inc., and Novotaste Corporation. She also conducted research in Dr. Ashraf Ismail's group, studying the structure-functionality relationship of whey proteins, as well as in Dr. Varoujan Yaylayan's group, investigating new methods to quantify a proprietary salt enhancer uptake by potatoes.

During her senior year at McGill, Jade was selected to be part of the second cohort of "The Next 36", a Canadian accelerator held at the University of Toronto, requiring its participants to found a technology business in less than nine months on top of completing a custom academic curriculum centered around entrepreneurship. After completing the program, Jade decided to go back to school and deepen her technical expertise in Food Science. She became a Master Student in Food Science & Technology at Cornell University in January 2013, working under the supervision of Dr. Carmen Moraru in Food Safety Engineering, while completing a course curriculum in Applied Statistics.

À ma famille:

Donald Proulx, Manon Daoust, Chloé proulx, Donald Proulx, Jr., Carlos Raul Pernalete Silva, Annie Gauthier, et Hélène Laberge

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These two years would not have been the same without the practical help and mental support of my fellow laboratory members, past and present: Dongjun Zhao, Yifan Cheng, Davin Dickson Bong, Guoping Feng, Iuliana Aprodu, Markus Walkling Ribeiro, Lee Cadesky, Emily Griep, Sheena Hilton, and Pedro Menchik. Just as important was the formidable help of the many undergraduate researchers who worked on this project: Karine Paradis, Genevieve Sullivan, Samantha VanWees, Luis Fernando Paulatti Marostegan, Anissa Taylor, and Jiahui Jian. Being able to share this project with you was both energizing and a privilege. I also thank Philip Carubia for sharing his expertise with the interferometric profiler at the Cornell Center for Materials Research and for helping me broaden my technical skills.

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CHAPTER 1
MECHANISM OF MICROBIAL INACTIVATION IN PULSED LIGHT TREATMENT: A REVIEW

Pulsed Light is a non-ionizing radiation technology that has been proven effective against vegetative bacteria, spores, yeasts, and molds on food and food contact surfaces (Elnmasser et al. 2007). Decontamination is achieved by bombarding microorganisms with short pulses of broad-spectrum light containing wavelengths ranging from the ultraviolet (UV) to near-infrared (NIR) regions. The pulses cause the light's intensity to be 20,000 to 100,000 times greater than that of sunlight at sea level (Dunn et al, 1995) and is then usually referred to as fluence which is expressed in J/cm^2. In 1996, the FDA has approved the use of Pulsed Light for the processing and handling of foods, given that the radiation source is a Xenon discharge lamp emitting the previously specified wavelength profile, and that the total cumulative treatment does not exceed 12 J/cm^2 (FDA, 1996).

The way such high intensity light is generated is by accumulating electrical energy in a capacitor over relatively long times –not more than fractions of a second– and releasing this stored energy to do work in much shorter times. A capacitor consists of a pair of conductive plates surrounding an insulator; when energy is passed through the plates, a potential difference develops between them which causes the positive charge to accumulate on one plate and the negative charge to accumulate on the other. A static electric field is thus formed across the insulator and can be stored as energy even though it is disconnected from its electrical power supply. The rate at which this stored energy is then subsequently released affects the light's power level (Fig 1.1).

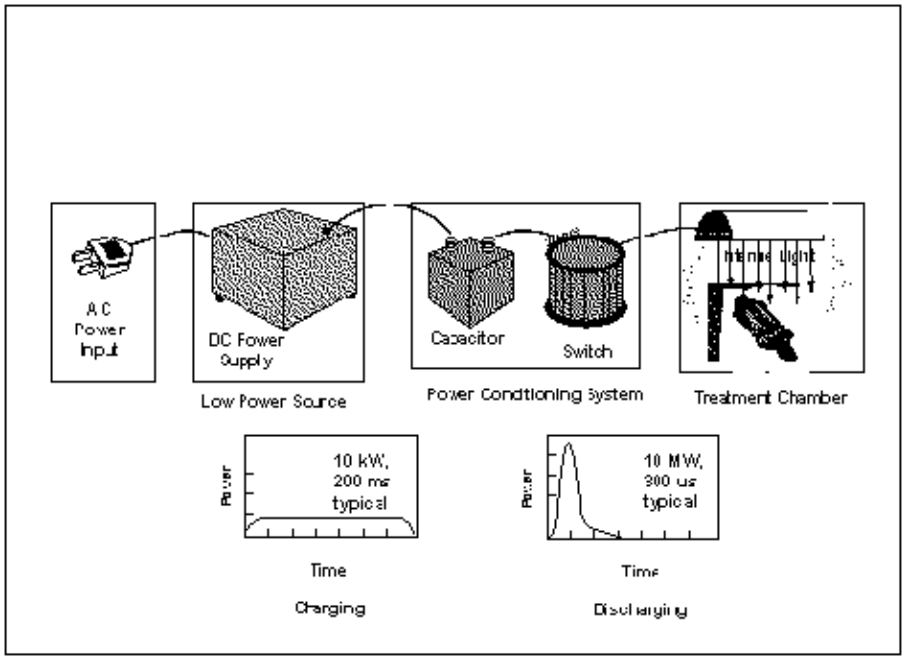

Figure 1.1 Experimental facility for bacterial inactivation using a pulsed light generator (Source: FDA, 2000)

Current explanation for Pulsed Light's microbial inactivation mechanism

The FDA approval process for Pulsed Light has been solely based on experiments showing satisfactory inactivation curves for a wide variety of microorganisms in a vast collection of food matrices (Dunn et al. 1996; Dunn et al. 1997; FDA, 2000). Even though the empirical evidence for Pulsed Light's bactericidal properties is undeniable, the microbial inactivation mechanism associated with it is still poorly understood.

The current conscensus is that Pulsed Light's decontamination power is mainly due to the UV portion of the spectrum, which has a known mechanism of inactivation. The germicidal properties of UV irradiation are attributable to photochemical reactions happening within the microorganism's DNA. Upon UV exposure,

neighbouring pyrimidine nucleoside bases crosslink and form cyclopyrimidine dimers on the same DNA strand. The alteration of those thymines and cytosines blocks the way to the enzymes responsible for DNA transcription and replication, which compromises cellular functions and eventually leads to cell death (Wang et al, 2005). This mechanism has been extensively studied and currently explains the bactericidal effect of other light based treatments, namely continuous UV.

Comparison between continuous UV and PL

Continuous UV is a non-thermal disinfection technology that is widely used for the treatment of water as well as other applications. It is often preferred to chemical disinfection which causes the formation of by-products of regulatory concern (Grapperhaus et al, 2007). UV light is created using a gas discharge lamp containing mercury, where an electric discharge ionizes the gas which radiates photons. Depending on the injected power density, the lamp's temperature rises due to ohmic heating up to a final temperature which plays a dominant role in the composition of the emitted spectrum (McDonald, 2000). The time it takes for the lamp to heat up before causing irradiation is referred to as the warm-up time. Commercially, low-pressure mercury lamps operating at 100 to 200Pa are the most common. They provide several emission lines in the visible region and two lines in the UV region at wavelengths of 185 and 254 nm. Since the only bactericidal emission line is the one at 254nm, it has quickly become the standard wavelength for commercial continuous UV applications (Wang et al, 2005).

Although both PL and continuous UV are using light in order to kill bacteria, there are several differences that deserve to be highlighted. First, the mercury lamps used in continuous UV applications are somewhat of a concern in the industry, which considers them as a potential health and environmental hazard in the case of a lamp failure. This problem is avoided in Pulsed Light by using Xenon instead of mercury, which also causes PL to emit broad spectrum light as opposed to monochromatic light. Another main difference is that continuous mercury lamps require the previously mentioned ‘’warm-up time’’ while Pulsed Light has instant-on capacities (Grapperhaus et al, 2007). This translates in overall treatment times that are significantly shorter for Pulsed Light (Fig 1.2).

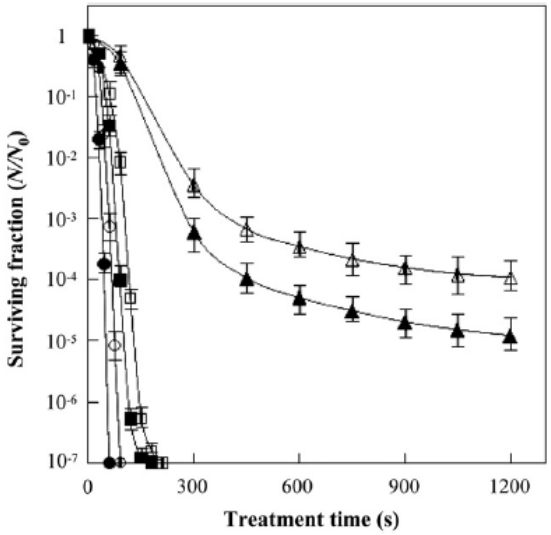

Figure 1.2. Inactivation characteristics of L. monocytogenes (open symbols) and E. coli O157:H7 (closed symbols) by UVC (triangles) and IPL treatment (squares/circles). (Source: Cheigh et al, 2012)

Finally, it has been observed that microorganisms treated with Pulsed Light were less susceptible to photoreactivation than those treated with continuous UV.

Photoreactivation is the phenomenon of breaking down the cyclopyrimidine dimers formed upon UV light exposure, and is catalyzed by the enzyme photolyase. The reason why photoreactivation is not as prominent in Pulsed Light is still unclear, but it has been suggested that the broader spectrum might play a role in inactivating photolyase (Gomez-Lopez et al, 2007).

The bottom-line is that these two technologies are extremely similar, and the reasons behind their differences remain poorly understood. This is particularly puzzling when trying to explain precisely why Pulsed Light offers such a gain in efficiency while it is supposed to rely on the exact same mechanism for microbial inactivation than continuous UV. Even though the photochemical effect's contribution to PL's lethality is certainly important – PL emissions consist of at least 25% UV radiation – it is reasonable to question whether it is the only mechanism at play (Takeshita et al, 2003). Other regions of the spectrum might be contributing to the lethality of the treatment, in which case the near-infrared region can easily be associated with some sort of heating : is it possible that PL also relies on a photothermal effect? Could it be that both photochemical and photothermal effects co-exist in synergy? All these questions have triggered a lot of scientific curiosity, and a considerable amount of research on the topic. However, Pulsed Light's mechanism for microbial inactivation is still subject to a fair amount of controversy, particularly as to whether or not a photothermal effect is induced by PL at the cellular level.

The Wekhof case for a photothermal effect

A publication by Wekhof (2000) tried to theoretically explain why a photothermal effect in Pulsed Light was plausible. He first reasoned that it should exist since after analyzing all the research that had been done with Pulsed Light, he noticed that PL had been efficient at killing bacteria that were usually UVC-resistant such as *Criptosporidium*, *Aspergillus niger*, and *Bacillus subtilis* spores. Wekhof then theorized that when energy exceeding 0.5 J/cm^2 is applied, bacterial disruption occurs due to temporary overheating caused by the absorption of all UV light from the flash lamp. To support his theory, he built a mathematical model calculating the amount of heat absorbed by bacteria in one pulse, and the amount of heat that had time to be released before the next pulse (Wekhof, 2000). He concluded that bacteria's cooling represented only 10% of its initial heating, thus proving his overheating theory. A summary of his calculations is presented below.

Sample Calculations for E.coli heating while in suspension:
Assumed bacteria size: 1.6 x 10^{-4} cm
Projected shadow surface: 3 x 10^{-8}cm^2
Bacteria weight: 3.3x10^{-12}g
Absorbance at 254nm: 0.2
Average dosage of a full UV component: 1 J/cm^2
Average absorption coefficient: 0.05
Average absorbance of full UV component:
1 J/cm^2 x (3 x 10^{-8}cm^2) x 0.05 = 1 x 10-^{9}J = 3.65 x 10^{-10} cal ≈ 365 cal/g
Sample Calculations for E.coli cooling while in suspension:
Average thermal conductivity of a generic organic compound mixture:
k= 0.003cal/sec/cm^2°C

Average thermal conductivity of water: k= 1cal/sec/cm^2°C

Q_{cool} = k x Sc x ΔT x time

For a pulse time of 10^{-3} sec:

Q_{cool} = 36cal

For every pulse cycle, bacteria's cooling represents only 10% of its initial heating.

It is difficult to objectively evaluate these calculations given that many numbers are not disclosed, averages are not accompanied with explanations as to how they were obtained, and that the model relies on numerous assumptions. For instance, it is assumed that bacteria's cooling is conductive, that bacteria's absorption of UV fluxes has the same wavelength dependence as one obtained from literature, and that a temperature value can be obtained for a specific spectral wavelength using the following correlation: *hv(eV)max=2. 82 T°(eV).* Nonetheless, these calculations were used to associate the estimated temperature increase of bacteria to different treatment conditions as a function of number of fluxes (Fig 1.3).

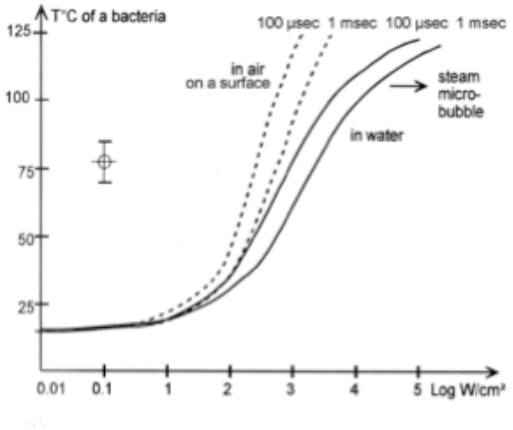

Figure 1.3 Calculated dependence of a bacteria temperature as a function of fluxes from a flash lamp, in log W/cm^2 (Source: Wekhof, 2000)

Aware that his calculations were only rough estimates, Wekhof's next steps were to capture an image of the steam microbubbles being formed while bacteria is overheating, in order to build stronger evidence for PL's photothermal effect. A year after, he published another paper showing spores of *Aspergillus niger* treated with Pulsed Light where the cells' disruption due to the formation of these microbubbles is clearly depicted (Fig. 1.4c).

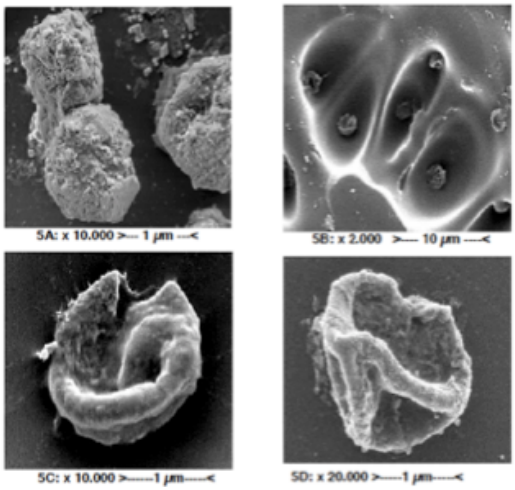

Figure 1.4 a Untreated spores of A. Niger **b** Spores of A. Niger treated with 2 pulses at 33kW/cm^2 **c** A single spore treated to 2 pulses of 33kW/cm^2 **d** A single spore treated to 5 pulses of 5kW/cm^2 each, open lamp. (Source: Wekhof et al, 2001)

The craters around the spores have also been used to confirm the temperature increase estimate calculations (Fig. 1.4b). The rationale was that the craters could only have been caused by the sinking of the spores into the melting surrounding substrate, in that case PET, and since the melting temperature of PET is 120°C, it showed empirical evidence that the bacteria followed the temperature curve from Figure 3 for treatments on a surface.

It is important to note that Wekhof associated the heating effect he demonstrated solely to the UV part of the spectrum generated by Pulsed Light. He clearly specified that according to his theory, the visible and near-infrared regions were not playing any role whatsoever in bacterial inactivation. Therefore, Whekof's work is only one possible explanation for a photo-thermal effect in PL, but it does not invalidate other potential mechanisms.

Furthermore, electron microscopy is a tricky tool that heavily relies on good sample preparation, and thus can hardly be used as the sole evidence for a theory. A good way to get around the skepticism those pictures may arise is to look for replication of the phenomenon in different lab settings, and see whether other researchers were able to accurately show the same photothermal effect using Pulsed Light.

Further evidence for a photothermal effect

At least three different studies were able to demonstrate a photothermal effect in a variety of microorganisms after treating them with Pulsed Light.

In 2003, the effects of both Pulsed Light and continuous UV on *Saccharomyces cerevisiae* were investigated (Takeshita et al, 2003). This study was a real leap forward towards confirming a possible photothermal effect of PL since it did not solely rely on electron microscopy to provide evidence. Rather, the study consisted of four distinct elements of investigation: cell viability, DNA electrophoresis, protein elution analysis, and finally transmission electron microscopy.

Both PL and continuous UV showed similar killing levels, reducing the initial yeast cell concentration of $7x10^6$ cfu/ml to 10 cfu/ml (See Fig 1.2), confirming that lethality is not a huge differentiating factor. The second element of investigation confirmed the presence of a photochemical effect in both treatments. UV endonuclease was used on isolated genomic DNA and helped detect the formation of CPD products after both treatments. Electrophoresis analysis revealed that although both PL and UV produced cyclopyrimidine dimers, DNA damage was slightly more pronounced in the samples treated with continuous UV. This suggests that killing levels are not perfectly correlated with DNA damage, and is a big indicator for the presence of a compensating mechanism in Pulsed Light (Takeshita et al, 2003).

The third and fourth elements of investigation are the biggest evidence for a photothermal effect in PL. After realizing that DNA damage could not be the only mechanism accounting for lethality in PL, levels of eluted protein were measured in both samples (Fig 1.5). It can be seen that almost no protein was eluted from the cells treated by continuous UV while high levels were detected in the PL samples. It was reasoned that if a higher level of proteins were eluted after irradiation, it could be an indication of potential cell membrane damage. The latter was confirmed by electron microscopy, where approximately 30% of yeast cells showed expanded vacuoles and cell membrane disruption (Fig 1.6).

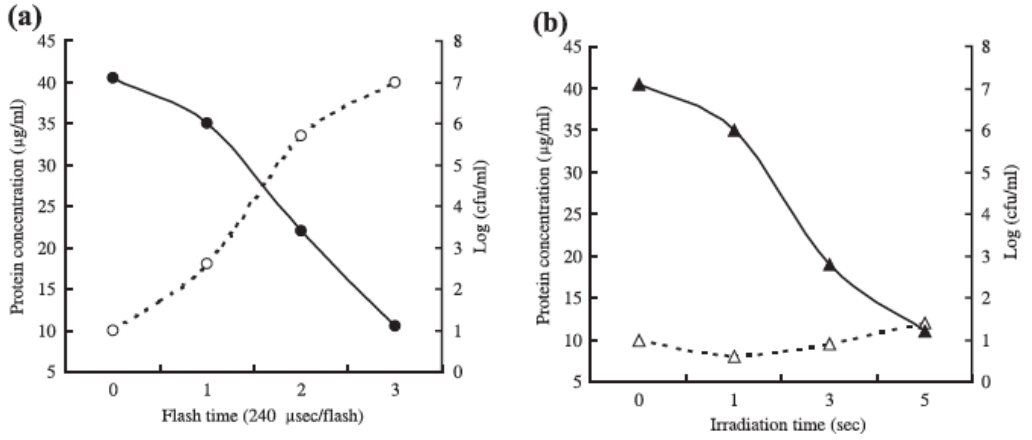

Figure 1.5 Eluted protein concentration (ug/ml, hollow shapes) and yeast cell viability (log cfu/ml, dark shapes) **a** Pulsed Light at 0.7 J/cm^2/flash **b** UV light at 60mW/cm^2/s (Source: Takeshita et al, 2003)

Another extremely similar study provided evidence for cellular disruption caused by PL on both *E.coli O157:H7* and *L.monocytogenes* (Fig 1.7) (Cheigh et al, 2012). The electron microscopy pictures were also backed by a DNA electrophoresis analysis leading to the same conclusions.

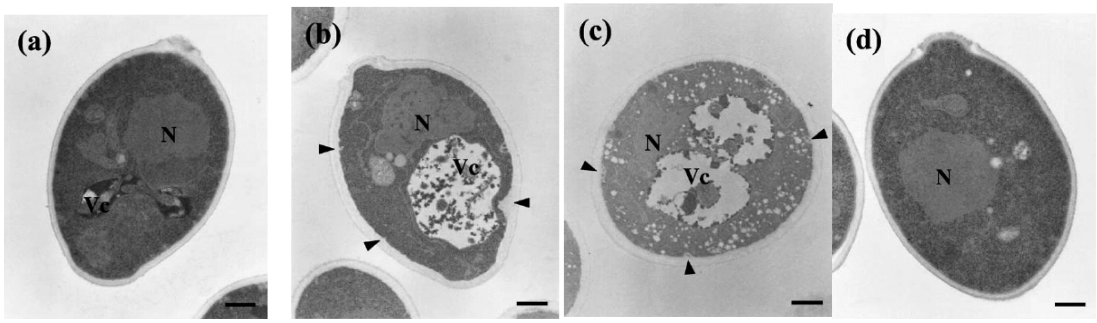

Figure 1.6 TEM of S. cerevisiae **a** Unirradiated, **b** irradiated with two flashes pulsed light at 0.7 J/cm2/flash and three flashes at 0.7 J/cm2/flash **c, d** irradiated with UV light (3 s at 60 mW/cm2/s). Bar corresponds to 0.5 Am. N; nucleus, Vc; vacuole. Arrowheads indicate damaged/ broken membranes. Magnification_40,000. (Source: Takeshita et al, 2003)

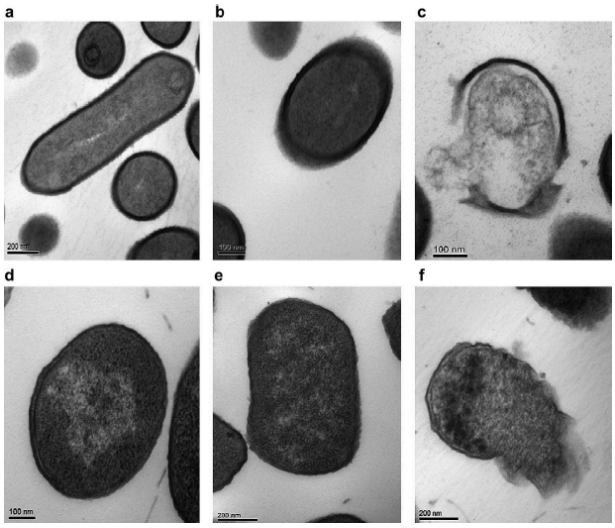

Figure 1.7 TEM of L. monocytogenes **a, b, c** and E. coli O157:H7 **d, e, f**. Images: **a** and **d** untreated control cells, **b** and **e** UVC treatment for 600 s, and **c** and **f** IPL treatment for 180 s at 376 W/m2. Bars correspond to 100 nm **b e d** and 200 nm **a, e, f**. (Source: Cheigh et al, 2012)

The results showed by Takeshita et al. and Cheigh et al prove that cellular damage occurs upon PL treatment, however it makes no mention of what part of the spectrum might cause it. While Wekhof was advocating for a photothermal effect caused by the UV region of the spectrum, another study conducted by Kathiravan Krishnamurthy from Alabama A&M University focused on comparing PL with infrared heating in order to elucidate this question.

The effect of PL on *Staphylococcus aureus* was compared with infrared heating by analyzing the treated cells using FTIR spectroscopy, a novel technique to determine the chemical and structural information of a target material based on vibration transitions (Krishnamurthy et al., 2010). The cells were also analyzed using transmission electron microscopy. *S. aureus*, a gram-positive microorganism usually more resistant to UV radiation than gram-negative bacteria, showed important

structural damage under both infrared and PL treatment. While the cells were submitted to PL for only 5 seconds, the results were comparable to those from the 20 min infrared heat treatment (Fig 1.8). Evidence from the FTIR analysis also supports these observations, reporting strong absorption peeks at 1,100cm^{-1} and 1,312 cm^{-1} for PL, corresponding to DNA/RNA and protein, respectively. This FTIR analysis thus confirms the findings by Takeshita et al showing stronger protein elution under PL treatment.

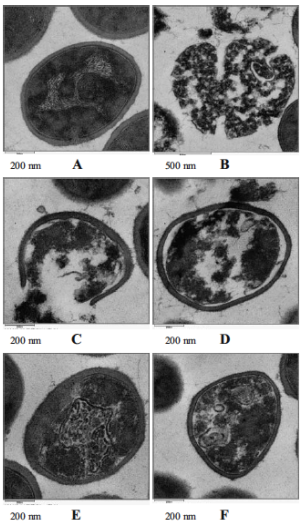

Figure 1.8. Evaluation of pulsed UV light (12-ml sample treated for 5 s at 8 cm below quartz window) induced damages in S. aureus by TEM: **a** control sample, **b** cell wall rupture, **c** lack of cell wall, **d** Cytoplasm shrinkage and cell wall damage, **e** cytoplasm shrinkage and membrane damage, and **f** cell wall damage and cellular content leakage. (Source: Krishnamurty, 2010)

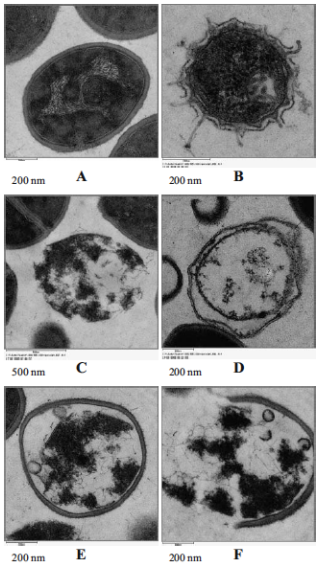

Figure 1.9 Microscopic evaluation of damages to S. aureus because of infrared heat treatment (5-ml sample treated at 700°C for 20 min): **a** control sample, **b** lack of cell wall, **c** cell wall breakage and cytoplasm content leakage, **d** cytoplasm shrinkage, **e** breakage in mesosome, and **f** cytoplasm damage (Source: Krishnamurty, 2010)

An interesting finding was that when monitoring temperature increase using a type K thermocouple, the maximum difference observed was only 2°C, suggesting that PL-induced structural damage might not be caused by heat as was previously hypothesized (Krishnamurthy et al, 2010). The author rather suggested that cytoplasmic membrane damage could be caused by physical disturbances exerted on the microbial cell due to the intermittent high intensity pulses. However, he did not completely dismiss the photothermal effect, reminding that this study was conducted on suspended *S. aureus* in phosphate buffer which was treated for only 5 seconds, conditions that are far from reflecting what would happen while disinfecting a real food matrix. Actual food samples require longer treatment to reach the same levels of inactivation, and it was thus suggested that for treatment times greater than 5 seconds,

a photothermal effect could indeed be observed.

This study was thus able to confirm structural damage induced by PL, but was incapable of demonstrating any causal relationship linking these observations to the near-infrared region of broad-spectrum light. This is despite the fact that actual infrared heating seems to induce similar structural damage.

Challenging evidence for a photothermal effect

Despite all the compelling evidence in favor of structural damage caused by PL, the latter has not been efficiently linked to a significant contribution to lethality. This is where it is interesting to look at challenging evidence, which helps put in perspective some of the seemingly convincing TEMs that were taken in other studies.

In order to understand the importance of UV emissions in Pulsed Light, a study was conducted on *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus* which compared the lethality effects of high versus low-UV emissions (Rowan et al, 1999). The experiment consisted of submitting bacteria to two different light sources, both of which emitted broad spectrum white light with a peak at 550nm, however one of them was approximately 800 times more intense near the UV region between 200 and 450nm. There was a drastic difference between the two treatments: 5 and 6 log reduction was achieved after treatment with 100 and 200 pulses, respectively with the higher UV light source, whereas 300 pulses of low-UV light gave only a 1 log reduction (Fig 1.10). These results indicate that lethality is in fact tightly correlated to the UV portion of the spectrum, and that any other effect would be highly marginal in

comparison. It was also determined that at one pulse per second, the average power consumption of the system was 3W and consequently, no discernable temperature change was observed during the treatment, further discarding the possibility of a photothermal effect having anything to do with inactivation.

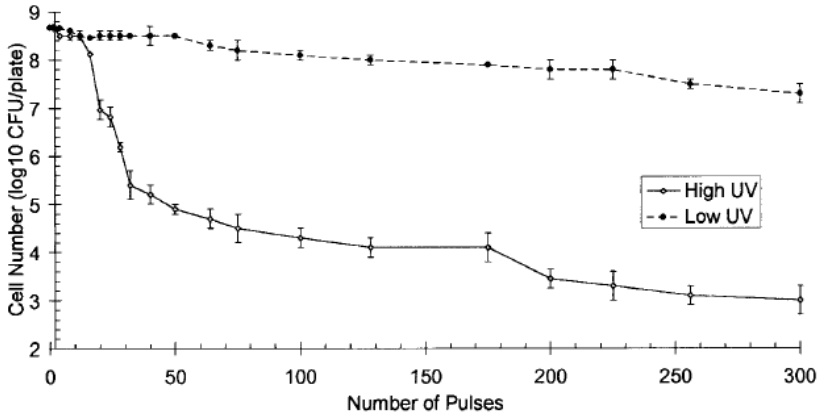

Figure 1.10 Pulsed-light inactivation of surface-inoculated E. coli using two light sources which contained either a low- or high-UV content (Source: Rowan et al, 1999)

The same findings were reflected in a more detailed study consisting of filtering Pulsed Light emission with three different optical filters : one UVA-transmitting filter (UVT), one UV blocking filter (UVB), and a cold-mirror allowing approximately 50% UV transmission (CM) (Woodling and Moraru, 2007). The different inactivation levels of *L.innocua* were observed, and no inactivation was achieved with the UV-blocking filter, while marginal reduction of less than 1 log was achieved with the UVA-transmitting filter and the cold mirror **(**Fig 1.11).

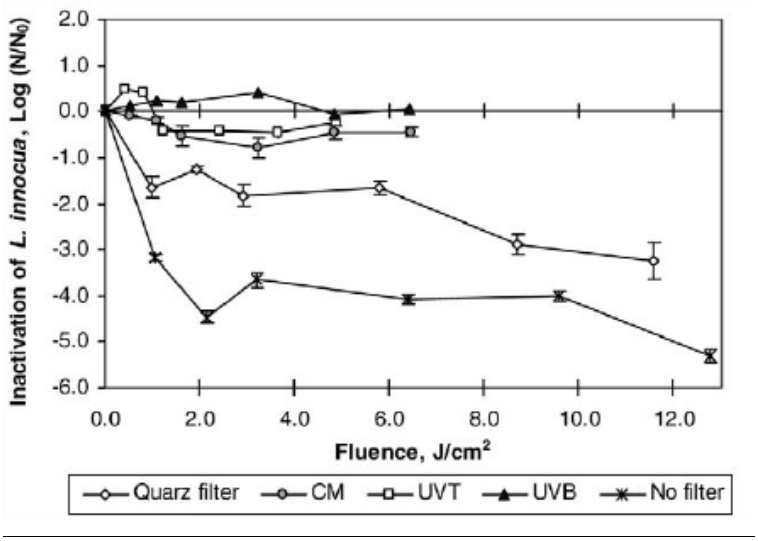

Figure 1.11. PL inactivation of L. innocua on the mill finish surface with and without filters (SPC data). Error bars represent standard errors calculated for each individual treatment (Source: Woodling and Moraru, 2007)

Finally, another study further confirmed how lethality was solely linked to the UV region of the spectrum by looking at the germicidal efficiency on *E.coli* of every 8 nm segment of a broad light spectrum using a monochromator (Wang et al, 2005). Their findings showed that lethality reached a peak at 270nm, and that no activation was achieved beyond 300nm (Fig 1.12-1.13).

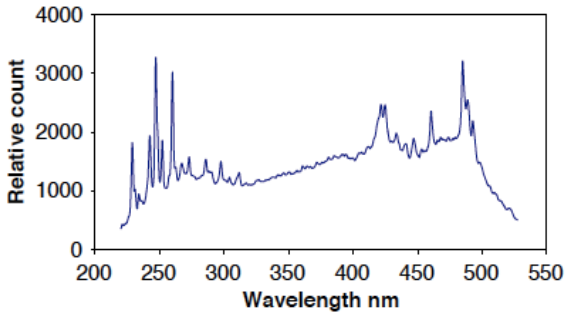

Figure 1.12 Spectrum of Xe flashlamp obtained with Ocean Optics spectrometer and 220-500nm grating (Source : Wang et al, 2005)

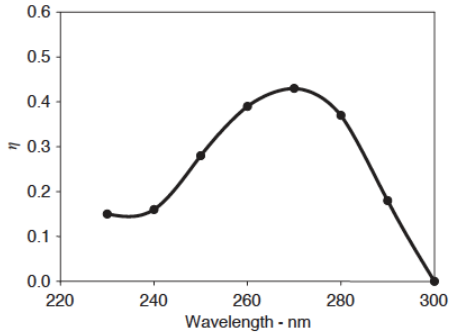

Figure 1.13 Germicidal efficiency n[$\log_{10}(N/N_0)$ per mJ/cm^2] for E.coli as a function of wavelength (Source : Wang et al, 2005)

While all these studies demonstrate that lethality is only achieved when the UV region of the spectrum is included in the Pulsed Light treatment, they do not specifically invalidate the possibility of bacteria absorbing UV light and instantly overheat, as was suggested by Wekhof. Moreover, they do not show strong counter-evidence for a potential synergy between a photochemical effect and a photothermal effect when compared to other treatments such as continuous UV.

A recent study tried to answer these questions by performing a gene expression analysis on *L.monocytogenes* treated with both Pulsed Light and continuous UV (Uesugi, 2012). The hypothesis was that if a photothermal effect indeed occurred in Pulsed Light, there would be a differential gene expression caused by the visible and NIR portions of the spectrum. The results revealed that 80 stress related proteins, motility genes, and transcriptional regulators had higher transcriptional levels for Pulsed Light, while continous UV caused only 39 such molecules to respond that way. This suggests that Pulsed Light indeed causes greater damage to the cells, but it does not clearly indicate how. The next steps of the study were to see if the VIS and NIR

regions of the spectrum were responsible for this greater stress response, and thus gene expression analysis was carried again on cells that were exposed to UV-blocked Pulsed Light (Uesugi, 2012). However, no stress response genes exhibited any transcriptional change, once again confirming that the microbicidal effect of PL are primarily caused by the UV portion of the spectrum.

Conclusions

There is an important body of evidence showing that Pulsed Light treatment induces structural damage in microbial cells. It has been demonstrated through higher protein elution levels (Takeshita et al, 2003), higher intensity protein peaks on FTIR spectra (Krishnamurthy et al, 2010), significantly higher transcriptional change of stress response genes (Uesugi, 2012), as well as multiple electron microscopy images (Wekhof et al, 2001; Takeshita et al, 2003; Krishnamurthy et al, 2010; Cheigh et al, 2012). It was also proven that PL does rely on a photochemical effect and the formation of cyclopyrimidine dimers in order to kill bacteria (Takeshita et al, 2003; Cheigh et al, 2012). The challenge is to know whether structural damage is caused by a thermal effect or not, and whether it contributes at all to the lethality of Pulsed Light treatment.

It was demonstrated that structural damage could hardly have been caused by the near-infrared region after being directly compared to infrared treatment (Krishnamurthy et al, 2010). The other regions of the spectrum were also discarded as a possible single source of inactivation after achieving no microbial reduction when omitting the UV region of the spectrum (Weigh et al, 2005; Woodling and Moraru,

2007; Uesugi, 2012). However, there is still the possibility of structural damage being caused either by heating due to UV absorption or to the pulsing effect of the light. It is also possible that even though it has no bactericidal effect on its own, structural damage could act in synergy with the photochemical effect.

Interesting topics for further research would be to confirm whether structural damage is caused by UV absorption, pulsing effects, or both, as well as looking into quantifying a potential synergistic effect between structural damage caused by PL and photochemical destruction of bacteria.

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CHAPTER 2

PULSED LIGHT INACTIVATION OF PATHOGENIC AND SPOILAGE BACTERIA ON CHEESE SURFACE

ABSTRACT

Cheese products are susceptible to post-processing cross-contamination that can lead to both food safety issues and significant losses due to spoilage. Pulsed Light (PL) treatment, consisting of short, high-energy light pulses, could represent a solution to address this issue since it is a non-destructive technology that can effectively inactivate microorganisms on surfaces. This study examined the effectiveness of PL on the inactivation of the spoilage microorganism *P. fluorescens* and the pathogen surrogates *E. coli* ATCC 25922 and *L. innocua.* The effect of inoculum level, cheese surface topography, and the presence of clear polyethylene packaging were evaluated in a full factorial experimental design. *L. innocua* was the least sensitive to PL treatment, with a maximum inactivation level of 3.37 ± 0.2 log, followed by *P. fluorescens*, with a maximum inactivation of 3.74 ± 0.8 log. *E. coli* was the most sensitive to PL, with a maximum reduction of 5.41 ± 0.1 log. All the PL inactivation curves exhibited similar characteristics, namely a non-linear inactivation curve and a plateau reached after 3 pulses (3.6 J/cm^2). PL treatments through packaging and without packaging consistently resulted in similar inactivation levels. This study suggests that PL has strong potential for decontamination of cheese surface.

INTRODUCTION

According to the Center for Disease Control (CDC) 90 cheese outbreaks were reported in the United States from 1998 to 2011, out of which 49% were caused by cheese made from pasteurized milk (Gould, 2014). These outbreaks were primarily caused by post-pasteurization cross-contamination of cheese when subjected to cutting, slicing or packaging, either at the processing plant or in retail environments. These outbreaks involved a variety of cheeses, such as Hispanic-style cheeses (Jackson et al, 2011), Gorgonzola cheese (Koch et al., 2010) and ricotta salata cheese (CDC,2012). In this last example, surface cross-contamination tripled the extent of damage caused by the source, a single contaminated ricotta salata wheel: out of 22 people infected, only 7 were found to have directly consumed the cheese responsible for the outbreak. The recurring pathogens in these outbreaks were *Listeria monocytogenes*, a ubiquitous, psychrotolerant, gram-positive bacterium, and *Escherichia coli O157:H7*, a gram-negative bacterium commonly associated with fecal contamination.

Microbial contamination of cheese surface can also involve spoilage microorganisms, which cause undesirable changes in appearance, texture, flavor, and odor. Such deterioration of product quality leads to lower consumer acceptance, shorter shelf life and significant losses for the cheese makers. *Pseudomonas spp*., some of the main spoilage bacteria in both raw and pasteurized milk (Ternstrom et al, 1993), are among the most common microorganisms implicated in food spoilage in general. *Pseudomonas fluorescens* has for instance caused a recall of 70,000

contaminated mozzarella balls in Italy (Nogarol, 2013) and a recall of Latin style fresh cheese (Queso Fresco) exhibiting blue discoloration in the US (Martin et al, 2011).

The main approach to avoid post-pasteurization microbial contamination of cheese is compliance with good manufacturing practices (GMPs) and proper sanitation. However, given the high number of outbreaks involving pasteurized milk cheeses still occurring, an additional kill step after processing and handling could be extremely beneficial. Pulsed Light (PL) treatment, consisting of short, high-energy pulses of broad-spectrum light could help address this problem. The high-intensity pulses are generated by a Xenon lamp that converts the high voltage, high peak current to light in the wavelength range from 200 to 1100 nm in mili- to microseconds (Dunn et al., 1995; Gomez-Lopez et al., 2007). PL is approved by the U.S. Food and Drug Administration as a process aid for the decontamination of food and food contact surfaces since 1996 (FDA, 1996). It is also a non-destructive treatment with very short treatment times, which makes it convenient to use in manufacturing, distribution, and retail environments. PL is also a non-thermal technology that leaves no chemical residue (Pereira and Vicente, 2010), which is aligned with current clean labeling trends.

The lethality of PL treatment is primarily attributed to the UV portion of its spectrum, and the mechanism of microbial inactivation is similar to continuous UV light, i.e. formation of pyrimidine dimers within the bacterial DNA, which block DNA transcription and replication, ultimately leading to cell death (Wang et al., 2005; Woodling and Moraru, 2007; Elmnasser et al., 2007; Kramer and Muranyi, 2013). Several studies show that PL can effectively inactivate microorganisms on various

foods and food contact surfaces, including 2 log reduction of *Listeria innocua* on fresh-cut mushrooms (Ramos-Villarroel et al., 2012), 2 to 4.5 log reduction of *Escherichia coli* in apple juice (Sauer and Moraru, 2009; Palgan et al., 2011), 1 log-reduction of *Salmonella typhimurium* on beef and tuna carpaccio (Hierro et al., 2012), and up to 7.2 ± 0.29 log-reduction of *L. innocua* on low density polyethylene (Ringus and Moraru, 2013).

The objective of this study was to examine the effectiveness PL for inactivating relevant spoilage and pathogenic microorganisms on cheese surface, including surrogates of *L. monocytogenes* and *E. coli* O157:H7 and the spoilage microorganism *P. fluorescens.* The effect of inoculum level, cheese type, PL dose, and the presence of UV-transparent polyethylene packaging on PL inactivation were evaluated for in a full-factorial design.

MATERIALS AND METHODS

Inoculum Preparation

The challenge microorganisms for this study included *L. innocua* FSL C2-008 obtained from the Food Safety Laboratory at Cornell University (Ithaca, NY), which has been demonstrated to behave as a surrogate for *L. monocytogenes* in PL treatment (Uesugi and Moraru, 2009; Lasabagaster et al., 2012). The second challenge microorganism was the gram-negative *Escherichia coli* ATCC 25922, a demonstrated surrogate for pathogenic *E. coli* O157:H7 for light-based treatments (Sauer and Moraru, 2009; Miller et al., 2012). The third organism was *P. fluorescens*, a gram-

negative rod associated with milk spoilage with the ability to synthesize a fluorescent blue pigment, pyoverdin, and to form biofilms.

A single colony of each bacterial strain was isolated from Tryptic Soy Agar (TSA) plates and inoculated into 10 mL Tryptic Soy Broth (TSB) for *E. coli* and *P. fluorescens*, or Brain Heart Infusion (BHI) for *L. innocua*. Cultures of *E. coli* and *L. innocua* were grown at 37 ºC with shaking at 225 rpm until stationary phase, typically for 20-24 h, while *P. fluorescens* was grown at 30 ºC with shaking at 225 rpm for 24-26 h. The stationary phase cultures were then diluted in Butterfield's Phosphate Buffer (BPB) to obtain initial inoculum levels of 5 or 7 log CFU per cheese slice.

Substrate Preparation

Sharp white cheddar cheese (Heluva Good, Sodus, NY) and white American singles (Kraft Foods Inc., Northfield, IL) purchased from a local retail store were chosen as cheese substrates, due to their different surface characteristics. A sterilized cheese cutter was used to obtain 2.5 x 5 cm (width x length) rectangular cheese slices of 1-3 mm thickness. The slices were individually spot-inoculated onto one face with the appropriate cell suspension using 10 droplets of 10 µL each. The inoculated cheese samples were subsequently stored at 4°C in individual sterile petri dishes overnight to allow the liquid portion of the inoculum to evaporate.

For the experiments performed through packaging, low-density polyethylene (LDPE) (2 mil polybags #S-951, Uline, Pleasant Prairie, WI) was used for cheddar cheese, and the original commercial plastic wrapper was used for processed cheese. Packaging materials were cut to the same dimensions as the cheese samples,

disinfected with 95% ethanol, air-dried, then placed on top of the inoculated cheese slices immediately prior to PL treatment.

Pulsed Light Treatment

PL treatments were conducted using a Xenon RS-3000C bench top unit (Xenon Corporation, Wilmington, MA) equipped with a Xenon flash lamp emitting 360 µs pulses at a frequency of 3 pulses per second. A single slice of cheese was placed centrally on an adjustable stainless steel shelf inside the PL unit at 5.8 cm from the surface of the lamp housing and subjected to 1, 3, 6, 9, or 12 pulses, corresponding to fluence levels of 1.2, 3.6, 7.0, 10.1, and 13.4 J/cm^2, respectively. Inoculated cheese slices that were not exposed to PL were used as controls.

Fluence Measurements

To monitor the PL dose, fluence measurements were taken using a pyroelectric head (PE25BBH) coupled with a Nova II display (Ophir Optronics Inc, Wilmington, MA). During the measurement, the pyroelectric head and its chord were covered in aluminum foil leaving only a 1-cm^2 circular opening exposed. Cumulative exposure was measured at a distance of 5.8 cm from the lamp housing, at a pulse width setting of 1.0 ms. Measurements for 1, 3, 6, 9 and 12 pulses were taken in triplicate. At least 1 min was allowed between measurements in order to prevent overheating of the pyroelectric head.

Recovery and Enumeration of Survivors

The treated cheese slices (along with the packaging material for the treatments performed through packaging) were placed into a sterile Whirl-pak stomacher bag (Nasco, Atkinson, WI) containing 1:10 mass/volume BPB solution, and stomached for

2 min at 230 rpm using a Seward Stomacher 400 Circulator (Seward, Davie, FL). Appropriate dilutions of the stomached sample were plated in duplicate on selective media (BD Difco, Bedford, MA), as follows: MacConkey agar for *E. coli*, Modified Oxford agar for *L. innocua,* and Pseudomonas Isolation agar for *P. fluorescens*. A minimum incubation time of 24 h at 37 ºC was used for *L. innocua* and *E. coli*, and at 30 ºC for *P. fluorescens*. Preliminary tests demonstrated an enrichment step was not necessary (data not shown). Survivors were enumerated by standard plate counting (SPC) and survivor ratios were quantified as Log (N/N_0), where N represents the number recovered survivors (log CFU) and N_0 represents the measured initial inoculum (log CFU). When plate counts fell below the 30 CFU/plate detection limit, the 3-tube most probable number technique, as described by Downes et al (2001), was used. All experiments were performed in triplicate.

Substrate Surface Characterization

Water Contact Angle Measurements. Dynamic water contact angles were measured using a Rame-Hart 500 Advanced Goniometer/Tensiometer (Ramé-Hart Inc., Succasunna, NJ) equipped with an automated dispensing system. The data was analyzed using the instrument's DROPimage software. Measurements were performed on 1 cm x 1 cm cheese samples, using the sessile drop method. As a microsyringe filled with deionized ultra-pure water (Mili-Q, Merck Milipore LTD, Billerica, MA) dispensed a 15-µl droplet on the sample, 40 stable advancing left and right angles were calculated and averaged. All measurements were performed in triplicate and average values of left and right contact angles were used as a measure of surface hydrophobicity. Values smaller than 90º indicate a hydrophilic surface, and values

larger than 90º indicate a hydrophobic surface. This measure of hydrophobicity was used to evaluate the potential for surface spreading of the liquid inoculum.

Surface Roughness Analysis. Surface roughness of the cheese substrates was determined to evaluate the potential for microbial hiding and light shading. Measurements were performed using a MicroXAM optical interferometric profiler with the MapVue™ mapping and analysis software (ADE Phase Shift Inc., Tucson, AZ), at the Cornell Center for Materials Research. The following roughness parameters were determined: S_a, the average roughness, S_z, the difference between the highest and lowest peak, S_{sk}, the surface skewness with values around zero indicating as many peaks as valleys, and S_{ku}, the surface kurtosis with a value of 3 indicating a Gaussian peak height distribution. Measurements were conducted on a 2.5 x 3.4 mm sample surface area, in triplicate, for each type of cheese.

Statistical Analysis

The data was analyzed with the general linear model up to a 3-way ANOVA using the statistical package JMP Pro 10 (SAS Institute, Cary, NC). The model included microorganism type, inoculum level, cheese type, presence of packaging, PL dose, every full factorial interaction terms as fixed effects, and replicates as a random variable. Backward variable selection was performed and Tukey's Honest Significant Difference (HSD) test was carried out on every significant effect to gain further insights on which factor level was most influential. The data for each microorganism was also analyzed separately.

RESULTS AND DISCUSSION

Levels of inactivation of at least 3-log reduction were achieved for all microorganisms and treatment conditions tested, at doses below 12 J/cm^2, which is the maximum allowed dose by the FDA (FDA, 1996). All the PL inactivation curves in this study exhibited similar characteristics, namely a non-linear inactivation curve and a plateau reached after 3 pulses (3.6 J/cm^2) (Fig. 2.1 - Fig. 2.3).

PL Inactivation on Cheese Surface: Effect of Microorganism Type

Statistical analysis showed significantly different responses to PL by each of the microorganisms tested ($p<0.05$). *L. innocua* was the least sensitive to PL treatment, with a maximum inactivation level of 3.37 ± 0.2 log (Fig. 2.1), followed by *P. fluorescens*, with a maximum inactivation of 3.74 ± 0.8 log (Fig. 2.2). In both cases, these maxima were achieved on processed cheese at an initial inoculum of 7 log CFU/slice, for treatments performed through packaging (Fig. 2.1d, Fig. 2.2d). Lasabagaster et al. (2012) observed the same order of bacterial sensitivity to PL on fish products. *E. coli* was the most sensitive to PL, with a maximum reduction of 5.41 ± 0.1 log, achieved on processed cheese at an initial inoculum of 7 log CFU/slice, without packaging (Fig. 2.3d).

The different responses to PL of the three bacterial species can be explained by their respective protective mechanisms against UV light. *L. innocua*, *P. fluorescens*, and *E. coli* have inherent physiological properties that offer varying levels of protection to their nucleus.

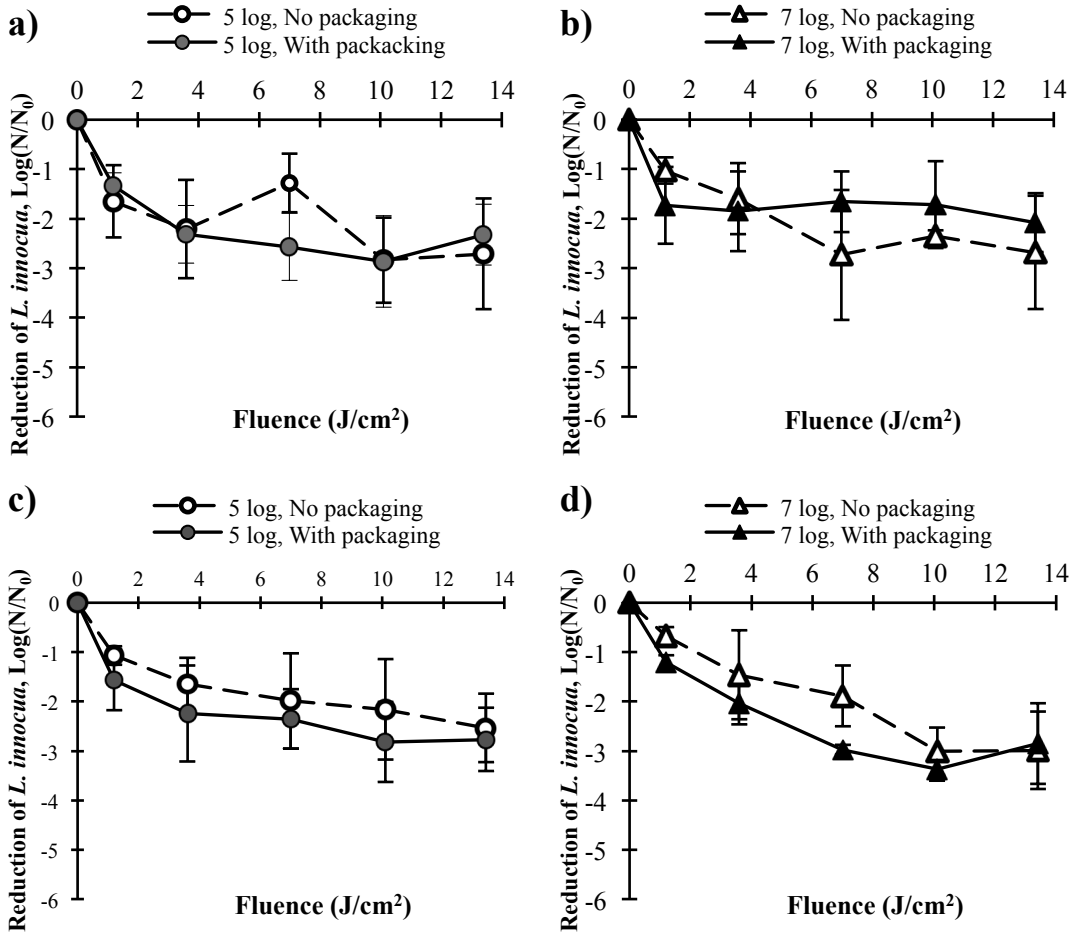

Figure 2.1 PL inactivation of *L. innocua* on (**a, b**) cheddar cheese and (**c, d**) processed cheese highlighting different combinations of initial inoculum levels (5 log CFU/slice, 7 log CFU/slice) and presence or absence of UV-transparent low density polyethylene packaging (no packaging, with packaging).

For gram-positive bacteria such as *L. innocua*, the cell wall's thick peptidoglycan layer shields the DNA located in the cell's nucleus from PL lethal light discharges. The relatively low inactivation levels obtained in this study are consistent with previous reports: a reduction of 1.37 log CFU/sample on Vienna sausages was obtained by Uesugi and Moraru (2009), 0.9 ± 0.09 log-reduction of *L. monocytogenes* on beef carpaccio was achieved by Hierro et al. (2012), and 1.41 ± 0.15 log-reduction

of *L. monocytogenes* on American cheese, under similar conditions was reported by Can et al. (2014).

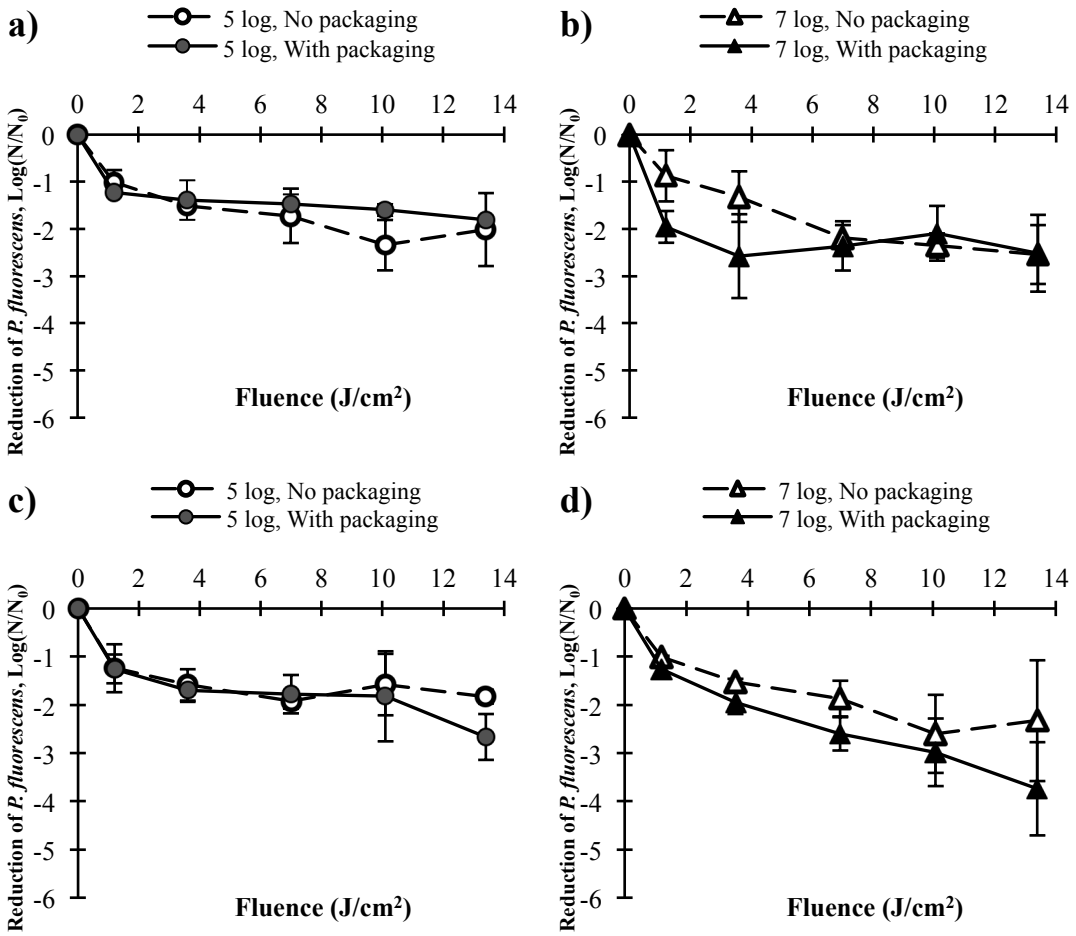

Figure 2.2 PL inactivation of *P. fluorescens* on (**a, b**) cheddar cheese and (**c, d**) processed cheese highlighting different combinations of initial inoculum levels (5 log CFU/slice, 7 log CFU/slice) and presence or absence of UV-transparent low density polyethylene packaging (no packaging, with packaging).

While *P. fluorescens* is a gram-negative bacteria and does not benefit from the protection of a thick peptidoglycan layer, it has the ability to synthesize pyoverdin, a fluorescent blue pigment that absorbs in the UV range (Brown and Luke, 2010), thus

reducing the effective PL dose. In addition to this potential defense mechanism, *P. fluorescens* has the ability to form biofilms, a complex, protective matrix of extracellular polymeric substances in which bacterial populations are enclosed. Garvey et al. (2014) have shown that PL is able to inactivate *P. aeruginosas* biofilms on surface-inoculated agar at very high doses, way above the FDA-allowed doses.

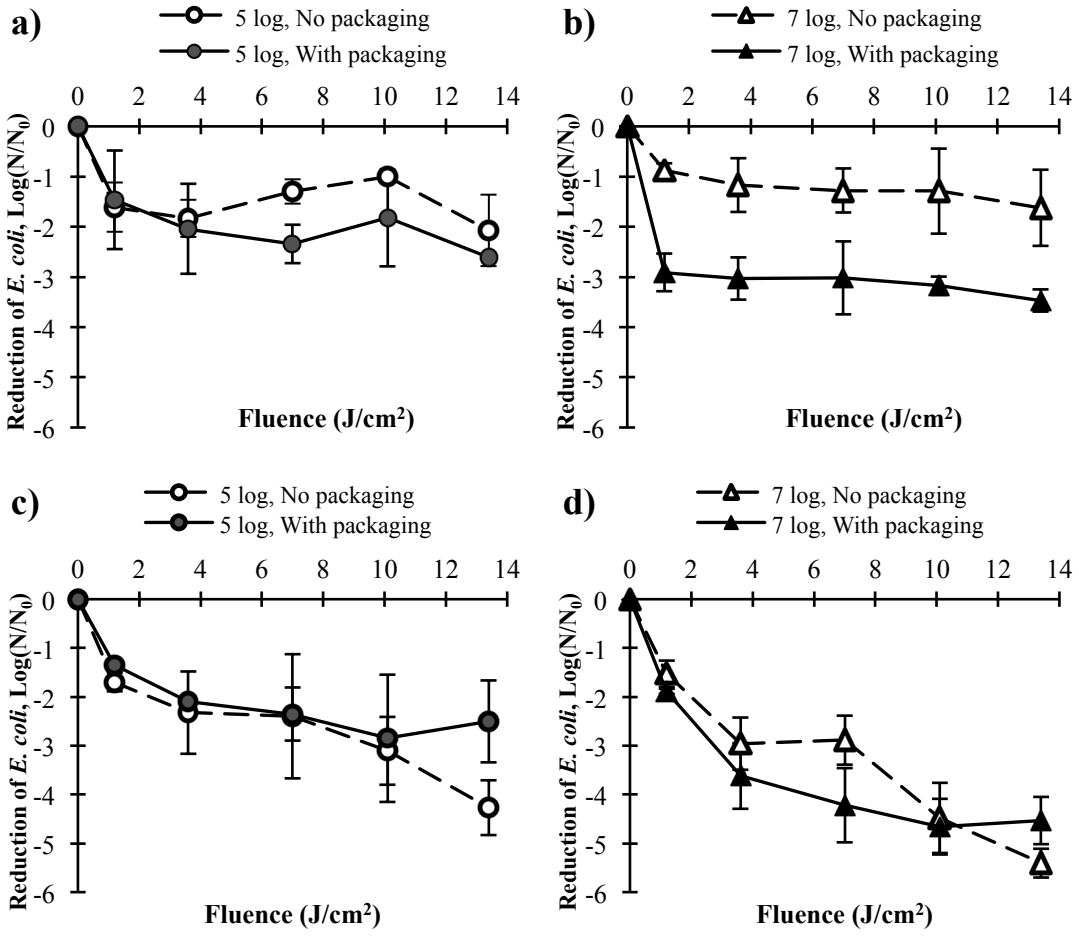

Figure 3. PL inactivation of *E. coli* ATCC 25922 on (**a, b**) cheddar cheese and (**c, d**) processed cheese highlighting different combinations of initial inoculum levels (5 log CFU/slice, 7 log CFU/slice) and presence or absence of UV-transparent low density polyethylene packaging (no packaging, with packaging).

Out of all the effects tested in the general linear model, PL inactivation of *L. innocua* and *P. fluorescens* was only affected by PL dose. For *E. coli,* multiple interaction effects were found to be significant (Table 2.1). *E. coli* is a gram-negative microorganism which was consistently shown to be more sensitive to PL when compared to other pathogenic bacteria in various food matrices like apple juice, orange juice (Pataro et al., 2011) and milk (Palgan et al., 2011). This greater sensitivity explains why *E. coli*'s response to PL was also the most influenced by the different treatment conditions.

Table 2.1 Significant predictors of *E. coli* ATCC 35922, *L. innocua*, and *P. fluorescens* inactivation by PL

Microorganism	Significant predictor of inactivation level	P-value
E. coli ATCC25922	Cheese Type*PL Dose	<0.0001
	Cheese Type*Packaging	<0.0001
	Inoculum Level *PL Dose	0.0369
	Inoculum Level*Packaging	<0.0001
	Inoculum Level*Cheese Type	0.0058
L. innocua	PL Dose	<0.0001
P. fluorescens	PL Dose	0.0036

A significant Cheese Type*PL Dose interaction was primarily due to the fact that, at high doses, greater inactivation is achieved on processed cheese, with a plateau at around 4-log reduction (Fig. 2.3c, 2.3d), than for cheddar cheese, for which a plateau was reached at 3 log-reduction (Fig. 2.3a, 2.3b). This difference was slightly more pronounced at high inoculum levels (Fig. 2.3b, Fig. 2.3d), as the significant Inoculum level*Cheese Type interaction highlights. Another interesting observation

was the dependence between the type of cheese and the presence of LDPE packaging (Table 2.1), which will be discussed in more detail below.

Effect of LDPE Packaging on PL inactivation

The results reported in this study indicate that the presence of LDPE (clear, UV transparent) packaging did not interfere with PL treatment regardless of the organism being treated. Moreover, the presence of a LDPE packaging layer seemed to enhance PL effectiveness against *E. coli* on cheddar cheese (Fig. 2.3b), although the reasons for this increase in effectiveness are not clear at this point and need further investigation.

The potential of PL to be used on packaged foods has been investigated before. Ringus and Moraru (2013) reported that PL treatment of *L. innocua* through transparent LDPE packaging was as effective as when applied directly on the inoculated side of the packaging material. Fernandez et al., (2009) reported that PL consistently achieved the same degree of inactivation of *L. monocytogenes* on inoculated agar either unwrapped or wrapped in polyethylene, polyamide/polyethylene/vinyl acetate co-polymer, or polyamide/polyethylene copolymer. PL inactivation of *L. monocytogenes* on polypropylene-packaged American cheese also did not show significant differences from unpackaged samples (Can et al., 2014). This demonstrates the potential for PL treatment on cheese pre-packaged in clear, UV transparent packaging material.

Effect of Cheese Surface Characteristics on PL inactivation

Similar inactivation levels were achieved on processed cheese for all three microorganisms tested. For cheddar cheese however, PL effectiveness against *E. coli* had a different behavior than for *L. innocua* and *P. fluorescens.* Several interaction terms involving cheese type were found to be significant for predicting the response of *E. coli* to PL (Table 1), which suggest an effect of cheese surface.

Although they appear somewhat different to the naked eye, surface roughness analysis indicated that cheddar cheese and processed cheese are not statistically different in terms of average roughness, highest to lowest peak height difference, surface skewness or surface kurtosis (Table 2.2).

Table 2.2 Surface characteristics of cheese samples

Substrate	Surface roughness parameters (μm)				Water contact angle (°)
	S_a	S_z	S_{sk}	S_{ku}	
Cheddar	3.8 ± 0.5	30.2 ± 4.2	0.64 ± 0.2	3.3 ± 0.5	58.6 ± 2.3[a]
Processed	4.5 ± 1.8	31.3 ± 6.8	0.35 ± 0.2	2.9 ± 0.9	45.6 ± 3.4[b]

[ab] Significant differences at significance level $p<0.05$

However, topographic maps obtained using an interferometric profiler revealed that the surface of cheddar cheese had a higher number of cavities per unit area (Fig. 2.4). This particular detail was not captured by surface roughness analysis, and could explain why higher inactivation is achieved on processed cheese at high doses. Specifically, the lower number of surface non-uniformities on the surface of processed cheese offered a decreased potential for microbial hiding and light shading, while the

larger number of surface non-uniformities on cheddar cheese caused more potential for microbial shading, especially at high inoculum concentration.

Another metric used to compare substrate surface characteristics was the water contact angle, as a measure of surface hydrophobicity. The surface of cheddar cheese was more hydrophobic than processed cheese, with water contact angles of 58.6 ± 2.3° and 45.6 ± 3.4°, respectively (Table 2). These differences may be explained by the respective fat content of the two cheeses, which was 32% for cheddar cheese vs. 21% for processed cheese, These variances in surface hydrophobicity indicate that the liquid inoculum tended to bead up on cheddar cheese, which resulted in more cell clustering and light shading effects. This was likely less pronounced on processed cheese, on which the liquid inoculum was able to spread more.

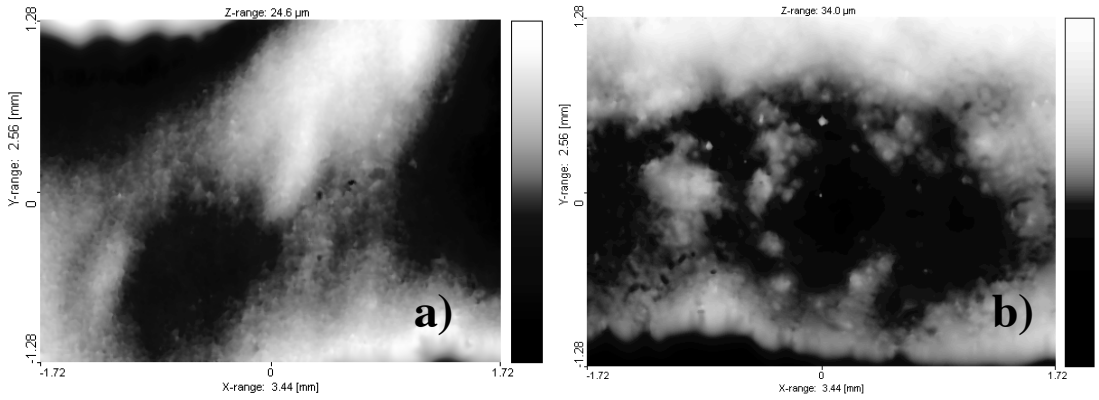

Figure 2.4 Topographic maps spanning 3.44mm in the x-range and 2.56 mm in the y-range generated with an interferometric profiler. The z-range indicates the highest peak in the spanned area. The white areas represent peaks and the dark areas represent valleys. **a)** processed cheese sample; **b)** cheddar cheese sample

CONCLUSIONS

PL treatment was able to consistently achieve over 2-log reduction of bacterial contaminants on cheese surface under a variety of conditions and at lower doses than the FDA approved limit. The lack of interference from LDPE packaging highlights the opportunity to use PL as a terminal treatment on cheese (or other foods) pre-packaged in clear, UV-transparent materials. PL could become a very attractive solution to mitigate surface contamination of cheese in manufacturing, distribution, and retail environments.

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CHAPTER 3

PULSED LIGHT TREATMENT COMBINED WITH NISIN AND NATAMYCIN FOR THE SURFACE DECONTAMINATION OF PASTEURIZED MILK CHEESES

ABSTRACT

Cheese products are susceptible to post-processing cross-contamination that can lead to both food safety issues and significant losses due to spoilage. Pulsed Light (PL) treatment, consisting of short, high-energy light pulses, could represent a solution to address this issue since it is a non-destructive technology that can effectively inactivate microorganisms on surfaces. This study examined the effectiveness of PL on the inactivation of *Pseudomonas fluorescens*, *Escherichia coli* ATCC 25922 and *Listeria innocua* in combination with the antimicrobials nisin and natamycin. The effect of inoculum level, cheese surface topography, and the presence of clear polyethylene packaging were evaluated in a full factorial experimental design. The order of treatment was also evaluated for *L. innocua*. Combination PL + antimicrobial treatments, with antimicrobials applied prior to PL, did not show significant differences ($p<0.05$) compared to PL alone for microbial inactivation. In certain cases, the use of nisin and natamycin even showed antagonistic effects with PL, which was attributed to absorption of UV by the antimicrobials. By reversing the order of treatments, increased *L. innocua* reduction levels as compared to PL treatment alone were achieved on processed cheese, but not on cheddar. This study suggests that PL

has strong potential for decontamination of cheese surface and that a hurdle strategy must take into account the order of treatment.

INTRODUCTION

Cheese products made from pasteurized milk are susceptible to post-processing surface contamination during cutting, slicing, packaging and handling. Pasteurized milk cheeses have caused 49% of all cheese outbreaks occurring in the United States from 1998 to 2011, leading to important health and economic consequences (Gould, 2014). The primary pathogen involved in these outbreaks was *Listeria monocytogenes*, a microorganism that is particularly difficult to control in food processing, distribution, and retail environments. *L. monocytogenes* is ubiquitous, psychrotolerant (George et al, 1988), resistant to relatively high levels of sodium (Conner et al, 1986), and is among the most heat resistant vegetative bacteria (Lovett, 1989), which limits greatly the possibility to prevent listerial contamination of cheese surface. Another pathogen involved in these outbreaks was *Escherichia coli* O157:H7, an enterohemorrhagic, gram-negative bacteria transmitted via the fecal-oral route.

A popular antilisterial solution is nisin, a generally regarded as safe (GRAS) bacteriocin that is approved as a food preservative in over 80 countries (Davies and Adams, 1994;). Nisin targets the cytoplasmic membrane of gram-positive bacteria by rendering the cell membrane permeable, leading to the complete loss of the proton motive force and potentially to cell content leakage (Stevens et al., 1991). Nisin is largely ineffective against gram-negative bacteria, which benefit from the protection of a partly anionic lipopolysaccharide outer membrane. Being hydrophobic, nisin is

unable to cross this barrier in order to reach and damage the inner cytoplasmic membrane (Helander and Mattila-Sandholm, 2000). Several studies have reported a significant effect of nisin on gram-negative *Salmonella spp.*, *Escherichia coli spp.*, and *Pseudomonas spp.* that had been pre-treated with cell-permeating agents like EDTA (Stevens et al., 1991; Delves-Broughton, 1993).

Microbial contamination of cheese surface can also involve spoilage microorganisms like the blue pigment-forming *Pseudomonas fluorescens* (Martin et al, 2011; Nogarol, 2013) and a wide variety of molds. Natamycin is extensively used as a natural preservative in cheese for its antifungal properties.

Pulsed Light (PL) treatment, consisting of short, high-intensity pulses of broad-spectrum light, has been shown to be effective against a wide variety of vegetative bacteria on food surfaces (Dunn et al, 1995; Dunn, 1997). The U.S. Food and Drug Administration has approved PL as a process aid for the decontamination of foods and food contact surfaces in 1996 (FDA Code 21 CFR179.41). It is a non-destructive treatment with very short treatment times, which makes it convenient to use in fast-paced industrial and commercial environments. PL lethality is attributed to the photochemical effect induced by the UV portion of the spectrum, namely to the formation of pyrimidine dimers that block DNA translation and replication (Dunn et al., 1995). Another hypothesis suggests that PL's mechanism of microbial inactivation could also be attributed to a photothermal effect consisting of an instantaneous, intracellular overheating of bacterial cells leading to cytoplasmic membrane damage, cell content leakage, and ultimately cell death (Wekhof, 2001; Takeshita et al., 2003; Cheigh et al., 2012).

PL used in combination with nisin has been demonstrated to show synergistic effects on listerial surface decontamination of Vienna sausages (Uesugi and Moraru, 2009), achieving 4.03 ± 0.15 log reductions compared to 1.37 ± 0.30 and 2.35 ± 0.09 log reductions achieved by PL and nisin alone, respectively. While there is no report of a synergistic effect between PL and natamycin, the extensive use of this antimicrobial in cheese grants further investigation of how the two treatments behave together.

The objective of this study was to examine the effectiveness of PL combined with nisin or natamycin for inactivating relevant spoilage and pathogenic microorganisms on cheese surface including surrogates of *L. monocytogenes* and *E. coli* O157:H7, and the spoilage microorganism *P. fluorescens.* The effect of inoculum level, cheese type, PL dose, and the presence of antimicrobials on bacterial inactivation were evaluated for in a full-factorial design. The order of PL and antimicrobial treatments was also evaluated for *Listeria spp.*

MATERIALS AND METHODS

Inoculum Preparation

The challenge microorganisms for this study included *L. innocua* FSL C2-008 obtained from the Food Safety Laboratory at Cornell University (Ithaca, NY), which has been demonstrated to behave as a surrogate for *L. monocytogenes* in PL treatment (Uesugi and Moraru, 2009; Lasabagaster et al., 2012). The second challenge microorganism was the gram-negative *E. coli* ATCC 25922, a demonstrated surrogate for pathogenic *E. coli* O157:H7 for light-based treatments (Sauer and Moraru, 2009;

Miller et al., 2012). The third organism was *P. fluorescens*, a gram-negative rod associated with milk spoilage with the ability to synthesize a fluorescent blue pigment, pyoverdin, and to form biofilms.

A single colony of each bacterial strain was isolated from Tryptic Soy Agar (TSA) plates and inoculated into 10 mL Tryptic Soy Broth (TSB) for *E. coli* and *P. fluorescens*, or Brain Heart Infusion (BHI) for *L. innocua*. Cultures of *E. coli* and *L. innocua* were grown at 37 ºC with shaking at 225 rpm until stationary phase, typically for 20-24 h, while *P. fluorescens* was grown at 30 ºC with shaking at 225 rpm for 24-26 h. The stationary phase cultures were then diluted in Butterfield's Phosphate Buffer (BPB) to obtain initial inoculum levels of 5 or 7 log CFU per cheese slice.

Substrate Preparation

Sharp white cheddar cheese (Heluva Good, Sodus, NY) and white American singles (Kraft Foods Inc., Northfield, IL) purchased from a local retail store were chosen as cheese substrates, due to their different surface characteristics. A sterilized cheese cutter was used to obtain 2.5 x 5 cm (width x length) rectangular cheese slices of 1-3 mm thickness.

Antimicrobial Treatment

When the antimicrobial treatment was performed before PL, cheese slices were first dipped into a 2.5% Nisaplin™ (nisin) or a 50ppm Natamax™ (natamycin) (Danisco, New Century, KS) solution for 2 min. The cheese slices were then removed, deposited on sterile LDPE coupons to maintain the sample's integrity during manipulation, and allowed to air dry for 2 min. Cheese slices were then individually spot-inoculated onto one face with 10 droplets of 10 μL of the appropriate cell

suspension. The slices were finally allowed to dry at room temperature for 15 min prior to PL treatment. The individual effect of antimicrobials on cheese slices was also monitored.

In the instance where the antimicrobial treatment was performed after PL, the cheese slices were first spot-inoculated as previously described. The inoculated cheese samples were then stored at 4 °C in individual sterile petri dishes overnight to allow the liquid portion of the inoculum to evaporate. After PL treatment, 3 mL of the appropriate antimicrobial solution was deposited onto the inoculated surface of the cheese in order to completely cover it and allowed to sit for 15 min.

Pulsed Light Treatment

PL treatments were conducted using a Xenon RS-3000C bench top unit (Xenon Corporation, Wilmington, MA) equipped with a Xenon flash lamp emitting 360 µs pulses at a frequency of 3 pulses per second. A single slice of cheese was placed centrally on an adjustable stainless steel shelf inside the PL unit at 5.8 cm from the surface of the lamp housing and subjected to 1, 3, 6, 9, or 12 pulses, corresponding to fluence levels of 1.2, 3.6, 7.0, 10.1, and 13.4 J/cm^2, respectively. Inoculated cheese slices that were not exposed to PL or antimicrobials were used as controls.

Fluence Measurements

To monitor the PL dose, fluence measurements were taken using a pyroelectric head (PE25BBH) coupled with a Nova II display (Ophir Optronics Inc, Wilmington, MA). During the measurement, the pyroelectric head and its chord were covered in aluminum foil leaving only a 1-cm^2 circular opening exposed. Cumulative exposure was measured at a distance of 5.8 cm from the lamp housing, at a pulse width setting

of 1.0 ms. Measurements for 1, 3, 6, 9 and 12 pulses were taken in triplicate. At least 1 min was allowed between measurements in order to prevent overheating of the pyroelectric head.

Recovery and Enumeration of Survivors

The treated cheese slices were placed into a sterile Whirl-pak stomacher bag (Nasco, Atkinson, WI) containing 1:10 mass/volume BPB solution, and stomached for 2 min at 230 rpm using a Seward Stomacher 400 Circulator (Seward, Davie, FL). Appropriate dilutions of the stomached sample were plated in duplicate on selective media (BD Difco, Bedford, MA), as follows: MacConkey agar for *E. coli*, Modified Oxford agar for *L. innocua,* and Pseudomonas Isolation agar for *P. fluorescens*. A minimum incubation time of 24 h at 37 ºC was used for *L. innocua* and *E. coli*, and at 30 ºC for *P. fluorescens*. Survivors were enumerated by standard plate counting (SPC) and survivor ratios were quantified as Log (N/N_0), where N represents the number recovered survivors (log CFU) and N_0 represents the measured initial inoculum (log CFU). When plate counts fell below the 30 CFU/plate detection limit, the 3-tube most probable number technique, as described by Downes et al (2001), was used. All experiments were performed in triplicate.

Statistical Analysis

The effect of hurdle treatments was analyzed separately for each microorganism with a general linear model up to a 3-way ANOVA, using the statistical package JMP Pro 10 (SAS Institute, Cary, NC). The model included inoculum level, cheese type, presence of antimicrobials, PL dose, every full factorial interaction terms as fixed effects, and replicates as a random variable. Backward

variable selection was performed and Tukey's Honest Significant Difference (HSD) test was carried out on every significant effect to gain further insights on which factor level was most influential. The order of treatment effect, which was only performed on *L. innocua* at an initial inoculum level of 7 log CFU/slice, was assessed using a two means t-test for each PL dose.

RESULTS AND DISCUSSION

Effect of antimicrobial treatments

As expected, the application of antimicrobials alone did not significantly affect the gram-negative bacteria included in this study. Nisin achieved less than 1-log reduction for both *E. coli* and *P. fluorescens*, while natamycin did not induce reductions of any of the microorganisms tested (Table 1). This can be explained by the fatc that natamycin works by inhibiting fungal ergosterol-dependent protein functions, a futile mechanism against bacteria.

Table 3.1. Inactivation of foodborne microorganisms by nisin and natamycin on cheese surface

Cheese	Microorganism	Antimicrobial	Reduction, $Log(N/N_0)$*
Cheddar cheese	*L. innocua*	Nisin	-1.30 ± 0.76^{c}
		Natamycin	-0.16 ± 0.26^{ab}
	E. coli	Nisin	-0.30 ± 0.20^{ab}
		Natamycin	-0.02 ± 0.08^{a}
	P. fluorescens	Nisin	-0.20 ± 0.09^{ab}
		Natamycin	-0.00 ± 0.08^{a}
Processed cheese	*L. innocua*	Nisin	-0.58 ± 0.22^{b}
		Natamycin	-0.03 ± 0.08^{ab}
	E. coli	Nisin	-0.03 ± 0.10^{ab}
		Natamycin	-0.15 ± 0.39^{ab}
	P. fluorescens	Nisin	-0.11 ± 0.08^{ab}
		Natamycin	-0.14 ± 0.16^{ab}

* Inactivation levels not connected by the same letter are significantly different ($p<0.05$)

The effect of nisin on the gram-positive *L. innocua* was modest, with 1.30 ± 0.76 log-reduction achieved on cheddar cheese, and 0.58 ± 0.22 log-reduction on processed cheese, after a 15 min surface exposure. This is consistent with previous reduction achieved by Uesugi and Moraru (2009), who reported 2.35 ± 0.09 log-reduction of *L. innocua* on Vienna sausages after a 48 h storage period, and Jung et al. (1992), who reported 1.63 log reduction of *L. monocytogenes* in 12.9% fat half-and-half milk.

Effect of hurdle treatments on the gram-negative E. coli and P. fluorescens

For *E. coli* on cheddar cheese, the combination of PL with either nisin or natamycin did not induce any significant increase in inactivation ($p<0.05$). Both combination treatment curves followed very closely the inactivation curve for PL treatment alone, for both inoculum levels tested, with a plateau being reached after 3.6 J/cm^2, at around 2 log-reduction (Fig. 3.1a, Fig. 3.1b).

On processed cheese, the hurdle treatment curves departed from the PL inactivation curves after 6 pulses (7 J/cm^2), and a significant interference ($p<0.05$) between PL and both antimicrobials was obtained (Fig. 3.1c, Fig 3.1d). Specifically, at PL doses exceeding 7 J/cm^2, the use of PL treatment in conjunction with previously added nisin or natamycin, diminished the effect of PL on processed cheese. It is interesting to note that the 2.17 ± 0.24 and 2.82 ± 0.47 log-reduction achieved by PL+nisin combination treatments at a fluence of 10.1 J/cm^2 is significantly higher than the inactivation levels achieved by nisin alone, namely 0.30 ± 0.20 and 0.03 ± 0.10 on cheddar and processed cheese, respectively.

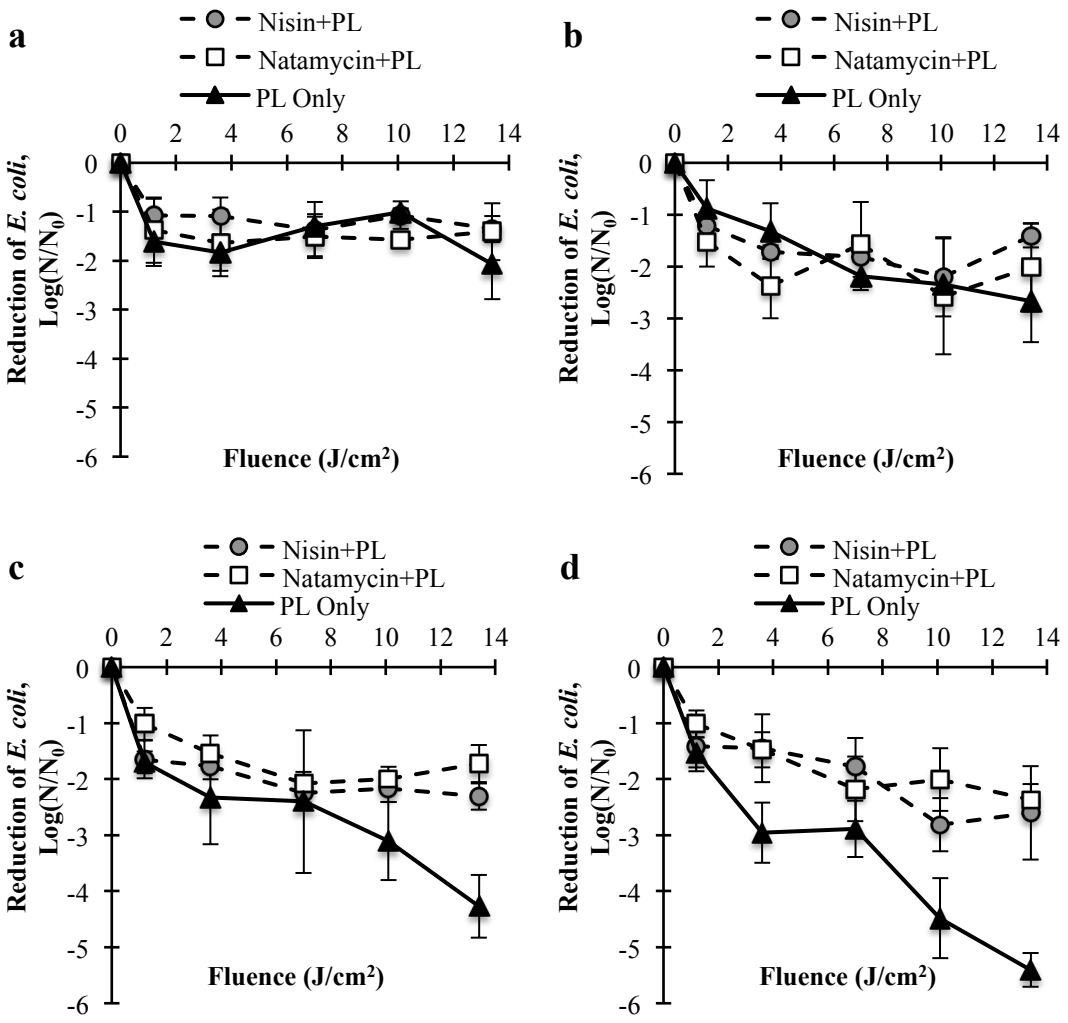

Figure 3.1 Inactivation of *Escherichia coli* on (**a, b**) cheddar cheese and (**c, d**) processed cheese at initial inoculum levels of 5 log CFU/slice (**a**, **c**) and 7 log CFU/slice (**b**, **d**) when being submitted to an antimicrobial dip followed by Pulsed Light treatment, compared to samples that have only been Pulsed Light-treated.

The same antagonistic effects between PL and antimicrobials were also observed for *P. fluorescens*, with the combination treatment showing significantly ($p<0.05$) lower inactivation levels than PL alone on cheddar cheese at high initial inoculum levels after a PL dose of 3.6 J/cm^2 (Fig. 3.2b). Specifically, PL alone

reduced *P. fluorescens* by 2.19 ± 0.27 log cycles after 7 J/cm^2, while combination treatments at the same PL dose yielded barely 1 log-reduction.

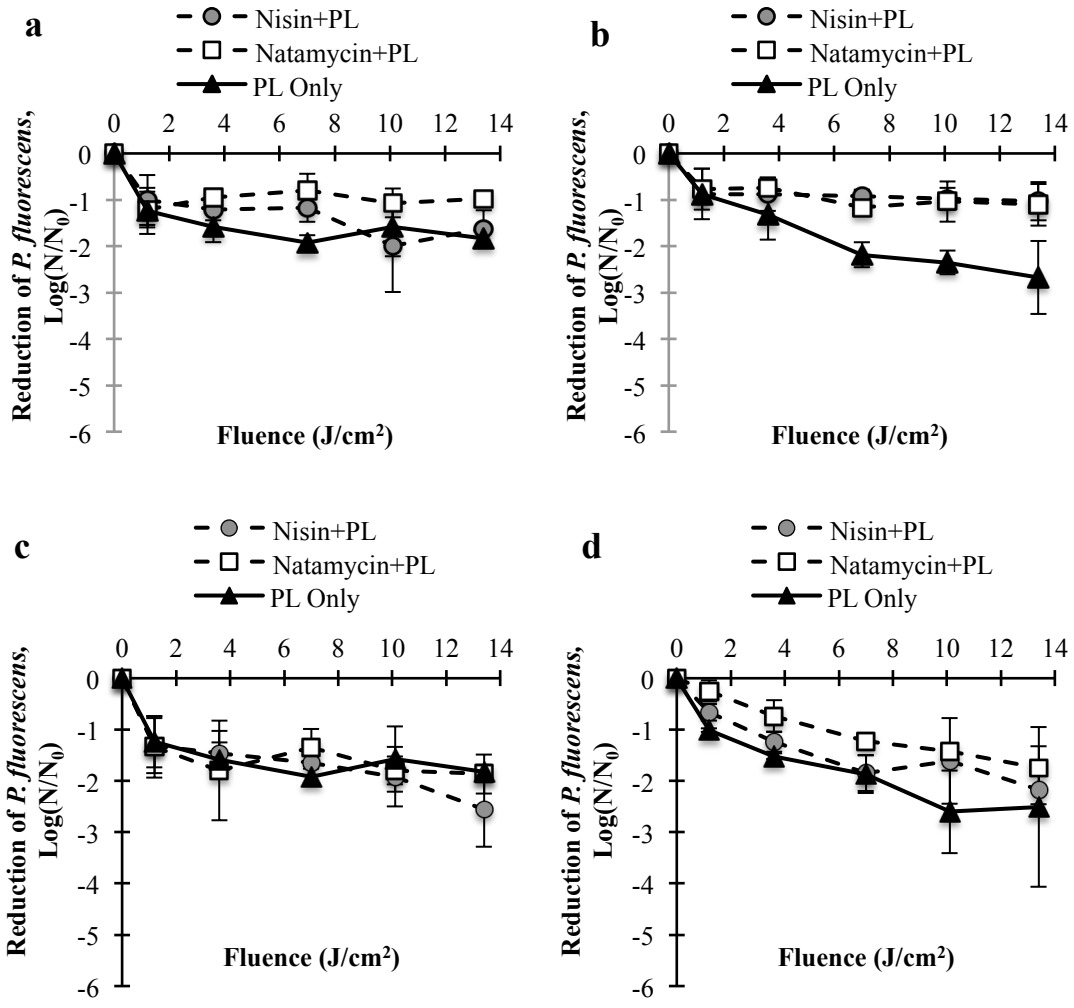

Figure 3.2 Inactivation of *Pseudomonas fluorescens* on (**a, b**) cheddar cheese and (**c, d**) processed cheese at initial inoculum levels of 5 log CFU/slice (**a**, **c**) and 7 log CFU/slice (**b**, **d**) when submitted to an antimicrobial dip followed by Pulsed Light treatment, compared to samples that have only been Pulsed Light-treated.

Effect of hurdle treatments on the gram-positive L. innocua

The combination treatments caused similar antagonistic effects on *L. innocua* to those observed for *E. coli* and *P. fluorescens*. On cheddar cheese, antagonism was

observed at doses of 3.6 J/cm^2 (Fig. 3.3a) while on processed cheese it was only observed at the highest dose tested, 13.4 J/cm^2 (Fig 3.3c). For *L. innocua* inactivation was diminished to 0.78 ± 0.01 log-reduction when using PL+nisin combination treatments as compared to 1.30 ± 0.76 log-reduction by nisin alone.

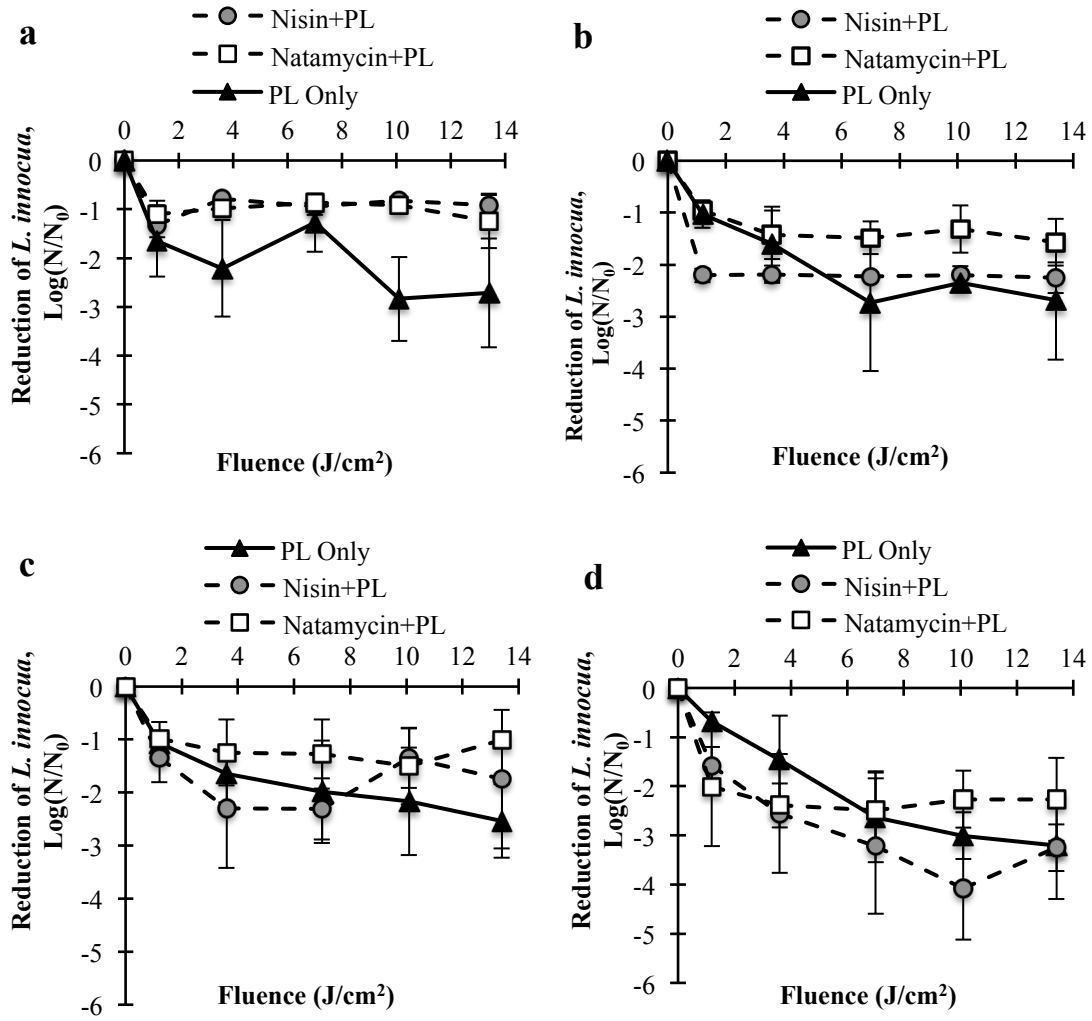

Figure 3.3 Inactivation of *Listeria innocua* on (**a, b**) cheddar cheese and (**c, d**) processed cheese at initial inoculum levels of 5 log CFU/slice (**a**, **c**) and 7 log CFU/slice (**b**, **d**) after being submitted to an antimicrobial dip followed by Pulsed Light treatment, compared to samples that have only been Pulsed Light-treated.

This antagonistic effect could be explained by the fact that both nisin and natamycin have the ability to absorb in the UV range. Polyene microlide antibiotics like natamycin are particularly sensitive to light relative to other antifungal agents and absorb the most at 220 nm (Thomas and Kubler, 1998). Similarly, nisin shows an absorption peak at 225 nm (Uesugi and Moraru; 2009). The UV absorption by both nisin and natamycin diminishes the antimicrobial potential of PL by lowering the bacteridical components of the treatment. Additionally, the possibility of degradation of antimicrobials by PL and formation of by-products should be monitored in the future.

Effect of order of treatment

A reversed order of the combination treatment was investigated to see if the antagonistic effects could be prevented. On cheddar cheese, which has a rougher surface, no improvement in inactivation was achieved as compared to applying the antimicrobials first (Fig. 3.4a, Fig. 3.4b). On processed cheese however, a synergistic effect was observed between PL and nisin, with a maximum inactivation of 3.73 ± 0.96 log-reduction obtained at 10.1 J/cm^2, compared to 1.30 ± 0.76 log-reduction by nisin alone or 3.01 ± 0.48 log-reduction by PL alone. This synergistic effect could be explained by the ability of PL to cause non-lethal cytoplasmic membrane damage in *L. monocytogenes* (Cheigh et al., 2012), which could help nisin's inherent mode of action that also targets the cell membrane.

The effectiveness of PL combined with natamycin on processed cheese was also improved by reversing the order of treatment. PL+natamycin combination treatment achieved statistically similar inactivation levels ($p<0.05$) as PL alone (Fig.

3.4d), namely 2.90 ± 0.69 log-reduction for combination treatments and 2.64 ± 0.91 log-reduction for PL treatment.

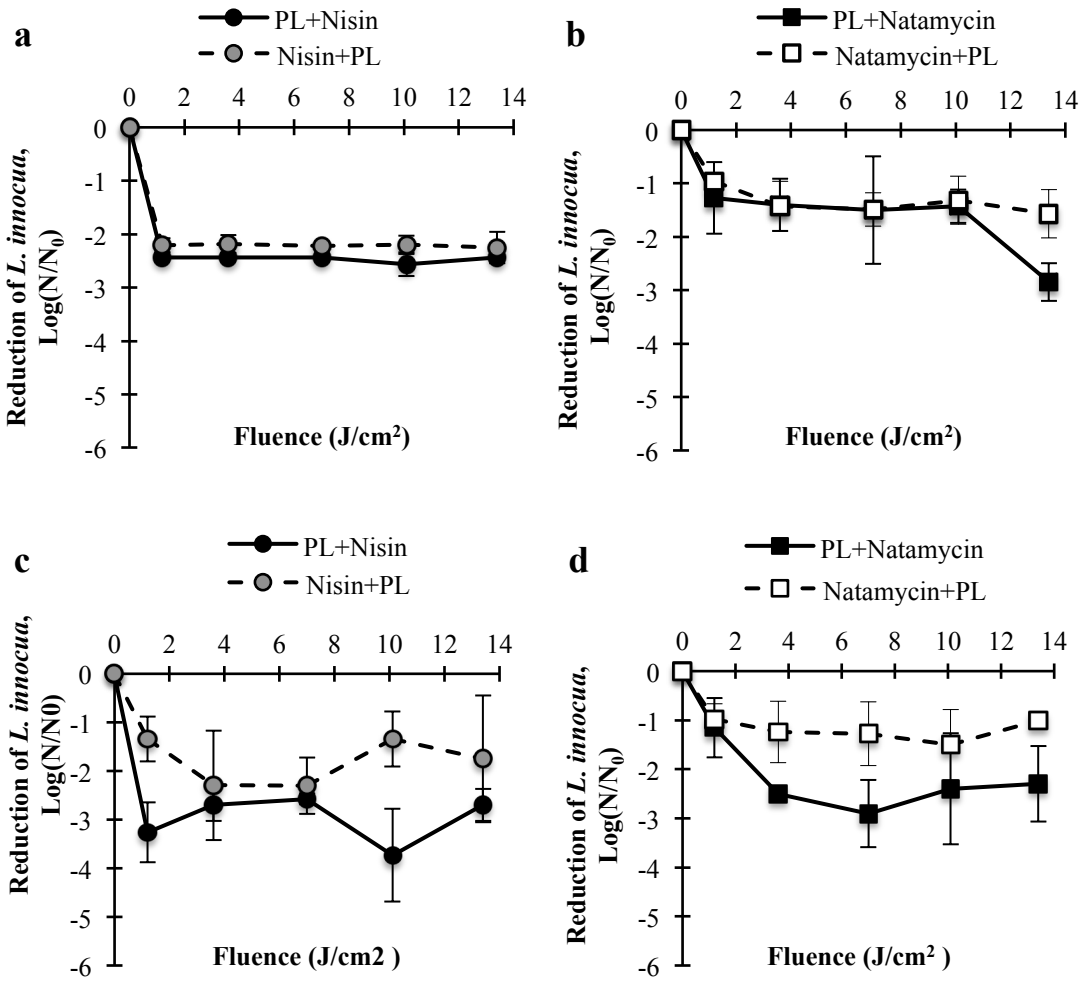

Figure 3.4. Effect of the order of Pulsed Light and nisin or natamycin treatments on *Listeria innocua* reduction on (**a,b**) cheddar cheese and (**c,d**) processed cheese, at initial inoculum level of 7 log CFU/slice. Error bars represent standard deviations of 3 replicates.

CONCLUSIONS

This study suggests that PL could be used in combination with nisin and natamycin and achieve higher inactivation levels of gram-negative bacteria *E. coli* and *P. fluorescens* than with antimicrobials alone, but lower inactivation than with PL alone. The bactericidal effect of nisin was counteracted by its destruction by PL when the antimicrobial was applied before PL, but showed a synergistic effect on processed cheese when applied after PL. Overall, PL combination treatments with nisin and natamycin can offer z marginal increase in microbiological reduction under specific conditions, namely when PL is applied before antimicrobials on processed cheese.

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CHAPTER 4

INFLUENCE OF PULSED LIGHT TREATMENT ON QUALITY PARAMETERS OF CHEDDAR CHEESE

ABSTRACT

Pulsed Light (PL) treatment, consisting of short, high-energy pulses of broad-spectrum light, has been shown to effectively inactivate microorganisms on cheese surface, but limited data is available on the effects of PL on cheese quality. The objective of this study was to examine the influence of PL on important quality parameters of cheddar cheese, including color, oxidative stability, and onset of molding, directly after the treatment and during refrigerated storage.

No significant change in color or increase in peroxide value ($p<0.05$) was observed for PL-treated samples compared to the untreated controls. PL was able to significantly delay surface molding during refrigerated storage. The largest effect was observed at a PL dose of 10.1 J/cm^2, which was able to delay onset of molding by 7 days. These findings suggest that PL will not significantly alter the physico-chemical properties of cheddar cheese and could become a very attractive solution to mitigate surface contamination of cheese.

INTRODUCTION

Cheese products are susceptible to post-processing cross-contamination that can lead to both food safety issues and significant losses due to spoilage (Gould 2014; Nogarol, 2013). Pulsed Light (PL) treatment, consisting of short, high-energy pulses of broad-spectrum light containing wavelengths ranging from the ultraviolet (UV) to near-infrared (NIR) regions, could represent a solution to address this issue. It is a non-destructive technology that can effectively inactivate pathogenic and spoilage microorganisms on surfaces, achieving 1.41 ± 0.15 log-reductions of *L. monocytogenes* on American cheese (Can et al, 2014) and almost 3 log-reductions of *L. innocua* on cheddar cheese (Proulx et al, 2015). PL has also been shown to be effective against a variety of yeasts and molds (Elmnasser et al, 2007; Gomez-Lopez et al, 2007). PL lethality is attributed to a photochemical effect induced by the UV portion of the spectrum, namely to the formation of pyrimidine dimers that block DNA translation and replication (Uesugi and Moraru, 2007).

Light is recognized as being detrimental to the quality of milk and other dairy products by initiating lipid oxidation, which can subsequently lead to product discoloration and the development of rancid off-flavors over extended storage periods (Kristensen et al., 2000). Light is also notorious for having the ability to degrade light-sensitive micronutrients like riboflavin (vitamin B_2) (Deger and Ashoor, 1987). While PL is an attractive solution from a microbiological inactivation perspective, limited data is available on its influence on the organoleptic properties of cheese. Moreover, since PL has the ability to inactivate yeasts and molds, this treatment has

the potential to extend shelf-life of cheese by preventing or delaying surface molding during storage. Therefore, the objective of this study was to assess the effect of PL on color change, oxidative stability, and onset of molding on cheddar cheese.

MATERIALS AND METHODS

Substrate Preparation and Storage

Sharp white cheddar cheese (Heluva Good, Sodus, NY) was purchased from a local retail store and cut to 2.5 x 5 cm (width x length) rectangular slices of 1-3 mm thickness using a sterilized cheese cutter. The exact weight of each slice was recorded and samples of 4 ± 0.2 g were kept for the experiments. After PL treatment, each slice was sealed in an individual polyethylene bag (Ziploc, SC Johnson, Racine, WI) and stored inside a commercial dairy display case equipped with a front low-UV emitting T-8 fluorescent lamp (True Manufacturing, O'Fallon, MO). The storage temperature was set at 6 ± 1°C and the samples were kept for up to one month.

Pulsed Light Treatment

PL treatments were conducted using a Xenon RS-3000C bench top unit (Xenon Corporation, Wilmington, MA) equipped with a Xenon flash lamp emitting 360 μs pulses at a frequency of 3 pulses per second. A single slice of cheese was placed centrally on an adjustable stainless steel shelf that had previously been covered with a sterilized, low-density polyethylene coupon, and was put inside the PL unit. During treatment, the samples lied 5.8 cm below the surface of the lamp housing. Cheddar samples were then subjected to 1, 3, 6, 9, or 12 pulses, corresponding to fluence levels of 1.2, 3.6, 7.0, 10.1, and 13.4 J/cm^2, respectively. For peroxide value

monitoring, cheese slices were only subjected to either a low (3.6 J/cm^2) or a high (10.1 J/cm^2) dose.

Fluence Measurements

To ensure consistent PL dose, fluence measurements were taken using a pyroelectric head (PE25BBH) coupled with a Nova II display (Ophir Optronics Inc, Wilmington, MA). During the measurement, the pyroelectric head and its chord were covered in aluminum foil leaving only a 1-cm^2 circular opening exposed. Cumulative exposure was measured at a distance of 5.8 cm from the lamp housing, at a pulse width setting of 1.0 ms. Measurements for 1, 3, 6, 9 and 12 pulses were taken in triplicate. At least 1 min was allowed between measurements in order to prevent overheating of the pyroelectric head.

Lipid Oxidation

Lipid Extraction

The lipid portion of cheese was extracted using an adapted method from Kristensen et al. (2001). The samples were first individually grated and transferred to a beaker containing 80 mL of chloroform-methanol (2:1 v/v). The mixture was homogenized using an IKA Ultra-Turrax homogenizer (IKA, Wilmington, USA) at 7,000 rpm for 60s. After $CaCl_2$ addition (1.0 mM; 16mL) and a vigorous 15 s shake, the mixture was centrifuged at 500×g using a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Thermo Scientific, Waltham, MA). The sediment (bottom layer) was transferred to an evaporation flask and left to dry under vacuum at room temperature until all the chloroform evaporated.

Peroxide Value Measurement

Lipid peroxides are used as indicators for early-stage oxidative changes (Shahidi and Zhong, 2005). In this study, peroxide value (PV) was monitored spectrophotometrically using the International Dairy Federation Standard method 74A (IDF, 1991). In this method, lipid peroxides oxidize ions to ferric ions, which then form a ferric-thiocyanate complex that absorbs at 500 nm. PV development was measured 3 times a week over a month of refrigerated storage on untreated cheddar and PL-treated cheese subjected to low (3.6 J/cm^2) and high (10.1 J/cm^2) PL doses. Measurements were performed in triplicate and values are reported as milliequivalents of O_2 per gram of lipid.

Color Measurement

The color of each cheese slice was measured prior to PL treatment, immediately after PL treatment, and then every 2 days over a 1-month refrigerated storage period. A Chroma C-400 colorimeter (Konica Minolta, Chiyoda, Japan) was used, after proper calibration. Color measurements were taken through the transparent polyethylene bag in which samples were stored, to minimize sample surface manipulations. Color change was expressed as CIELAB $L^*a^*b^*$-values, where L^* is a measure of increasing lightness ranging from 0 to 100, a^* is a measure of the sample's color on the CIELAB green-red axis, and b^* is a measure of the sample's position on the CIELAB blue-yellow axis (Witzel et al, 1973). The CIE74 ΔE-metric was also calculated as a more global measure of perceptual color change, according to the following formula (Lawless and Heymann, 2010): $\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$. All measurements were performed in triplicate.

Onset of molding

Cheese slices were cut from the outer portion of a commercial brick of cheddar cheese, to ensure that the surface exposed to PL treatment had been previously exposed to air and typical handling in a commercial cheese making facility. Recorded fungal development thus originated from spores present in the processing environment. A set of 30 cheese slices was prepared for each PL treatment dose. The onset of molding was monitored daily by visually inspecting the samples; the time until the first appearance of fungal growth on each cheese slice was recorded.

Statistical Analysis

A one-way ANOVA was performed on ΔE-CIELAB values for each storage day. If a significant effect of PL dose on ΔE was found significant on a given day, Tukey's Honest Significant Difference (HSD) test was carried out to determine which factor level differed from the others, especially from the control. The same procedure was followed to determine significant differences in PV values between untreated and PL-treated cheddar samples. The data was analyzed using the statistical package JMP Pro 10 (SAS Institute, Cary, NC).

Survival analysis, a biostatistics method identified as a useful tool for food shelf life studies (Marquenie et al, 2002) was used to analyze the time until onset of molding on PL-treated cheddar cheese. An advantage of this method is that it is able to account for incomplete datasets, in this particular case for cheese slices that will not have molded by the time the study ended, otherwise called censored observations (Kleinbaum and Klein, 2005). The non-parametric Kaplan-Meier estimator $\hat{S}(t)$, which approximates the probability of a cheese sample to remain mold-free until a

certain time *t*, was calculated and used to describe general molding patterns of PL-treated cheese. A semi-parametric Cox Proportional Hazards model was then used to assess the effect of PL dose on the onset of molding. A goodness of fit test based on Schoenfelds' residuals confirmed that the Cox Proportional Hazards assumptions were respected. Survival analysis was performed using Small Stata 13.1 (StataCorp, College Station, TX).

RESULTS AND DISCUSSION

Effect of PL on cheddar cheese color

Over a 30-day refrigerated storage period, PL-treated samples exhibited stable CIELAB lightness parameters L^*, around 75, a slight increase in the green-red hue, going towards neutrality, and a small decrease in the yellow-blue hue (Fig 4.1). It is important to note that these slight changes in hue did not translate into a perceptible change in color to the naked eye. This is consistent with other work where the authors were only able to see significantly altered cheese color at higher storage temperatures of 37 ºC (Kristensen et al, 20001).

When evaluating the global ΔE color difference metric encompassing L^* a^* and b^* values, there was no significant difference ($p<0.05$) between the PL-treated cheese samples and the untreated controls. The only exception was a significant difference found after 10 days of refrigerated storage, where cheese samples that were untreated along with samples treated with the highest dose (13.4 J/cm^2) had ΔE values that were significantly different from the others (Table 4.1). This single discrepancy may be

attributed to a wider range of a* values observed around 10 days of refrigerated storage, but this was an isolated event that did not reflect an overall trend.

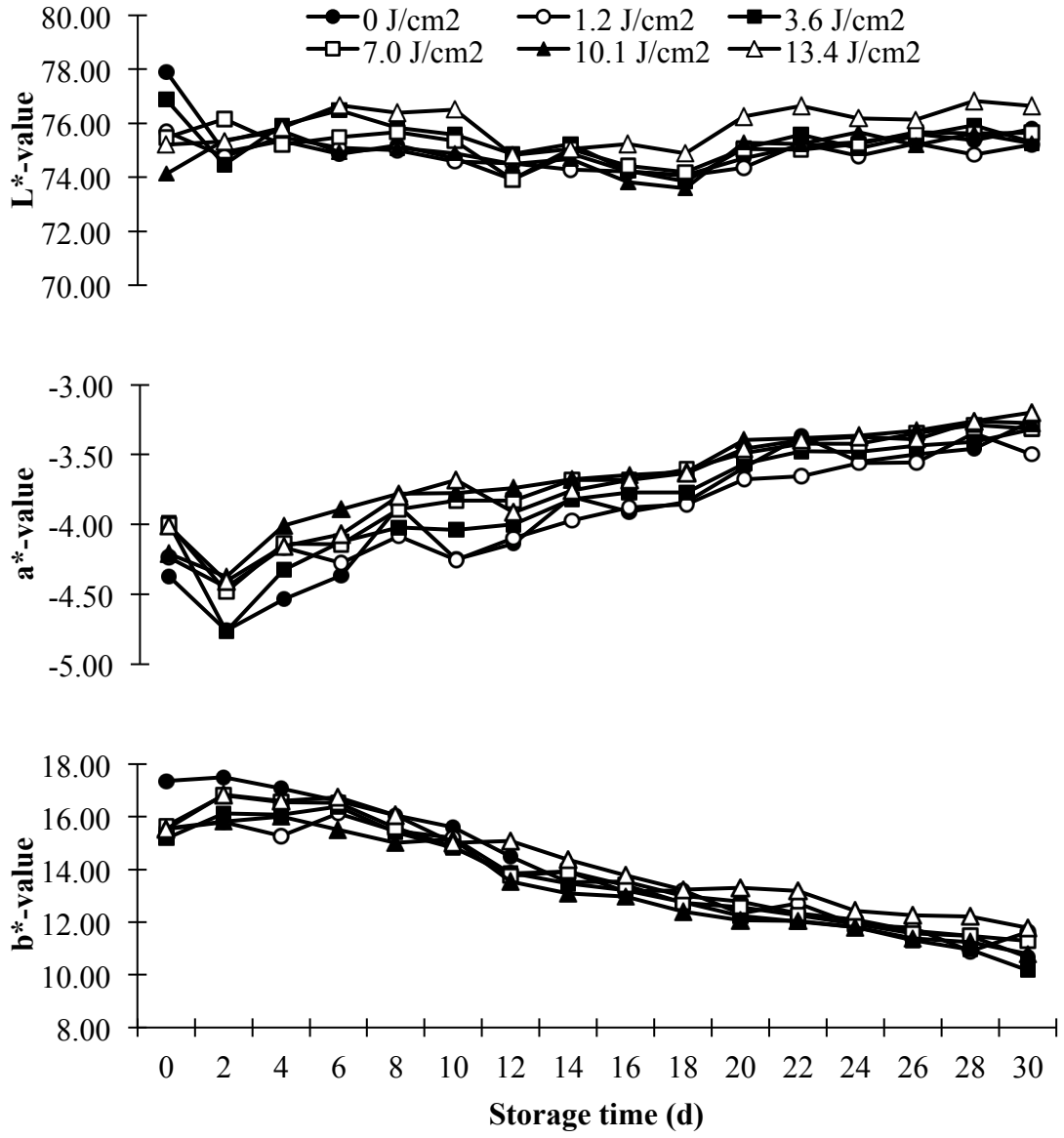

Figure 4.1 Color change expressed in CIELAB L*a*b* values monitored on PL-treated cheddar cheese stored at 6ºC for one month. Each data point represents the mean of 3 separate measurements.

Table 4.1 CIE74 ΔE values of PL-treated cheddar cheese after 10 days of refrigerated storage

PL Dose (J/cm^2)	ΔE^*
0	1.70 ± 0.40^a
1.2	3.38 ± 0.67^b
3.6	2.63 ± 0.31^{ab}
7	2.21 ± 0.72^{ab}
10.1	3.13 ± 0.77^{ab}
13.4	1.79 ± 0.41^b

* Values marked with different letters are significantly different from each other ($p<0.05$)

Effect of PL on oxidative stability of cheddar cheese

The untreated, control samples experienced a PV value increase from 0.07 ± 0.03 at day 0 to 0.19 ± 0.05 after a month of refrigerated storage in a commercial dairy case fluorescent light. No significant increase in PV value ($p<0.05$) compared to the controls was observed for neither the low-dose treated cheddar slices nor the high-dose treated cheddar slices (Fig. 4.2). At 3.6 J/cm^2, PV value increased from 0.06 ± 0.04 to 0.22 ± 0.02 after a month of storage. Similar increases were observed at 10.1 J/cm^2, from 0.09 ± 0.02 to 0.23 ± 0.02. These results show that there is no significant effect of PL dose on lipid peroxide formation in cheddar cheese relative. In comparison, Kristensen et al. (2001) reported significantly higher PV values for processed cheese samples exposed to fluorescent light at a 2000 lx intensity after one month of storage at 5 ºC relative to untreated samples, with PV values quickly exceeding 1.

An important parameter that could change this non-effect of PL dose on cheddar oxidative stability is the surface to volume ratio of the sample being treated given that PL is a surface treatment.

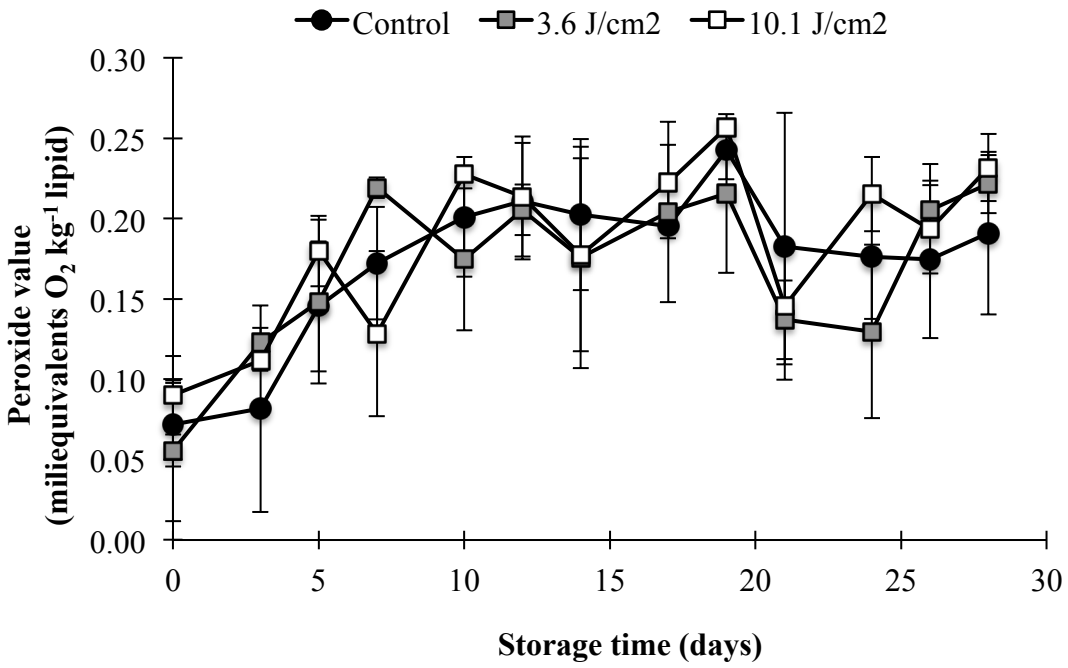

Figure 4.2 Peroxide value development as a measure of primary lipid oxidation products in PL-treated cheddar cheese stored for one month at 6ºC

Effect of PL on onset of molding on cheddar cheese surface

Survival analysis that evaluated the time until the first mold growth on PL-treated cheddar cheese occurred revealed that a PL dose of 10.1 J/cm^2 could delay the first mold appearance by 7 days relative to the control (Fig. 4.3). The median survival times obtained from the Kaplan-Meier estimates also reveal that after 21 days, half of the control samples were moldy. In contrast, it took 28 or 30 days for samples treated at low to medium PL doses for half the samples to mold, while high PL doses of 10.1 J/cm^2 and 13.4 J/cm^2 caused more than half of the samples to remain mold-free after the one-month refrigerated storage period.

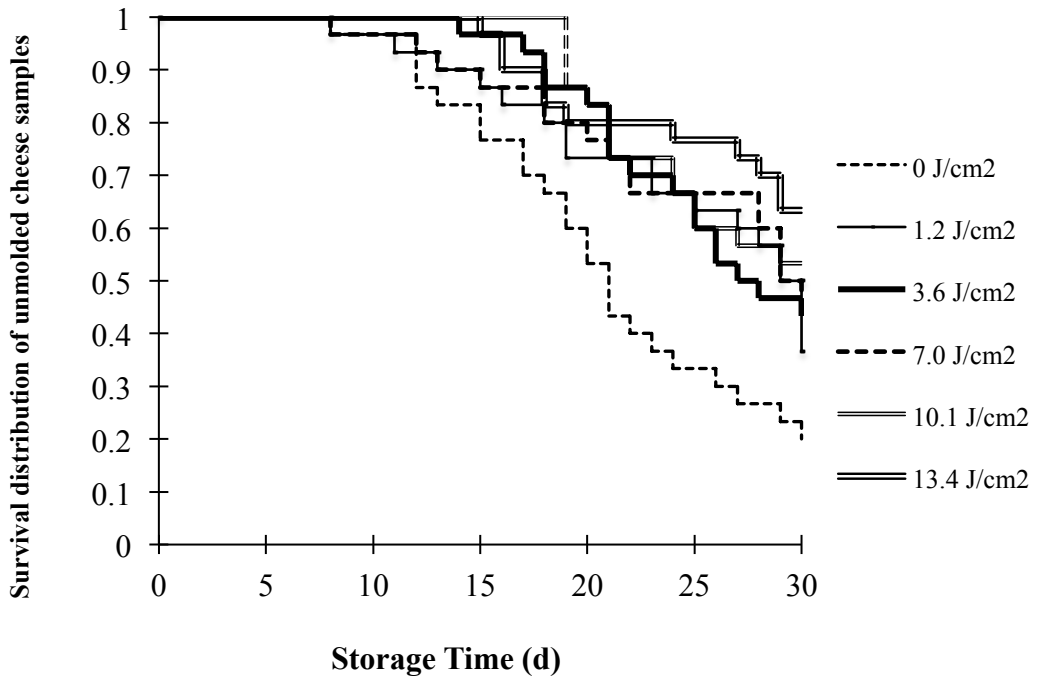

Figure 4.3 Kaplan-Meier survival curves of 180 cheddar samples treated with varying PL doses. Each curve associated with a particular dose represents the onset of molding in a set of 30 cheddar slices treated under the same conditions. (Survival of 1= 0% of cheese samples are molded, Survival of 0 = 100% of cheese samples are molded.)

A Cox Proportional Hazard model was used to establish whether these apparent differences between the controls and PL-treated cheddar slices were significant. A univariate Wald test revealed that PL dose had a highly significant effect on the time until onset of molding ($p<0.001$). With a hazard ratio of 0.93, for every 1-unit increase in PL dose, the risk of obtaining moldy cheese is reduced by 107%.

CONCLUSIONS

No significant change in color or increase in peroxide value ($p<0.05$) was observed for PL-treated samples relative to the controls, and a PL dose of 10.1 J/cm^2 was able to delay onset of molding by 7 days. These findings suggest that PL will not significantly alter the physico-chemical properties of cheddar cheese and could become a very attractive solution to mitigate surface contamination of cheese. A sensory evaluation of these samples, as well as monitoring of secondary products of lipid oxidation, will be required in order to further understand possible detrimental changes caused by PL treatment of cheese surface.

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