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Danielle M. Gunter-Ward
Tennessee State University

Ankit Patras
Tennessee State University

Manreet Singh Bhullar
Tennessee State University

Agnes Kilonzo-Nthenge
Tennessee State University

Bharat Pokharel
Tennessee State University

See next page for additional authors

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Efficacy of Ultraviolet (UV-C) Light in Reducing Foodborne Pathogens and Model Viruses in Skim milk

Danielle M. Gunter-Ward[a], Ankit Patras[a] , Manreet Singh Bhullar[a] Agnes Kilanzo-Nthenge[a], Bharat Pokharel[a], Michael Sasges[b]

[a] Food Biosciences Program, Department of Agricultural and Environmental Sciences, College of Agriculture, Human and Natural Sciences, Tennessee State University, Nashville, TN 37209, USA

[b]Aquafine Corporation, Valencia, California, CA, 91355, USA

Corresponding author:
Ankit Patras, Ph.D.
Assistant Professor, Food Science
Department of Agricultural and Environmental Sciences
Optical Technologies Research Program
College of Agriculture, Human and Natural Sciences
Tennessee State University
Nashville TN 37209
USA
Email: apatras@tnstate.edu

Tel: 1-615-963-6007
Fax: 1-615-963-5436

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Abstract

The efficacy of low wavelength ultraviolet light (UV-C) as a disinfection process for a scattering fluid such as skim milk was investigated in this study. UV-C inactivation kinetics of two surrogate viruses (bacteriophages MS2 and T1UV) and three pathogenic bacteria *Escherichia* coli ATCC 25922, *Salmonella* Typhimurium ATCC 13311, *Listeria monocytogenes* ATCC 19115 in buffer and skim milk were investigated. UV-C irradiation was applied to stirred samples, using a collimated beam operating at 253.7 nm wavelength. A series of known UV-C doses (0 - 40 $mJ \cdot cm^{-2}$) were delivered to the samples except MS2 where higher doses (0 – 150 $mJ \cdot cm^{-2}$) were delivered. Biodosimetry, utilizing D values of viruses inactivated in buffer, was carried out to verify and calculate reduction equivalent dose (RED). At the highest dose of 40 $mJ \cdot cm^{-2}$, the three pathogenic organisms were inactivated by more than 5 $\log_{10}$ ($p<0.05$). Results provide evidence that UV-C irradiation effectively inactivated bacteriophage and pathogenic microbes in skim milk. The inactivation kinetics of microorganisms were well described by log linear and exponential models with a low root mean squared error (RMSE) and high coefficient of determination ($R^2>0.96$). Models parameterized for predicting log reduction as a function of UV-C irradiation dose were significant ($p < 0.05$) with low standard error and high coefficients of determination (R^2). This study clearly demonstrated that high levels of inactivation of pathogens can be achieved in skim milk, and suggests significant potential for UV-C treatment of treating fluids that exhibit significant scattering.

Keywords: UV-C, irradiation, biodosimetry, microbial, inactivation, bacteriophage, kinetics

Practical application

This research paper provides scientific evidence of the potential use of UV technology in inactivating pathogenic bacteria and model viruses in skim milk. UV-C doses were validated and verified using biodosimetry. UV-C irradiation is an attractive food preservation technology and offers opportunities for dairy and food processing industries to meet the growing demand from consumers for safer food products. This study clearly shows the potential for using UV-C treatment for treating highly scattering fluid such as skim milk. Results from this work will be used to further develop continuous flow-through UV-C systems based on dean or turbulent flow patterns for uniform UV dose delivery.

1. Introduction

Microbial safety of food products remains a critical public health issue as food borne outbreaks consistently occur as a result of pathogenic contamination (CDC, 2016). Despite widespread adoption of Hazard Analysis and Critical Control Points (HACCP), food borne illnesses still pose a great public health risk (United States Food and Drug Administration, 2015). Milk product safety in the United States is generally very high, driven by the 1924 Pasteurized Milk Ordinance. Since this federally mandated quality standard for milk processors was adopted, outbreaks from milk have gone from approximately 25% of foodborne outbreaks to < 1% (USPHS/USFDA, 2009).

Skim milk is prone to spoilage by many pathogenic bacteria and viruses due to its rich nutrient content of lipids, proteins, vitamins, and saccharides (Bandla et al., 2012; Oliver et al., 2005; Dega et al., 1972). The primary pathogens that have been associated with skim milk and dairy products are the following: *Coxiella burnetii, Mycobacterium tuberculosis, Brucella, Listeria monocytogenes, Salmonella spp., Shiga toxin–producing Escherichia coli O157:H7, Campylobacter jejuni, Yersinia enterocolitica, Staphylococcus aureus, Aeromonas hydrophila, Serratia marcescens, Clostridium perfringens*, norovirus, and *Bacillus cereus* (Langer et al., 2012; Claeys et al., 2013).

Thermal pasteurization constitutes the most extensively used and approved method for the inactivation of skim milk borne pathogens (Jayarao et al., 2006), but it has a number of drawbacks. Thermal pasteurization has high energy costs, can denature proteins, and often alters the taste of products (Islam et al., 2016a; Cappozzo et al., 2015). Not all liquid foods can tolerate the heat treatments in the pasteurization process as they contain heat-sensitive bio-molecules. As

a result, there is interest in alternative, non-thermal methods for disinfecting skim milk and other liquid foods.

Ultraviolet light in the germicidal range (UV-C) from 200 to 280 nm, is being investigated as an alternative to thermal treatment of skim milk for inactivating pathogens and improving shelf life and safety of skim milk (Cappozzo et al., 2015; Bandla et al., 2012). Ultraviolet (UV-C) light is well proven as a non-thermal method of disinfecting drinking, waste, and recreational water (Beck et al., 2015) and is applied extensively in the US and around the world. It is well documented in the literature that the primary mode of UV-C disinfection occurs when nucleic acids absorb UV-C light and form cross-linked pyrimidine dimers. These dimers, when present in sufficient numbers and critical locations, lethally inhibit DNA repair and prevent the organism from replicating, rendering it non-pathogenic (Bintsis et al., 2000; Guerrero- Beltrán and Barbosa-Cánovas, 2004; Shama, 1999; Hamkalo and Swenson, 1969). Recent advances in science and engineering have clearly demonstrated that UV-C technology holds considerable promise as an alternative to traditional thermal processes such as pasteurization for food preservation (Park et al., 1999; Hanes et al., 2002; Matak et al., 2005).

There is regulatory guidance for applying new disinfection technologies to juices and excludes skim milk. The United States Food and Drug Administration (USFDA) and United States Department of Agriculture (USDA) have concluded that the usage of UV-C light at 253.7 nm for food processing is safe and has further approved the usage as an alternative treatment to reduce pathogens and other microorganisms (US FDA, 2000). USFDA issued Code 21CFR179.41, which approved the use of UV-C light in the production, processing, and handling of food.

Although several researchers have investigated the application of UV-C disinfection to liquid foods, the varying experimental methods and quantification of UV-C dose makes it difficult or

impossible to draw meaningful conclusions. For example, a recent study conducted by Crook et al. (2015) suggested that extremely high UV-C dose in the range with 1450–2000 J/L was required to achieve 5 log reduction of selected pathogens including *E. coli* 0157:H7. Note that the dose required for a log reduction of *E coli*, established by a number of independent studies (Sommer et al., 2000; Quek et al., 2008; Wilson et al.,1992; Rattanakul et al., 2014) is only 2 $mJ \cdot cm^{-2}$. The units of UV-C reported by Crook et al. (2015) (J/L) differ from UV-C industry approach that uses J/m^2 (Sommer et al., 2000.)

For those fluids (e.g. skim milk) that have high optical absorption coefficients, it is difficult to obtain a uniform fluence. When a liquid is opaque or turbid, such as skim milk, UV-C photons cannot penetrate deeply into the liquid. The effective penetration depth for skim milk at 254 nm is only about 0.003 cm (Koutchma, 2009). This creates design challenges for UV-C equipment and for laboratory tests that must ensure uniform and known UV-C delivery.

In order to calculate the UV-C dose in a given system, the optical properties of the fluid must be correctly determined. Light propagation in an opaque fluid is governed by the Radiation Transport Equation (RTE). For a scattering fluid, such as skim milk, the RTE must include absorption coefficient, scattering coefficient, and anisotropy of scattering:

μ_a: absorption coefficient

μ_s: scattering coefficient

g : anisotropy factor

Scattering and absorption properties of these opaque fluids contribute to the attenuation of the light through the samples (Ghosh et al., 2001). Light scattering by fat globules and casein micelles causes skim milk to appear turbid and opaque. These two components scatter light differently based on differences in size, number, and optical properties such as the index of

refraction of the particles (Walstra and Jenness, 1984). Unlike highly diluted mixtures where conventional spectroscopy methods based on the well-known Beer-Lambert's law apply, optically thick fluids like skim milk require appropriate mathematical methods to separate the reduced scattering coefficient (μ_s) from the absorption coefficient (μ_a).

A common weakness in many UV-C irradiation studies is that they do not consider the optical absorbance of the fluid (Unluturk et al., 2010; Caminiti et al., 2012). This oversight means that the data cannot be related to any desired disinfection result, or compared against studies of a different fluid with different optical properties (Unluturk et al., 2010; Caminiti et al., 2012).

The purpose of the present study was to investigate the effectiveness of UV-C irradiation for the inactivation of *Salmonella* Typhimurium ATCC 13311, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 1911, and two model viruses (bacteriophages MS2 and T1UV) in skim milk.

2. Material and Methods

2.1 **Bacteriophage and Biodosimetry**

Two bacteriophages were used as surrogates for viral pathogens: MS2 (Single Stranded RNA virus) and T1UV (Double stranded RNA virus). The cultures were obtained from GAP EnviroMicrobial Services Limited (London, Ontario, Canada). Cultures were kept at -4ºC until further use. The concentrations of MS2 and T1UV (10^{11} PFU/ml and 10^{10} PFU/ml respectively) were determined by GAP EnviroMicrobial Services (ON, Canada). The bacteriophages were diluted to a concentration of 10^6 PFU/ml. The optics (absorption and scattering coefficients) of the fluid are accounted for, and dose delivery is verified through biodosimetry, ensuring that

target levels of disinfection are achieved, and allowing direct comparisons with other UV-C treatment studies (Islam et al, 2016a).

2.2 Bacterial strains and culture conditions

Three strains of bacteria were used in this study: *Escherichia coli* ATCC 25922 (EC), *Salmonella* Typhimurium ATCC 13311 (ST) and *Listeria monocytogenes* ATCC 19115 (LM). The bacterial cultures were stored in 25% glycerol in cryovials at -80^{o} C. To verify the purity of EC, ST, and LM, each bacterium was plated on specialty media EMB Agar, Levine (Hardy Diagnostics, Santa Maria, CA), XLT4 Agar Base (Becton, Dickson, and Company, Sparks, MD), and *Listeria* Selective Agar Base (Oxoid Ltd., Basingstoke, UK) respectively. EC and ST strains were grown by two successive transfers of individual strains incubated at 37 °C for 18h in 15 ml Tryptic Soy Broth (TSB) (Oxoid Ltd., Basingstoke, UK). LM was also subjected to two successive transfers in tubes containing 15 ml Buffered *Listeria* Enrichment Broth (LEB) (Oxoid Ltd., Basingstoke, UK) and incubated for 24h at 37°C. These cultures were used as the adapted inoculum. After incubation, EC and ST cultures were transferred into 15 ml of TSB (Oxoid Ltd., Basingstoke, UK) and incubated for 18 h at 37°C to stationary phase. LM culture was also transferred to 15 mL LEB (Oxoid Ltd., Basingstoke, UK) and incubated for 24h at 37°C. The bacterial cells were harvested by using a Sorvall ST16R centrifuge (ThermoScientific) at 3000 × g for 15 min. Cell pellets were washed twice in 0.1% (w/v) Phosphate Buffer Saline (PBS, Becton Dickinson, New Jersey, US) and re-suspended in 50 ml of PBS. To enumerate the original population densities in each cell suspension, appropriate dilutions in peptone water (in 0.1% PW) were plated in duplicate onto Tryptic Soy Agar (TSA) (Oxoid Ltd., Basingstoke, UK) plates for EC and ST suspensions and incubated for 24h at 37°C. LM *suspensions* were plated on

Listeria Selective Agar Base (LAB) (CM0856; Oxoid Ltd., Basingstoke, UK) plates with incubation at 48h at 37°C (Baumann et al. 2005).

2.3 Buffer and Humic Acid Preparation and Inoculation

Buffered water (buffer) was prepared by combining KH_2PO_4 (SigmaAldrich) at a concentration of 0.24834M, $MgCl_2$ (Acros Organics) at a concentration of 0.399M, and organic humic acid (SigmaAldrich) (pH 7, concentration of 1%). (The humic acid was added to the buffer to increase the absorbance for optical measurements for subsequent UV Dose calculations.) Aliquots of 100 ml of buffer were inoculated individually with each of the three bacterial cultures (*EC*, *ST*, and *LM*) with a concentration of 10^8 CFU/ml (confirmed by plating as described previously). To determine the original concentration of EC and ST titers, inoculated buffer was plated on TSA (Oxoid Ltd., Basingstoke, UK) plates and incubated for at 37 °C for 24 h. Buffer inoculated with LM was plated on LAB (Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 48 h.

2.3 Skim milk inoculation

The skim milk samples were obtained from a local grocery store located in Nashville, Tennessee. The commercial pasteurized skim milk samples were immediately placed in a refrigerator at 4 °C and stored for a period of no longer than 3 weeks. The skim milk was examined for background microbial populations of EC, ST, and LM. These microbes were not present in the skim milk prior to inoculation.

Aliquots of 100 ml of skim milk were inoculated individually with each of the three bacterial cultures. After harvesting, the inoculum was plated to determine the bacterial concentrations

(Baumann et al. 2005). The inoculum concentrations for EC, ST and LM were 8.85 CFU/ml, 11.65 CFU/ml, and 6.87 CFU/ml respectively. To determine the original EC and ST concentrations in inoculated skim milk, untreated inoculated control samples were plated on TSA (Oxoid Ltd., Basingstoke, UK) plates and incubated for at 37 °C for 24h. Skim milk inoculated with LM was plated on LAB (Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 48h.

2.4 Optical Measurements of Skim milk

Optical properties of the fluids were measured in a double beam Cary 100 Spectrophotometer (Varian, USA) equipped with a 6-inch single Integrating Sphere (Labsphere, DRA-CA-30, USA) to include scattered light which measured at 254nm wavelength (Prerana et al., 2008). Measurements were done in thin quartz cuvettes (1mm path-length) (H. Baumbach & Co Ltd, UK). The total transmitted and reflected (diffuse reflectance) light was collected by the integrating sphere when the sample was placed at the entrance and exit ports respectively. An eight-degree reflectance port was used to measure the total reflectance. The calibration of the Cary100 – IS system was verified using National Institute of Standards and Technology (NIST)-certified potassium dichromate standards (concentrations of 20 mg/L to 100 mg/L) with certified absorbance at 350, 313, 257 and 235nm, obtained from Starna, UK. The system scale was set by setting the 100% baseline using the blank provided with the NIST standards and then 0% baseline by blocking the beam. All scans were done in triplicate to reduce the random error. Due to a potential impact of light losses in the quartz cuvettes, and depending on the wavelength of interest, the amount of light absorbed by the quartz cuvettes was also quantified as quartz optical depth. Ultraviolet transmittance (UV-CT %/cm), which is a measure of the fraction of incident light transmitted through a material over 1 cm path, was calculated as per equation 1.

$$\text{UV-CT (\%/cm)} = [10^{-A}] \times 100 \qquad (1)$$

where A represents the absorbance (base $_{10}$) of the test fluid at 254nm for a 1cm path. Optical properties of the test fluids are shown in Table I.

2.5 UV-C irradiation treatments

Irradiation of buffer and skim milk samples was conducted using a bench top Collimated Beam System (CB) (Trojan Technologies, London, Ontario, Canada). This apparatus was designed to provide uniform, quantified irradiation to liquid samples and the associated methods. Calibration, fluence determination, and quality assurance protocols have been developed and standardized in the field of water disinfection (Bolton and Linden, 2003). The CB approach is based on using well-mixed samples in a quantifiable UV-C radiation field. For treatment application herewith, smaller sample volumes were used to ensure thorough mixing. The irradiated volumes were 5ml in a 10ml beaker with continuous stirring (for the duration of preparation and selected treatment times) with a magnetic stir bar so as to ensure thorough mixing of the test fluid. The standard method accounts for all relevant optical factors such as beam divergence, reflection, and non-uniformity. It assumes that the mixing is sufficient to ensure that all fluid elements are exposed equally and that the geometric average UV-C intensity may therefore be used to calculate total fluence. This approach overcomes the problems in other studies in which stagnant, highly absorbent fluid samples were irradiated. In those cases, the UV-C fluence measured at the surface was not representative of the average delivered to the sample.

The UV-C doses delivered (ranging from 0 – 40 mJ·cm^{-2}, with the exceptions of MS2 which required higher doses) to the suspension is calculated using a standardized approach (Bolton and

Linden, 2003) where the average intensity (fluence rate) in the well-mixed sample is expressed as:

$$E'avg = E_0 \times P_f \times (1-R) \times \frac{L}{d+L} \times \frac{1-10^{-Ad}}{Ad\ln(10)} \quad (2)$$

where E_0 is the radiometer meter reading at the center of the dish and at a vertical position such that the calibration plane of the detector head is at the same level as the top of the solution. The average germicidal fluence (UV-C dose) ($J.m^{-2}$ or $mJ \cdot cm^{-2}$) is then given by the product of E'_{avg} and the exposure time t ~s, Pf is termed the Petri factor defined as the ratio of the UV-C intensity measured at the center of the sample surface to the average intensity measured across the sample surface, 1-R is termed the reflection factor where R is the fraction of UV-C light at 253.7 nm reflected at the air-surface interface (typically R = 0.025), L/(d+L) is termed the divergence factor where L is the distance from the lamp centerline to the sample surface and d is the sample depth, and (1-10-Ad)/(Ad ln(10)) is termed the absorbance factor where A is the UV-C absorbance coefficient ($base_{10}$) at 253.7 nm of the fluids.

The CB apparatus used in this study incorporates a 25 W Philips low pressure mercury lamp, with nearly monochromatic output at 253.7nm as shown in Figure 1. The UV-C lamp is cooled by a fan to help maintain a constant output. The lamp was switched on for 20 minutes before use to stabilize the UV-C output. The irradiance at the sample location was measured using an International Light Technologies (Peabody, USA) IL-1700 radiometer with an SED 240 detector and a NS254 filter. The radiometer and detector were calibrated by International Light and are accompanied by NIST traceable certifications. The sample holder was adjustable to locate the surface of the microbial suspension at the same level with each irradiation and to position the calibration plane of the radiometer detector at the same level as the surface of the microbial

suspension (Bolton and Linden, 2003). The distance between liquid and UV-C lamp was 28.05 cm. UV-C radiation treatment was carried out at room temperature (≈22 ° C) and relative humidity of about 55%, unless otherwise stated. Experiments were conducted in duplicate on two different working days. Based on the D_{10} (dose required for 1 log reduction) values of the microbes, UV-C doses required for 5 log reduction of microbes were selected. Irradiation treatments were performed in triplicate.

2.6 Verification of UV-C fluence

Verification of UV-C fluence was conducted with a challenge organism as per the method described by Islam et al. (2016a). In order to determine the actual UV-C fluence values delivered to skim milk, a viral clearance test was conducted using as a challenge organism MS2, bacteriophage, with an icosahedral shell approximately 27 nm in diameter. This organism is used extensively in validation of UV-C disinfection systems for drinking water (Pirnie et al., 2006).

The UV-C sensitivity of the MS2 was first established using an inactivation test in buffer water. MS2 bacteriophage was suspended in buffer and irradiated in a CB to different UV-C fluence values. The buffer used in this characterization had a high transmittance at 254 nm, typically 90% at 1 cm path-length, so that the UV-C intensity gradient in the fluid was small, and therefore, the uncertainty in the UV-C fluence was small. Irradiance measurements and optical correction factors were applied according to the method of Bolton and Linden (2003) as described above. The samples were stirred during irradiation to ensure that all organisms were exposed to the same integrated UV-C fluence over the course of the irradiation. After irradiation, each sample was diluted serially and aliquoted into culture tubes containing 1 mL EC broth culture and 20 mL of molten tryptone yeast extract glucose agar containing triphenyl tetrazolium chloride. The mixtures were mixed by inversion and plated into sterile petri dishes. The agar was

allowed to solidify and the plates were incubated at 35 ±0.5 ° C for 18–24 h before performing a plaque assay. The plates were then evaluated to determine the number of plaque forming units. By comparing with the control (non-irradiated) sample, the relationship between UV-C dose and the log-reduction of this population of MS2 was established.

With the UV-C sensitivity of the organism determined, the organism was then used to quantify the UV-C dose delivered to a UV-C-scattering fluid. UV-C dose applied to the skim milk was determined by using MS2 as the dose indicator. This is sometimes denoted as the reduction equivalent dose (RED) or fluence (REF), since it is inferred from the log reduction of a well-characterized challenge organism. MS2 from the characterized population was inoculated into a sample of skim milk. The optical properties of the skim milk were used to calculate the irradiation times necessary to achieve a desired UV-C dose in the CB apparatus. The samples were then irradiated for the prescribed time, and then serially diluted and cultured as described above. The log reduction in numbers of active MS2 was used to calculate the UV-C dose delivered in the skim milk in each irradiation by using the sensitivity of the MS2 as established by the buffer tests (Pinjuv et al. 2006).

2.7 Organism sensitivity test

In order to determine the UV-C sensitivity of the organisms, UV-C irradiations were performed in buffer (pH 7) combined with organic humic acid (SigmaAldrich) (pH 7, concentration of 1%) using a CB irradiation device as described above. (The humic acid was added to the buffer in order to increase the absorbance for optical measurements for subsequent UV Dose calculations.) This approach, with high optical transparency, minimizes the intensity gradient in the fluid sample, reducing the mixing required to ensure uniform average dose delivery, reducing the uncertainty in the delivered RED. The Dose-Response curves for these organisms may be seen in

RED. The data were found to be well-described by a first order model, with R-squared values of 0.88 or greater (considering the linear part of the curve). From the slope of these plots, the UV-C dose per log inactivation, or D_{10} values, were calculated, and are shown in Table II.

2.8 Enumeration of pathogens in skim milk after UV-C treatments

After UV-C treatment, decimal dilutions of the treated samples and control were prepared in 0.1% peptone water (Oxoid Ltd., Basingstoke, UK). The EC, ST and LM inoculated skim milk samples were diluted to between 10^0 and 10^{-8}. EC and ST viable cell counts were obtained by culturing on appropriate agar plates as described previously. Plate counts within the range of 25-250 were considered for analysis. Bacteria colonies were counted and transformed into log CFU mL^{-1} of undiluted skim milk.

2.9 Statistical analysis

Log-linear and non-linear models were fitted in R Statistical Computing Environment (R Development Core Team 2016) after subsequent UV-C induced log reductions were recorded. Model fit statistics including R^2 and RMSE were compared among the competing models. Independent sets of data were collected for three bacteria, and model performance parameters were evaluated for each model. The magnitude of bias, precision, and accuracy were assessed using an independent dataset by generating a suite of validation statistics such as average bias, relative error, mean absolute error, and model efficiency. Finally, the parameters were estimated by combining both training and validation datasets.

2.10 Inactivation kinetics

Log-Linear model

Log-Linear model has been widely accepted and used to describe the microbial inactivation resulted from application of both thermal and non-thermal processes. This model provides a good fit to data in which the inactivation exhibits first order kinetics. The model is given in the following equation (Van Boekel, 2002):

$$Log\ Reduction = \alpha + \beta \cdot dose \qquad (3)$$

where, α and β are parameter estimates, dose is UV-C dose in $mJ \cdot cm^{-2}$. Log reduction is calculated as $log\left(\frac{N}{N_0}\right)$. Classical D_{10} value is calculated from the reciprocal of the first order rate constant ($D_{10}=1/\beta$, units in $mJ \cdot cm^{-2}$). Eq. (3) is also known as Chick Watson linear equation (Marugán et al., 2008).

Non-linear model

For cases where inactivation does not follow a simple first order function, the exponential model was fitted and model fit statistics were evaluated. A bacterial or viral strain may provide different shapes of inactivation curves due to heterogeneity among the cells of a population even if the population is pure. In some cases, inactivation curves may be sigmoidal and exhibit concavity or convexity behaviors. The exponential model usually describes this behavior very well. Exponential function is composed of two parameters (α and β) given in the following model form:

$$Log\ Reduction = \alpha \cdot e^{\left(\frac{\beta}{dose}\right)} \qquad (4)$$

where, α and β are parameter estimates, dose is UV-C dose in $mJ \cdot cm^{-2}$. Log reduction is calculated as $log\left(\frac{N}{N_0}\right)$. In this function dose is non-linear in the exponent. If the both parameters $\alpha<0$ and $\beta<0$, the shape of the curve is downward concavity of the inactivation curve, which indicates stress adaptation of target microorganism with increasing resistance as dose is increased.

3. Results and Discussion

3.1 UV-C inactivation results

Optical and physical properties of skim milk are illustrated in Table I. The optical data indicates that skim milk was a strong absorber and scatterer of UV-C light. From the measured optical data, it is quite apparent that the UV-C light has very little transmission through skim milk. This is due to the presence of micro-molecules (amino acids) compounds, organic solutes or suspended matter, and could result in a reduced efficiency of UV-C disinfection. Soluble and suspended solids are responsible for low penetration of UV-C light (Koutchma et al., 2004). In liquids with absorption and significant scattering, the irradiation system must be designed to overcome this short penetration depth. For this reason, a CB with stirred samples is commonly used for delivering UV-C dose in the laboratory (Islam et al., 2016a, Islam et al., 2016b, Kuo et al., 2003; Qualls et al., 1983). This technique has evolved as a standard method. The incident intensity at the surface is not the intensity at any other depth. In this current study, disinfection in a scattering fluid (i.e. skim milk) was studied using a bench UV system and scattering in skim milk was accounted for in the dose calculations. Scattering reduces the performance of the

reactor and needs to be accounted for. This is the first study which accounts for the reduced scattering in the UV dose calculations. It also calculates the UV energy required for 99.999% reduction of pathogen reduction. Measuring irradiance with appropriate sensors is very critical. In most cases, the authors quantify only UV-C incident dose at the surface of system, measuring the incident intensity at the surface of the liquid with a radiometer (Assatarakul et al., 2012).

To our knowledge, no USFDA recommendations regarding the use of UV-C irradiation as an alternative to conventional pasteurization to inactivate bacterial pathogens, specifically microorganisms in skim milk, are available and said treatments are considered novel, requiring validation (USFDA, 2015; EFSA, 2016). However, general approval for the use of UV-C irradiation for the decontamination of food surfaces and for the treatment of water and liquid foods under specific conditions has been reported (US FDA CFR, 2000). USFDA recommendations for fruit juices state that the UV-C process must guarantee that at least a 5-log reduction (99.999%) of the more resistant microorganism present be achieved, under specific operating conditions (US FDA, 2000).

Inactivation kinetics for EC, ST and LM may be seen in Figure 2. The inactivation curves were constructed by plotting the log reduction ($Log_{10}[N/N_0]$) versus UV-C dose. In all cases, the inactivation may be described as first order up to about 6 log_{10} inactivation. At higher inactivation levels, the inactivation curves show tailing for EC and ST, with an apparent increase in resistance at high dose. (The tailing effect refers to the shape of the graph transitioning from linear inactivation until a high dose, to a decrease of inactivation resulting in a curvature on the end.) Similar results were observed for buffer which has a transparency of more than 90% (Figure 3). This curvature is consistent with inadequate mixing, or with clumping of organisms in the solution, or with attachment to the (relatively dark) surface of the dish. The tailing affect

was quite apparent at doses higher than 15 mJ·cm^{-2}. Similar to skim milk results no tailing was observed for LM in buffer. The gram-positive organism (LM) was the least resistant to UV-C treatment in comparison to the gram-negative organisms (EC and ST). But for all three organisms, a dose of 30 mJ·cm^{-2} was sufficient to achieve ≈6 log inactivation, which is greater than the USFDA guidance for juice treatment.

The UV-C sensitivity of EC in skim milk in our study is consistent with the literature. EC in skim milk exhibited a D_{10} of about 2.3 mJ·cm$^{-2,}$ based on 4.39 log inactivation at a dose of 10 mJ·cm^{-2}. Studies have reported D_{10} values for EC close to 2 mJ·cm^{-2} (Sommer et al., 1998; Zimmer & Slawson, 2002; Chang et al., 1985) D_{10} value of 1.795 mJ·cm^{-2} in buffer was observed in our study (Figure 3 and Table II).

Some authors have found very high resistance of EC in opaque test fluids, but these values were likely the effect of improper dose calculation and poor dose distribution in opaque test fluids. A study by Unluturk et al. (2008) exposed assorted liquid egg products (LEP) contaminated with non-pathogenic *Escherichia coli* K-12 (ATCC 25253) and pathogenic *Escherichia coli* O157:H7 to UV-C via a CB apparatus. At the maximum dose of approximately 98 mJ·cm^{-2}, the bacteria were inactivated by 0.675-log CFU/ml and 0.316-log CFU/ml reduction for liquid egg yolk and liquid whole egg respectively. This would correspond to a D_{10} of more than 145 mJ·cm^{-2}, an extraordinarily high resistance to UV-C. In a separate study, Unluturk et al. (2010) exposed liquid egg whites (LEW) contaminated with non-pathogenic *Escherichia coli K-12 (*ATCC 25253*)* and pathogenic *Escherichia coli O157:H7* to UV-C via CB. At an approximate 26.44 mJ·cm^{-2} dose the treatments resulted in only 0.896 and 1.403 log CFU/ml reduction respectively, corresponding to a D_{10} of 19 mJ·cm^{-2} or more. These values are lower than the earlier study by the same group, but still much higher than consensus value from literature.

UV-C irradiation effectively inactivated ST in skim milk as may be seen in Figure 2. The D_{10} value of ST in buffer resulting from our study was 3.6 $mJ \cdot cm^{-2}$. The D_{10} from the 5.43 log inactivation in skim milk at a dose of 15 $mJ \cdot cm^{-2}$, the end of the first-order portion of the disinfection curve, corresponds to a D_{10} of 2.76 $mJ \cdot cm^{-2}$. This is close to the values reported in literature by Yuan et al. (2003) and Wilson et al. (1992) that have a consensus value of about $2mJ \cdot cm^{-2}/log_{10}$. In the conventional approach of using CB apparatus it is assumed that the mixing is perfect and so all the micro-organisms accumulate the same UV-C-dose. One of the assumptions used in the calculation of UV-C dose-response data is that all the fluid elements receive the same dose, achieved by strong mixing. In contrast, our experimental data presented in the paper indicate that this assumption can fail, resulting in significantly less log removal. It is quite apparent that this curvature is indicative of inadequate mixing at higher doses. It is believed that for very sensitive microorganisms the microbial inactivation is more strongly impacted by the lower limit of the dose distribution. The deviation from apparent first-order behavior is attributable to insufficient mixing at low UV Transmittance (UVT) fluids such as skim milk. As a result of this effect, the dose-response curve has tailing, even though the underlying kinetics are actually first order. The organism appears to be more resistant to UV-C at higher doses.

For more resistant organisms, such as MS2, the impact of mixing in the CB is negligible (Figure 4). The assumption of perfect mixing for MS2 in this investigation is reasonable. When conducting CB tests with sensitive organisms, lower UV-CT values and higher doses, the mixing in a standard CB apparatus may not be sufficient to yield accurate inactivation results. To this point, Gross et al. (2015), conducted a UV-C inactivation comparison study of *Escherichia coli* DSM 498 in water continuously mixed versus stagnant samples. As hypothesized, the mixed samples yielded a collective log reduction of 4.28 as opposed to the stagnant which only yielded

2.80 log reduction. The magnitude of this effect will be dependent on the apparatus in question, and will be exacerbated by non-uniform irradiance on the sample (especially intensified in the case of highly opaque, viscously turbid fluids such as skim milk), poor collimation, and less aggressive mixing (Crofcheck et al., 2002).

The efficacy of UV-C irradiation in inactivating LM is reported in Figure 2. The LM inactivation showed first order kinetics throughout. This suggests that the bacteria were not present in clumps, did not become coated and protected by skim milk constituents, and did not attach to the dish. The D_{10} value of Listeria in buffer was 2.46 $mJ \cdot cm^{-2}$, which agrees well with literature. Matak et al. (2005) reported *Listeria monocytogenes* in skim milk samples required treatment with a UV-C dose of 15.8±1.6 $mJ \cdot cm^{-2}$ to achieve 5 log reduction using a continuous flow through reactor, corresponding to a D_{10} of approximately 3 $mJ \cdot cm^{-2}$. Another study reported 0.960 log CFU reduction of Listeria after administering 1.314 mW.cm via CB (Unluturk et al., 2010), corresponding to a D_{10} of only 1.4 $mJ \cdot cm^{-2}$. Other values from literature are those of Collins (1971) who found a D_{10} of 1 $mJ \cdot cm^{-2}$, and Yousef (1988) who reported a D_{10} of 2.4 $mJ \cdot cm^{-2}$.

Phage, which are viruses that infect bacteria and are therefore safe for humans, are often used as viral surrogates in disinfection studies. Bacteriophages MS2 and T1UV were selected as model viruses in this study. Dore et al. (2000) conducted a study investigating Norovirus (NoV) contaminated oysters which demonstrated that an F-specific RNA bacteriophage is an indicator organism for NoV. MS2 phage is an F-specific group I RNA coliphage in the family Leviviridae (Calender, 1988). Similar to NoV, MS2 is a single-stranded, positive sense RNA virus with icosahedral symmetry that is adapted to the intestinal tract. MS2 and NoV have similar size i.e.,

26 nm diameter. The MS2 genome houses 3569 single-stranded RNA nucleotides (Fiers et al., 1976).

Figure 4. depicts the inactivation of MS2 and T1UV by using UV-C-irradiation in skim milk. Higher doses induced greater levels of MS2 and T1UV inactivation in skim milk. The inactivation pattern displayed on the graph was linear (first order) for both T1UV and MS2. As expected, the UV-C-resistant phage MS2 required approximately 150 $mJ \cdot cm^{-2}$ to achieve 5 log inactivation. The resulting reduction equivalent dose vs. target dose has been plotted (Figure 5). The excellent agreement between target dose and RED shows that the optical model and measured optical properties are valid for skim milk. This result shows that the target doses up to 150 mJ·cm-2 can be applied to the skim milk. Figure 6. shows the amount of absorbed energy for microbial inactivation. There is a linear relationship between target dose and reduction equivalent dose (RED) showing that even relatively high UV-C doses can be accurately delivered to opaque fluids such as skim milk, as long as optical properties are properly measured and accounted for in the method.

The inactivation of T1 phage in skim milk may be seen in Figure 4. The viral surrogate T1 is less resistant to UV-C, making it an appropriate surrogate for more sensitive organisms such as EC, ST, and LM. Our calculated D_{10} in this test was 6.2 $mJ \cdot cm^{-2}$/log, similar to other results reported in the literature.

3.2 Modeling inactivation kinetics

The inactivation curves of microorganisms in skim milk exposed to UV-C irradiation exhibited tailing in some cases and linear behavior in others (Figure 2.). As a result, UV-C inactivation

kinetics in this study were modeled by using both Log-Linear and exponential models to take into account the tailing effect.

The applicability of non-linear model (exponential model) to experimental data was tested by plotting the double logarithms $\log_{10}(N/N_0)$ against UV-C dosage. The exponential model was able to capture the tailing through the scale parameter (α) and the shape parameter (or concavity index) (β) (Table III). These parameters may indicate stress adaptation of target microorganism with increasing resistance as dose is increased, or may represent the impact of non-uniform dose distribution through poor mixing or clumping. It was observed that EC and ST followed an exponential model whereas LM, MS2, and T1UV followed log linear model. Parameter estimates and goodness of fit for the models are listed in Tables III and IV.

Considering only the inactivation up to 5 log, the D_{10} values for the three organisms are: EC: 1.8; ST: 3.6; LM: 2.4. This is consistent with results of Gabriel and Nakano (2009) and of Guerrero-Beltrán and Barboza-Cánovas (2004). It is felt that this initial inactivation is likely more representative of intrinsic disinfection kinetics. Since 5 log is the disinfection level required by FDA for fresh juices, this is the relevant portion of the inactivation curve for disinfection. Higher inactivation in skim milk required significantly more UV-C dose, as the inactivation curves demonstrated increasing UV-C resistance at higher log inactivation.

The parameter estimates are presented in Table III. Log linear model fit the experimental data for *L. monocytogenes*, MS2 and T1UV with the correlation coefficient (R^2) higher than 0.95. This was also true for exponential models fitted for *Escherichia coli* and *Salmonella* Typhimurium experimental data. In these experiments, an independent set of data to validate the model were used. We calculated model validation statistics (Eq 4-10) for each model (Table V). Model

prediction errors for each bacterium and viral surrogates were estimated by using Equation (4) by calculating the difference between the observed/experimental and predicted values.

$$e_i = (Y_i - \hat{Y}_i) \quad (4)$$

Where e_i is error, Y_i is observed value and $\hat{Y}_i$ is predicted value. The validation statistics were calculated using following equations.

$$\text{Average Bias } (\bar{e}) = \frac{\sum_{i=1}^{n} (Y_i - \hat{Y}_i)}{n} \quad (5)$$

$$\text{Average Bias Percent } (\bar{e}\%) = \frac{\sum_{i=1}^{n} (Y_i - \hat{Y}_i)}{\sum_{i=1}^{n} Y_i} * 100 \quad (6)$$

The value of an average bias of zero indicates the model with no prediction bias. Negative average bias is linked with an over-prediction by the model, whereas a positive value is associated with under-prediction by the model. Average bias percent reflects a relative measure of the magnitude of bias. The magnitude of bias from model prediction was determined separately for each bacterium. It was found that the EC model was more biased compared to ST and LM as listed in Table V. Parameter estimates and goodness of fit for the models are listed in Table IV and Table III.

The MAE, PRMSE and RE% were estimated by:

$$\text{Mean Absolute Error (MAE)} = \sqrt{\frac{\sum_{i=1}^{n}(|Y_i - \hat{Y}_i|)}{n}} \quad (7)$$

$$\text{Prediction Root Mean Square Error (PRMSE)} = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2}{n}} \quad (8)$$

$$\text{Relative Error (RE\%)} = \frac{\sqrt{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2 / n}}{\sum_{i=1}^{n} Y_i / n} * 100 \quad (9)$$

Mean Absolute Error (MAE) and Prediction Root Mean Square Error (PRMSE) were used to describe the model prediction uncertainties. It can be inferred from Table V that the model for LM has the highest MAE followed by EC and ST. Relative Error Percent (RE%), also known as coefficient of variation, indicates the relative size of model prediction uncertainty; which also follows the same trend as MAE among three microbes. Model efficiency (EF) as described by Pinjuv et al. (2006), also known as correlation index squared (I^2) as described by Kozak and Kozak (2003), indicates an overall goodness of fit (Mayer and Butler, 1991). The Model Efficiency had the highest value for *Salmonella* Typhimurium and *Escherichia coli* followed by *Listeria monocytogenes* as in Fig 6. This fit statistic was estimated by:

$$\text{Model Efficiency (EF)} (I^2) = 1 - \frac{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2}{\sum_{i=1}^{n}(Y_i - \overline{Y})^2} \quad (10)$$

where, $\overline{Y}$ is the average observed value and other variables are as described earlier. A maximum value of EF equal to 1 indicates a model of "near-perfect" (Mayer and Butler 1991), while with a

value of zero indicates a model with poor fit. The average value represents a moderate relationship being captured by the model. To confirm the adequacy of the fitted models, studentised residuals versus run order were tested and the residuals were observed to be scattered randomly, suggesting that the variance of the original observations were constant for all responses. Consequently, based on the validation statistics obtained from using independent set of experimental data, the predictive performance of the established model may be considered acceptable.

Conclusions

Results from this study displayed that UV-C doses successfully reduced the microbial load in skim milk, suggesting that UV-C irradiation treatment is a plausible disinfection method to inactivate microbes in skim milk. The inactivation rate in skim milk was assessed by using non-pathogenic microorganisms including bacteriophages. This study confirms the importance of the quantifying optical properties, namely absorption and scattering values, of highly opaque fluids (i.e. skim milk) in order to efficiently disinfect food borne pathogens. The inactivation kinetics of these tested microorganisms were best described by log linear and non-linear kinetics. Inactivation UV-C doses for a 5 log reduction (99.999%) were around 40 mJ. cm^{-2} for EC and T1UV, and around 20 $mJ \cdot cm^{-2}$ for ST and LM. In contrast, doses around 150 $mJ \cdot cm^{-2}$ were required for 5 log reduction of MS2 population. Therefore, UV-C irradiation could be used as a potential alternative to traditional thermal pasteurization for control of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Typhimurium populations to help ensure the safety of skim milk. Scale-up of the UV-C device, toxicity, and sensory changes in skim milk will be the subject of further investigations. Scale up equipment has already been developed by the research team and

its efficacy in inactivating microorganisms and other viral surrogates (Norovirus) in skim milk on a larger scale will be subject to future investigation.

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List of Tables:

Table I. Optical properties and pH values for skim milk.

Parameters	Values
pH	7.08 ± 0.19
UVT ($base_{10}$)	0.52 ± 0.22
Absorbance ($base_{10}$)	2.31 ± 0.17
Scattering ($base_{10}$)	0.89 ± 0.03

Table II. UV-C sensitivity or D_{10} values of *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, and *Listeria monocytogenes* ATCC 19115.

Microbe	**D_{10} value**[a]
Escherichia coli ATCC 25922	1.795±0.041
S. typhimurium ATCC 13311	3.602±0.167
L. monocytogenes ATCC 19115	2.460±0.118

[a]D_{10} value expressed as $mJ.cm^{-2}$

Table III. Parameter estimation table for each model.

Microbes	Parameter estimates		Model
	α	β	
E. coli	-9.4479	-6.70262	Equation 4
S. typhimurium	-10.2454	-8.98181	Equation 4
L. monocytogenes	0.024678	-0.3534	Equation 3
MS2	-0.14574	-0.03469	Equation 3
T1UV	-0.10368	-0.1499	Equation 3

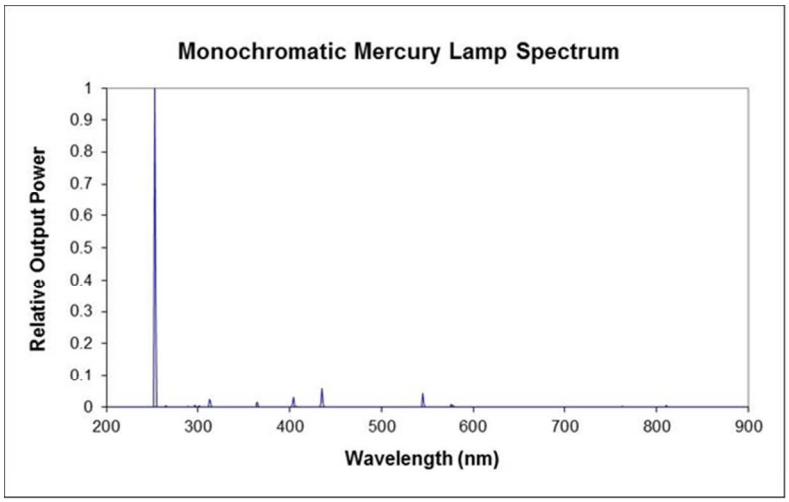
Note: parameters were estimated using both training and validation datasets.

Table IV. Goodness of fit

Microbe	R_{Square}	RMSE
Escherichia coli ATCC 25922	0.9970	0.1638
Salmonella typhimurium ATCC 13311	0.9857	0.3409
Listeria monocytogenes ATCC 19115	0.9657	0.5080
MS2	0.9850	0.2348
T1UV	0.9934	0.1855

Table V. Validation statistics using independent set of data

Validation Statistics	EC	SL	LS
Average Bias (AB)	0.29	-0.14	0.09
Mean Absolute Error (MAE)	0.29	0.27	0.33
Mean Square Error (MSE)	0.12	0.11	0.23
Root Mean Square Error (RMSE)	0.34	0.32	0.48
Average Bias Percent (PBIAS %)	-6.4	4.1	-3
Relative Error Percent (RE%)	-7.5	-9.1	-16.2
Model Efficiency (I^2)	0.99	0.99	0.97



Peak mission of low pressure lamp.

69x38mm (300 x 300 DPI)

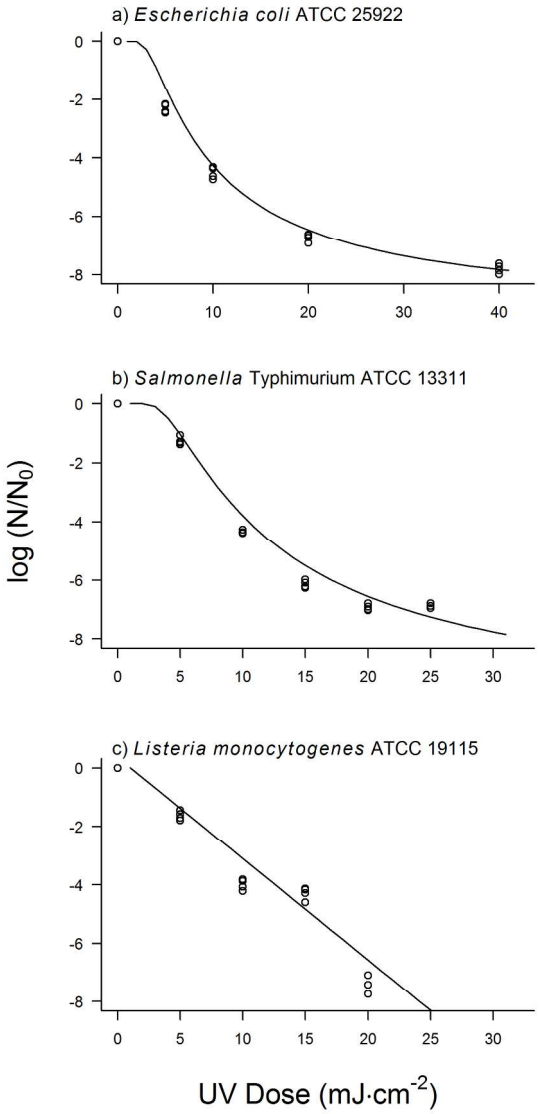


Figure 2. Inactivation of *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311, and *Listeria monocytogenes* ATCC 19115 in skim milk.

215x407mm (300 x 300 DPI)

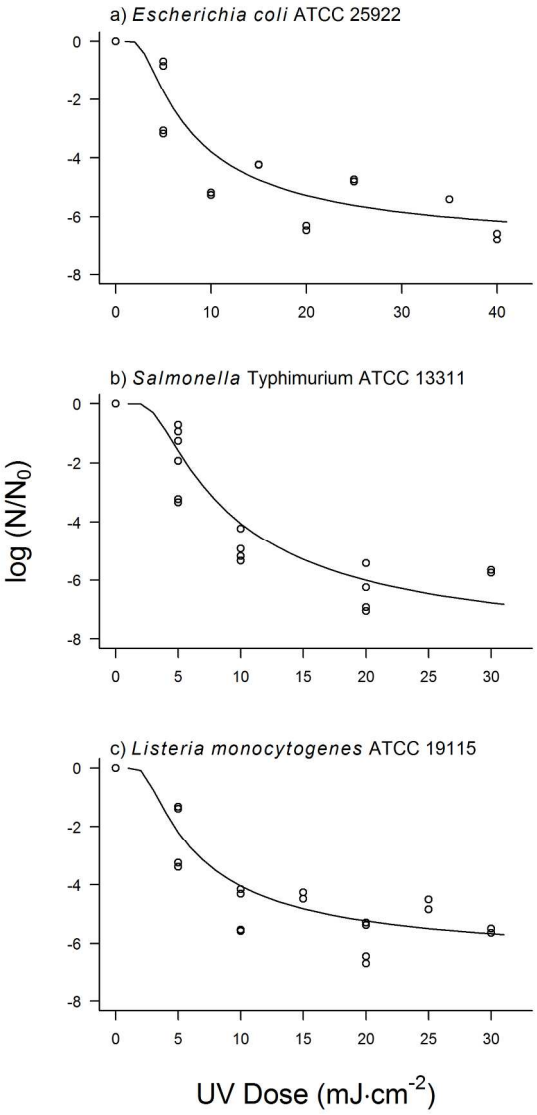


Figure 3. D10 values (experimental values) for microbial log inactivation of *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311 and *Listeria monocytogenes* ATCC 19115 in buffer.

215x407mm (300 x 300 DPI)

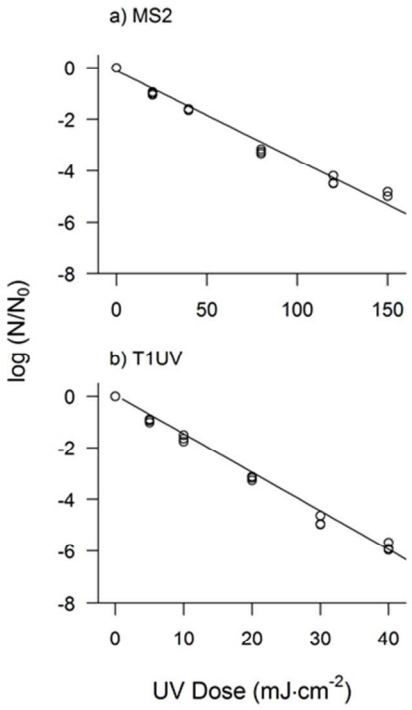
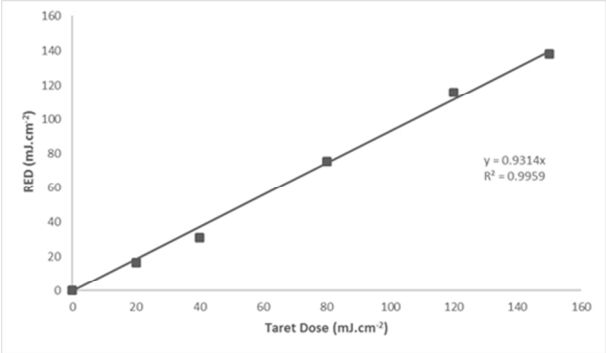


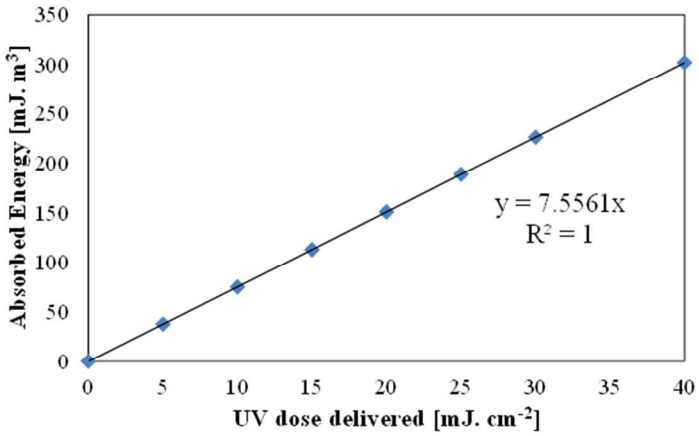
Figure 4. Inactivation rate results of MS2 and T1UV-C in skim milk.

69x52mm (300 x 300 DPI)



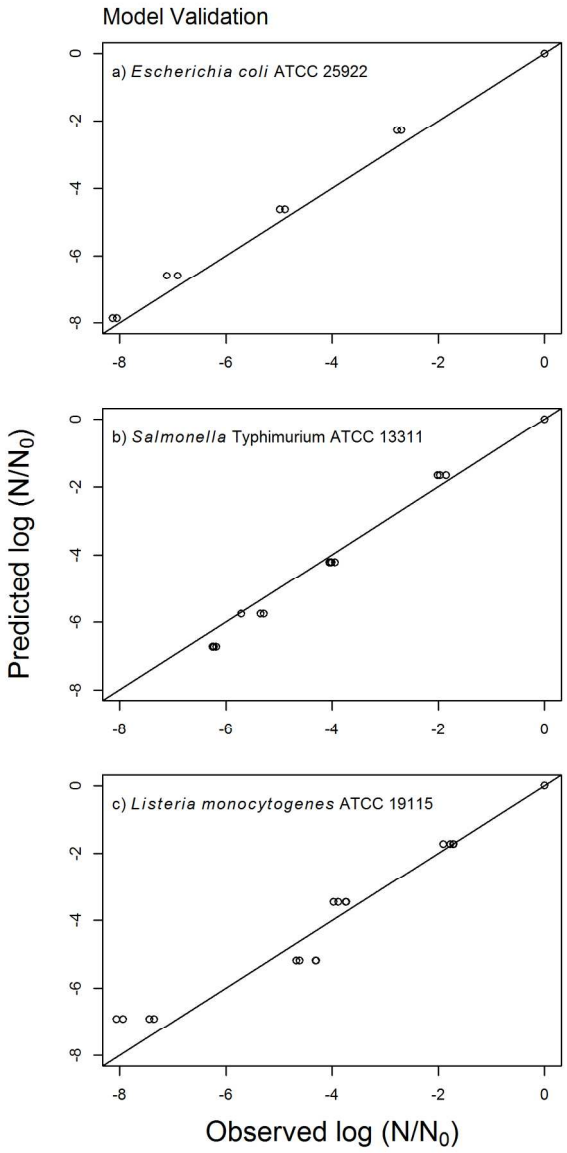
UV-C fluence delivered into milk measured by bioassay (MS2 bacteriophage) vs. target value. The reduction equivalent dose (RED) applied to milk was determined by well-characterized MS2 phage as the dose indicator (Target dose).

69x38mm (300 x 300 DPI)



Absorbed energy for microbial inactivation.

69x38mm (300 x 300 DPI)



Predicted and actual (experimental values) for microbial log inactivation *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311 and *Listeria monocytogenes* ATCC 19115.

114x215mm (300 x 300 DPI)