Inactivation of *Escherichia coli* in milk and concentrated milk using pulsed-light treatment

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ABSTRACT

Pulsed light (PL) treatment has been viewed as an alternative to thermal treatments for the inactivation of pathogenic and spoilage microorganisms in recent years. The objectives of this study were to quantify the effectiveness of PL on inactivating *Escherichia coli* in cow milk and to evaluate the effect of total solids and fat content on inactivation. Samples of reconstituted milk with variable total solids levels (9.8, 25, and 45%) and commercial cow milk with different fat contents (skim milk, 2% fat, and whole milk) were inoculated with nonpathogenic *E. coli* ATCC 25922 at a concentration of 10^7 cfu/mL. One milliliter of the inoculated sample was placed in a thin layer in a glass chamber and exposed to PL doses of up to 14.9 J/cm^2 , both in static mode and turbulent mode. Survivors were quantified using standard plate counting. All experiments were performed in triplicate. Pulsed light treatment of the concentrated milks of 25 and 45% solids content resulted in reductions of less than 1 log, even in turbulent mode, whereas for the milk with 9.8% solids content, reduction levels of 2.5 log cfu were obtained after treatment with 8.4 J/cm^2 in turbulent mode. In the skim milk, a 3.4 log cfu reduction at 14.9 J/cm^2 was obtained and a plateau of the inactivation curve typical of PL treatment was not achieved. Under the same conditions, both 2% and whole milk attained inactivation levels greater than 2.5 log cfu. These data indicate that PL is effective for the inactivation of *E. coli* in milk, but has limited effectiveness for microbial inactivation in concentrated milk, due to the absorption of light by the milk solids and shielding of the bacteria in the concentrated substrates. Milk fat also diminishes the effectiveness of PL to some extent, due to light-scattering effects.

Key words: pulsed light, *Escherichia coli*, concentrated milk, fat

INTRODUCTION

Foodborne illness outbreaks caused by the consumption of contaminated foods continue to be a major concern in the United States and worldwide (Scallan et al., 2011). Dairy products still contribute to outbreak statistics, despite the highly regulated and widely implemented pasteurization of milk. Milk must be pasteurized before interstate sale in the United States, according to the Pasteurized Milk Ordinance of the US Food and Drug Administration (FDA; US FDA, 2009). Nonetheless, illnesses due to the consumption of milk still occur. Eighty-three fluid milk-borne outbreaks were reported between 1990 and 2006 in the United States, resulting in 3,621 illnesses. Of these outbreaks, 44.6% were associated with the consumption of pasteurized milk (Newkirk et al., 2011). A large listeriosis outbreak was reported in Germany in 2006 to 2007, in which 189 patients were affected by eating a commercial cheese made from pasteurized milk contaminated with *Listeria monocytogenes* (Koch et al., 2010). In one of the largest dairy-borne outbreaks in Japan, milk powder contaminated with *Staphylococcus aureus* enterotoxin A affected 13,420 people (Asao et al., 2003).

Although HTST treatment of milk is known to cause minimal undesirable changes in sensory properties and quality, continued pressure exists from consumers for non-heat-treated foods, including milk (Zink, 1997). As pointed out by Oliver et al., (2009), some consumers believe that non-heat-treated milk has better taste, nutrition, and health benefits, however unsubstantiated these claims may be. Pulsed-light (PL) treatment has emerged in recent years as a viable alternative to heat treatment for the inactivation of microorganisms in foods, on food packaging, on food contact surfaces, and even in waste streams (Woodling and Moraru, 2007; Moraru and Uesugi, 2009; Sauer and Moraru, 2009; Harrington, 2011). Some interest also exists in the dairy industry to explore light-based treatments (UV and PL) as an alternative to heat treatment (Nieburg, 2010).

Pulsed-light treatment was approved by the FDA for the treatment of foods and food surfaces, provided that the radiation source is a xenon flashlamp that emits

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broadband-spectrum radiation in the wavelength range of 200 to 1,100 nm, with a pulse duration of less than 2 ms, and a total treatment not exceeding 12 J/cm² (US FDA, 1996). Although the exact mechanism of inactivation has not been fully elucidated, it is generally agreed that most of the inactivation effect comes from the UV range [wavelength (λ) <300 nm] of the PL spectrum (Rowan et al., 1999; Woodling and Moraru, 2007). It has been suggested that PL is more effective than continuous UV treatment due to higher penetration depth and emission power, with high peak power of pulses, although this is still debated (Oms-Oliu et al., 2010). Most likely, inactivation occurs through dimer formation and helix distortion of microbial DNA (Rowan et al., 1999). A recent study found that, in addition to the DNA damage induced by PL, inactivation was also achieved through a photothermal and photophysical effect, which caused cell wall and membrane rupture, leading to leakage of the cytoplasm, and ultimately to cell death (Cheigh et al., 2012).

Pulsed-light and UV treatments have been shown to be effective at reducing the bacterial load in liquid dairy foods in previous work. Krishnamurthy et al. (2007) found reduction of up to 7.26 log cfu of *Staph. aureus* in raw milk at 2 passes in a PL unit at a distance of 11 cm from the lamp. Matak et al. (2005) reported a 5.45 log cfu reduction of *L. monocytogenes* in raw goat milk when exposed to 16.9 mJ of UV light/cm² in a flow-through system. Reinemann et al. (2006) observed a 3-log reduction in total viable bacterial count in raw cow milk using a circulating UV treatment system at a dose of 15 kJ/L. Variations in treatment effectiveness between these studies can be mostly attributed to the substrate, as absorption, reflection, and scattering of light by the substrate can reduce the effectiveness of the light-based treatment. No systematic information is available, however, on how the effectiveness of PL treatment in cow milk is affected by milk components. Therefore, the main objectives of this study were to evaluate the effectiveness of PL treatment for the inactivation of *Escherichia coli* in milk and to evaluate the effect of fat and TS content on inactivation.

MATERIALS AND METHODS

Substrates

To evaluate the effect of solids on inactivation, commercially available nonfat instant milk powder was reconstituted to the desired solids content. Three different levels of TS were chosen for the reconstituted milk: 9.8 (package instructions to produce skim milk), 25, and 45%. The appropriate amount of powdered milk was weighed and added to autoclaved distilled water

to produce the desired levels of TS. The reconstituted samples were then stored in a refrigerator at 4°C for up to 22 h to allow full hydration. Prior to the PL treatment, the samples were removed from the refrigerator and immediately placed in an ice bath to maintain the sample temperature at approximately 4°C.

For the evaluation of fat percentage on PL effectiveness, commercially available cow skim milk, 2% milk, and whole milk (3.25% fat) were used. The milk samples were stored at 4°C for less than 48 h until use and then transferred to an ice bath at approximately 4°C immediately before treatment.

Composition of the Milk Powder

The milk powder used for substrate preparation was analyzed at Dairy One Inc. (Ithaca, NY) for fat, total nitrogen (TN), and lactose content, using ether extraction (AOAC International, 2000; method 989.05; 33.2.26), Kjeldahl (AOAC International, 2000; method 991.20; 33.2.11), and enzymatic analysis (AOAC International, 2000; method 984.15; 33.2.24), respectively. The TS content of the powders was measured by forced-air oven drying (AOAC International, 2000; method 990.20; 33.2.44). The milk powder had an average of 0.5 g of fat, 34.8 g of total protein, 96.1% TS, 7.8 g of ash, and 53.1 g of lactose.

Challenge Organism and Inoculum Preparation

The challenge microorganism used in this work was nonpathogenic *E. coli* ATCC 25922 in the stationary growth stage. This microorganism was previously proven to be an appropriate surrogate organism for the pathogenic *E. coli* O157:H7 for continuous UV light treatment (Quintero-Ramos et al., 2004). *Escherichia coli* O157:H7 is not frequently a cause of foodborne outbreaks in dairy products, with only 1 of 38 raw milk-associated outbreak reported to the Centers for Disease Control and Prevention (Atlanta, GA) between 1973 and 1992, and 6 of 46 unpasteurized milk outbreaks from 1990 to 2006 being attributed to *E. coli* O157:H7 contamination (Headrick et al., 1998; Newkirk et al., 2011). Earlier work demonstrated that *E. coli* ATCC 25922 was an appropriate surrogate organism for *E. coli* O157:H7 in terms of response to PL treatment (Sauer and Moraru, 2009). Therefore, this strain was used in the current study as a model for studying the resistance of gram-negative microorganisms to PL inactivation in milk substrates.

Escherichia coli ATCC 25922 was maintained on Trypticase soy agar (TSA; Becton Dickinson, Sparks, MD) at 4°C. Prior to the PL treatments, a single colony was transferred onto a TSA plate and incubated for 22

± 2 h at $37 \pm 1^\circ\text{C}$. A single colony was then transferred into Trypticase soy broth (TSB; Becton Dickinson) and incubated at $37 \pm 1^\circ\text{C}$ for 20 ± 2 h, under shaking (at 230 rpm). An aliquot of 1 mL of inoculum was transferred into 9 mL of milk substrate, resulting in a starting population (N_0) of approximately 10^7 cfu/mL.

PL Treatments

One-milliliter volumes of the inoculated liquid milk substrates were placed in sterile, transparent Lab-Tek II Chamber Slide 1 well slides (Nalge Nunc International, Naperville, IL) with dimensions of $23.2 \times 53.3 \times 11.0$ mm. The bottom of the chamber was made of glass, and the side walls were made of polystyrene. Previous work has shown that substrate geometry is a very important factor for the effectiveness and uniformity of PL treatment, and that fluence decays proportionally to the second power of distance from the light source (Hsu and Moraru, 2011). Because the thickness of the liquid layer is critical for liquid substrates, a constant thickness of the liquid layer was maintained in the current study. Specifically, 1 mL of liquid substrate was used, which resulted in a thickness of 1.3 mm.

Pulsed-light treatments were performed using a RS-3000C SteriPulse system (Xenon Corp., Woburn, MA). The system consists of a controller unit and a treatment chamber that houses a Xenon flash lamp. To generate turbulence during the shaking treatments, an MS 3 basic orbital shaker (IKA Works Inc., Wilmington, NC) was placed in the center of the PL treatment unit atop a stainless steel shelf, at a distance of 55.9 mm (2.2 in) under the lamp. The shaker was completely wrapped in aluminum foil to prevent the change of the light path inside the PL unit during treatment. The chamber slides were kept in place using a universal attachment of the shaker. For the treatments performed under turbulence, the shaker was used at a speed of 3,000 rpm. Static treatments were performed in the same manner, with the exception that the shaker was not powered on during these treatments.

Inoculated reconstituted milk and cow milk samples were exposed to treatment for 3 to 21 pulses of light, at a fluence of 0.71 J/cm^2 , which resulted in total energy doses of 2.14 to 14.85 J/cm^2 , respectively. All PL treatments were performed in triplicate.

PL Fluence Measurements

The dose of PL treatments, (fluence, expressed in J/cm^2) was quantified for all treatments. Measurements of fluence were taken using a pyroelectric head (PE25BBH-DIF) with a Nova II display (Ophir Optics Inc., Wilmington, MA). The pyroelectric head

was placed centrally atop a stainless steel shelf at a height of 55.9 mm (2.2 in) underneath the focal point of the Xenon lamp. A stainless steel aperture was used to cover the top surface, leaving a 1-cm^2 circular opening exposed centrally on the detector surface. For the measurements, a pulse width setting of 1.0 ms was used, along with a wavelength setting of $<0.3 \text{ nm}$. Fluence measurements were performed in triplicate, with pauses of at least 60 s between measurements to prevent overheating of the pyroelectric head.

Enumeration of Survivors

Following PL treatment, the treated 1-mL sample was transferred into 7 mL of Butterfield's phosphate buffer (BPB) and the glass chamber was rinsed twice with 1 mL each of BPB, adding the rinse BPB to the treated inoculum. This yielded a volume of 10 mL of recovery liquid. The recovery liquid was serially diluted in BPB, after which $100\text{-}\mu\text{L}$ volumes were spread plated in duplicate on both TSA and MacConkey agar (Becton Dickinson), and incubated for 22 ± 2 h at $37 \pm 2^\circ\text{C}$. For the cow milk samples, the recovery liquid was produced and plated in the same manner. Survivors (N) were then enumerated using the standard plate counting method. Because similar counts were found on both TSA and MacConkey agar (selective media for *E. coli*), only counts determined on TSA will be reported in this paper. Levels of microbial reduction were calculated as $\log(N/N_0)$, and inactivation curves were drawn by plotting reduction against fluence.

Statistical Analysis

For each treatment, the mean and standard deviation of survivor ratios were calculated. Analysis of variance was used to determine significant differences at $P < 0.05$, using JMP 8.0 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Effect of TS Levels on Microbial Reduction by PL Treatment

The level of reduction of *E. coli* ATCC 25922 by PL was correlated with the level of fluence used in the treatment. At the lowest fluence of 2.14 J/cm^2 , reductions (means $\pm$ SD) of 0.21 ± 0.03 , 0.13 ± 0.05 , and $0.03 \pm 0.30 \log \text{ cfu}$ were found, for 9.8, 25, and 45% TS, respectively. When operating at a fluence of 8.41 J/cm^2 , inactivation of *E. coli* ATCC 25922 in the 9.8% TS sample reached a slightly higher reduction level ($0.41 \pm 0.04 \log \text{ cfu}$; Figure 1). As the concentration of TS increased to 25 and 45%, the reduction levels after

Figure 1. Inactivation of *Escherichia coli* in concentrated milks with 3 different TS contents by pulsed-light (PL) treatment in static mode. Plotted are mean ($n = 3$) values ± 1 SD. N = number of survivors; N_0 = number in starting population.

treatment with 8.41 J/cm^2 were found to be $0.30 \pm 0.07 \text{ log cfu}$ and $0.28 \pm 0.29 \text{ log cfu}$, respectively. No statistical difference between the different substrates was observed. These levels of inactivation are extremely low, almost negligible from a microbiological point of view, indicating that PL treatment of concentrated milks in static mode is rather ineffective.

Use of turbulence enhanced the inactivation of *E. coli* by PL treatment. In this study, turbulence was achieved by using a variable-speed orbital shaker inside the PL unit, to mimic turbulence created in a commercial flow-through treatment system. At a fluence level of 8.41 J/cm^2 , a $2.05 \pm 0.11 \text{ log cfu}$ reduction (means $\pm$ SD) was achieved for the reconstituted milk with 9.8% TS (Figure 2). At the same treatment dose, significantly lower reduction levels were observed for the higher TS content milks, with reductions in *E. coli* of $0.62 \pm 0.07 \text{ log cfu}$ and $0.45 \pm 0.40 \text{ log cfu}$ for 25 and 45% TS, respectively. The difference between the reduction levels for the 2 TS concentrations was statistically insignificant. Whereas the 9.8% TS reconstituted milk reached inactivation levels similar to that of HTST pasteurization (Grant et al., 1996), the 2 samples with larger percentages of TS failed to reach this level of reduction. This can mostly be attributed to the solids in the substrate reflecting and absorbing the light, which causes lower light penetration and, thus, lower levels of PL inactivation.

Effect of Fat Content on PL Inactivation of *E. coli* in Milk

For the treatment of fluid milk samples of varying percent fat, fluence levels of up to 14.85 J/cm^2 were

investigated, which was above the FDA-mandated limit of 12 J/cm^2 (US FDA, 1996). For the milk samples, increasing fluence increased the reduction levels of *E. coli* ATCC 25922 by PL, until a plateau was reached; a plateau was not observed for the turbulent treatments.

In the static treatments, inactivation levels (means $\pm$ SD) of up to $0.35 \pm 0.08 \text{ log cfu}$ and a plateau of the inactivation curve were reached, with no statistically significant differences between skim, 2% fat, and whole milks (Figure 3). By comparison, Palgan et al. (2011) found a maximum reduction of 0.99 log for *E. coli* in full-fat milk at a distance of 2.5 cm from the light source in static mode at a fluence of 14 J/cm^2 , which was higher than in the current study.

Pulsed-light treatment effectiveness for these samples increased when the treatments were performed under shaking. Under turbulent conditions, the inactivation levels for *E. coli* in skim milk reached $3.36 \pm 0.21 \text{ log}$ at 14.85 J/cm^2 , and a plateau was not achieved (Figure 4). The effect of turbulence decreased from skim milk to the samples with higher fat content; for 2% fat and whole milk samples, inactivation levels of $2.89 \pm 0.06 \text{ log}$ and $2.94 \pm 0.35 \text{ log}$ at a fluence of 14.85 J/cm^2 were attained, respectively, (Figure 4). No statistical difference existed between inactivation levels for 2% fat milk and whole milk under turbulent conditions. This data suggests that PL treatment effectiveness was hindered by the presence of milk fat, most likely due to scattering of the incident light by the fat globules. However, it should be noted that inactivation levels comparable to HTST pasteurization (over 2 log cfu) were attained for all fat levels at about 12 J/cm^2 , which is the maximum level of fluence accepted by the FDA (US FDA, 1996).

Figure 2. Inactivation of *Escherichia coli* in concentrated milks with 3 different TS contents by pulsed-light (PL) treatment, under shaking. Plotted are mean ($n = 3$) values ± 1 SD. N = number of survivors; N_0 = number in starting population.

Figure 3. Inactivation of *Escherichia coli* in cow milk with varying fat levels by pulsed-light (PL) treatment in static mode. Plotted are mean ($n = 3$) values ± 1 SD. N = number of survivors; N_0 = number in starting population.

Inactivation Kinetics for *E. coli* ATCC 25922

When designing microbial inactivation treatments, it is helpful to be able to do theoretical calculations and predict the treatment dose necessary to achieve a desired level of inactivation. In thermal treatment, first-order kinetics are typically used to do process calculations (i.e., to determine what treatment dose is required to achieve a certain inactivation level). However, it has been shown that the use of first-order inactivation kinetics is not appropriate for PL inactivation (Uesugi et al., 2007). This is confirmed by the results obtained in the current study, as the inactivation curves for *E. coli* had both concave and linear shapes (Figures 1–4).

The Weibull model has been used before to successfully model microbial inactivation by PL (Uesugi et al., 2007; Sauer and Moraru, 2009). The Weibull model is a nonmechanistic model that uses a power function to describe the change of survivor ratio as a function of the intensity of the treatment. For PL treatment, the Weibull function has the general form (Uesugi et al., 2007)

$$\log(N/N_0) = -\alpha \cdot F^\beta, \quad [1]$$

where N is the number of survivors after PL treatment (cfu); N_0 is the number of *E. coli* before PL treatment (cfu); α is the scale parameter; β is the shape factor, which describes the shape of the curve; and F is the PL fluence (J/cm^2).

To obtain Weibull parameters, Equation 1 was linearized, the logarithm of $\log(N/N_0)$ was plotted against the logarithm of fluence, and the regression equation

$y = \beta \cdot x + n$ was used to obtain the regression parameters β and n , where $n = \log(\alpha)$. For *E. coli* ATCC 25922 in 9.8% TS concentrated milk, the Weibull equation took the form

$$\log(N/N_0) = 0.48 \cdot F^{0.14}. \quad [2]$$

The calculated shape and scale parameters (β and α , respectively) for the PL inactivation of *E. coli* ATCC 25922 in the other substrates are presented in Table 1. As shown by the coefficient of determination values in Table 1, the Weibull model was able to accurately calculate PL inactivation for most conditions, with a few exceptions.

Although the current study was concerned with microbial inactivation effects of PL, it is important to make some comments regarding the effects of this treatment on the quality and sensory properties of milk treated by PL. To date, only a few sensory studies of milk treated by other light-based methods have been published. Krishnamurthy (2006) conducted a sensory evaluation of pasteurized milk that was treated by PL. A consumer panel of 29 evaluated samples for overall acceptability, and it was found that the acceptability decreased about 4 points on a 9-point hedonic scale between the control and PL-treated milk, although the level of treatment was not indicated (Krishnamurthy, 2006). Matak et al. (2007) explored the sensory changes (odor) of goat milk when exposed to 15.8 ± 1.6 mJ/cm² of continuous UV irradiation. A significant negative difference was found in the odor of the milk treated

Figure 4. Inactivation of *Escherichia coli* in cows' milk with varying fat levels by pulsed-light (PL) treatment, under turbulence. Plotted are mean ($n = 3$) values ± 1 SD. The dotted line indicates the maximum fluence of $12 J/cm^2$ allowed for treatment of foods, as mandated by the Food and Drug Administration (US FDA, 1996). N = number of survivors; N_0 = number in starting population.

Table 1. Weibull parameters for the inactivation of *Escherichia coli* ATCC 25922 in concentrated milk and cow milk substrates

Substrate type	Substrate	Mode	No. of data points	Shape parameter β	Scale parameter α	Goodness of fit (R^2)
Concentrated milk	9.8% TS	Turbulent	5	1.04	0.22	1.00
		Static	5	0.48	0.14	0.97
	25% TS	Turbulent	5	1.10	0.06	1.00
		Static	5	0.54	0.08	0.84
	45% TS	Turbulent	5	0.67	0.13	0.83
		Static	5	1.41	0.01	0.65
Cow milk	Skim	Turbulent	8	0.98	0.25	0.99
		Static	8	0.13	0.27	0.61
	2%	Turbulent	8	1.08	0.17	1.00
		Static	8	0.31	0.15	0.94
	Whole	Turbulent	8	1.03	0.18	1.00
		Static	8	0.30	0.17	0.97

with 12 passes of UV irradiation (15.6-mJ/cm² dose) compared with the raw goat milk (Matak et al., 2007). On the other hand, Elmnasser et al. (2008) found that only minimal chemical changes in terms of lipids and proteins were induced in milk by PL treatment. A systematic study of the effect of PL treatments that are effective for microbial inactivation on the overall quality of treated milk products will be required, and this will be the subject of a subsequent study.

CONCLUSIONS

Inactivation of *E. coli* in highly concentrated milks by PL treatment does not present an effective alternative to conventional pasteurization, as illustrated by the less than 1-log reduction reached. Even with the addition of turbulence, the concentrated milk samples failed to reach sufficient levels of inactivation to be an adequate replacement of conventional pasteurization. Pulsed-light treatment could, however, be an effective alternative to pasteurization for fluid milk, based on levels of total inactivation obtained in this study (higher than 2-log reduction). Although this shows potential for the use of PL for the treatment of fluid milk, before a recommendation is made to consider PL as a viable alternative to HTST pasteurization, the effectiveness of this treatment against the pathogens of concern in milk (*Mycobacterium tuberculosis* and *Coxiella burnetii*) needs to be tested. In addition, the effect of the treatment on the chemical and sensory properties of the treatment milk also needs to be investigated.

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