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RESEARCH ARTICLE

Inactivation of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 9027) in Skimmed Bovine Milk using UltraViolet-C Irradiation

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Abstract

The present study showed the effectiveness of UV-C irradiation on the inactivation of 3 strains of bacteria; *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 9027) inoculated in skim milk (0.1% fat content). The treatment chamber was made of stainless steel having 3 germicidal UV-C lamps. The treatments were carried out at different distances from source light: 5, 15, 25 and 45 cm at several treatment times (5, 10 and 15 min) and a constant treatment temperature (26.5 ± 2°C). The maximum inactivation extents were 1.8, 1.95 and 2.05 log reductions for *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. Different inactivation kinetic models have been developed to determine the model that best represents the results obtained in this work. Weibull distribution was shown as the model that most accurately represented the data.The most sensitive strain to UV-C treatment was *P. aeruginosa*, followed by *E.coli* and *S. aureus*.

INTRODUCTION

Milk is the most consumed food product worldwide since it provides all the nutrients needed for humans being and animals to grow and stay in good health (FAO, 2015). However, milk is a perishable product that can be contaminated by pathogenic and spoilage microorganisms because it has an optimal pH for microbial growth for *Listeria monocytogenes, Escherichia coli* O157:H7*, Salmonella Enteritidis*, etc.,.Therefore, to ensure the safety of the milk, the dairy industries process the milk products by using conventional thermal pasteurizations and/or sterilizations.This method consists of subjecting the product to temperatures between 60° and 140°C for different times ranging from few seconds to several minutes. Although effective for microorganism inactivation, the level of the temperatures (>60°C) used in this technology can adversely affect the organoleptic properties and nutritional values of milk. Consumer demand for milk that retains the flavor, taste and nutritional values of the fresh form has pushed researchers to develop reduced-temperature food processing techniques (Yu et al., 2014).

Innovating and emerging non-thermal technologies such as Pulsed Electric Field (PEF), Ultrasound (US), High Hydrostatic pressure (HHP) and Ultraviolet-C (UV-C) are promising technologies that can replace or supplement heat pasteurization in a hurdle process. The UV-C method consists in the exposition of the milk to low electromagnetic spectrum of 200-280 nm (Choudhary and Bandla, 2012). Several studies showed that the optimum inactivation happens approximately within the range of 254 to 264 nm because typical mercury UV lamps deliver at 254 nm. The wavelength of 253.7 nm was reported to have the most efficient bactericidal effect. This was due to microorganisms' DNA that greatly absorbs photons at this wavelength (Harm, 1980). The destruction of microorganisms may be due to the penetration of UV-C light into the outer membranes of the cells leading to damage DNA or RNA owing to the formation of pyrimidine dimers which can interfere the transcription and replication, thus eventually resulting to cell death (Miller et al. 1999; Cutler and Zimmerman, 2011). The efficiency of the UV-C lights to inactivate microorganisms is also influenced by the optical properties of the food products. The UV penetration is depending on the liquid absorbability, which depends on color, transparency and soluble and/or suspended solids content as proteins and fats (Choudary and Bandla, 2012). Therefore, UV-C light cannot penetrate turbid liquid foods such as milk unless to be presented to the system as a thin layer. Some studies have confirmed the effectiveness of the UV-C radiations to inactivate microorganisms such as *Staphylococcus aureus* (Krishnamurthy et al., 2007; Peireiraet al. 2014; Engin and Yuceer, 2011), *Pseudomonas aeruginosa* (Lu et al., 2011) and *Escherichia coli* (Peireiraet al. 2014; Choudharyet al, 2011, Engin and Yuceer, 2011) in milk medium. Recent studies (Choudharyet al., 2011a; Bandlaet al., 2012) reported more than 7 log cycle reduction of *E. coli* W 1485 in skimmed milk against only 4 log cycles of the same bacteria in raw milk. Therefore, higher UV-C dose for raw cow milk than for skimmed milks is required due the lower UV transmission of raw cow milk (Choudary and Bandla, 2012). The UV-C light sensitivity of microorganisms varies depending of the species. In general, Gram-negative bacteria are the most sensitive to UV-C radiations compared to Gram-positive ones, yeast, bacterial spores, molds and viruses (Sastryet al., 2000; Shama, 1999; Gayanet al., 2011).

The effectiveness of UV light on the inactivation of suspended cells in liquid mediums could be increased by minimizing the sample depth, reducing distance from the sample and/or increasing the treatment time. Therefore, there is a need for optimizing the experimental parameters to achieve the target inactivation level for specific applications.

Various kinetic modeling approaches have been reported in the literature to predict UV-C microorganism inactivation and all of them have a first-order model behavior (Severinet al., 1983; Kowalski, 2001; Koutchma, 2009). This models assume that the inactivation rate changes with respect to pathogen concentration, N, and fluence rate, E, such as in

$$\frac{dN}{dt} = -k_1 \cdot E \cdot N \qquad (1)$$

Where, k is the first-order inactivation constant in cm^2/mJ, k_1 is based on the fluence absorbed by the liquid or Einsteins absorbed by the liquid and delivered to molecule or organism and indicates the amount of radiant energy required to drive the reaction and ideally does not depend on absorbance (Koutchma, 2009). Fluence rate is the appropriate term when, for example, a microorganism is being irradiated byUV light emanating from many different directions ~e.g., in a multilamp array, whereas UV dose'' is utilized almost universally in UV disinfection literature (Bolton and Linden, 2003).

There are two main deviations from first-order UV inactivation kinetics. At low UV fluences, no inactivation of microorganisms was observed whereas at higher UV fluences, a normal log-linear relationship was noticed. The first behavior is called shoulder effect and the deviation coming right after linear kinetics where it is no further increase in inactivation at higher fluences is called tailing effect (Koutchma, 2009).

The objectives of this study were: (a) to investigate the effect of UV-C treatment on the inactivation of Gram+, Gram-, pathogenic and spoilage bacteria (*Staphyloccus aureus, Escherichia coli and Pseudomonas aeruginosa*) inoculated in skim milk and (b) to develop a kinetic modelof inactivation during the UV-C treatment.

Material and Methods

Milk products

Ultra-High-Temperature (UHT) skimmed milk (0.2%) (SarlTchinlait-Candia, Bejaia, Algeria) was purchased from a local supermarket and maintained at 4°C in a refrigerator until use.

Microorganisms and samples preparation

The milk products were inoculated with three (03) bacterial strains:

- *Staphylococcus aureus* (ATCC 6538) obtained from Eurl Envirozone laboratories, Beni-Messous, Algiers, Algeria.
- *Escherichia coli* (ATCC 25922) obtained from Microbiology Laboratory of Birtraria Hospital, El Biar, Algiers, Algeria.
- *Pseudomonas aeruginosa* (ATCC 9027) obtained from Microbiology Laboratory of SPA, Saidal, El Harrach, Algiers, Algeria.

Each strain was inoculated and grown to the early stationary growth phase at a temperature of 37°C for 18 to 24h depending of the bacteria, grown into 15 ml sterile centrifuge tubes containing Brain Heart Infusion Broth (BHIB). The culture was spun at 3,500 rpm for 15 min using acentrifuge (Ultra-8TL, LW Scientific, Lawrenceville, GA, USA) to harvest the cells and supernatant was discarded. The cell pellets were washed three times by re-suspension in saline water. Washed pellets were finallyre-suspended in 10 ml skimmed milk to achieve aninitial cell concentration of about 10^8 to 10^9 CFU/ml. The samples were prepared in filling and spreading 2 mL of inoculated skimmed milk into 90 mm petri dishes resulting in 1mm thickness.

Viable cells were evaluated before and after UV treatment by plating on selective and non-selective agar and incubating at 37°C for 24 hrs. The selective agar was Mannitol-Salt-Agar (MSA) for *S.aureus* and Violet-Red-Bile-Glucose-Agar (VRBGA) for the other bacteria (*E.coli* and *P.aeruginosa*). The non-selective agar for the three strains was Plate-Count-Agar (PCA). All culture media agars used in this study were purchased from Envirozone laboratory (Beni-Messous, Algiers, Algeria) which were imported from Quélab laboratory (Montreal, QC, Canada). Priorto plating on the selective media, the processed milk samples were maintained at 4°C for about 4-12 h to repair any injured cells. This method has been shown to be an effective technique for resuscitation of injured cells compared to plating on a non-selective medium and followed by overlaying with the appropriate selective media (Mussa et al.,1999). The mean count was reported for all milk samplings and the three plates were used for each dilution.

UV-C apparatus and processing

Custom-made UV apparatus was modified and designed according to Palgan et al. (2011). The apparatus consisted of a stainless steel parallelepiped (100x90x70 cm^3) treatment chamber equipped with 3 UV-C germicide lamps (Model: G8T5/OF, 8 Watt, 56V, Sylvania, Osram, Denver, MA, USA) mounted on top (Fig.1). In order to avoid any increasing temperature during the treatment because of UV-C radiation, a fan was placed on the left lateral face of the chamber. The air was blowing from left to right perpendicularly with UV radiation to maintain the treatment temperature as constant. The temperature was measured and controlled before and after the treatment to 25.6±2°C using a mercury thermometer installed inside the chamber. The milk samples were mixed carefully before and after UV treatment to homogenize the bacterial cells in all treatment surfaces.

The chamber was subdivided in 4 levels of treatments: 5, 15, 25 and 45 cm distance from the UV light. The treatment times were 5, 10 and 15 min.

The UV light dose was determined using the following equation

$$D = I_0 t \qquad (2)$$

Where, D is the UV-C dose (mJ/cm^2), I_0 is the UV-C intensity emitted on the treatment surface (mW/cm^2), and t is treatment time.The UV-C radiation during the treatment varied from 113.2 up to 339.53 J/cm^2.

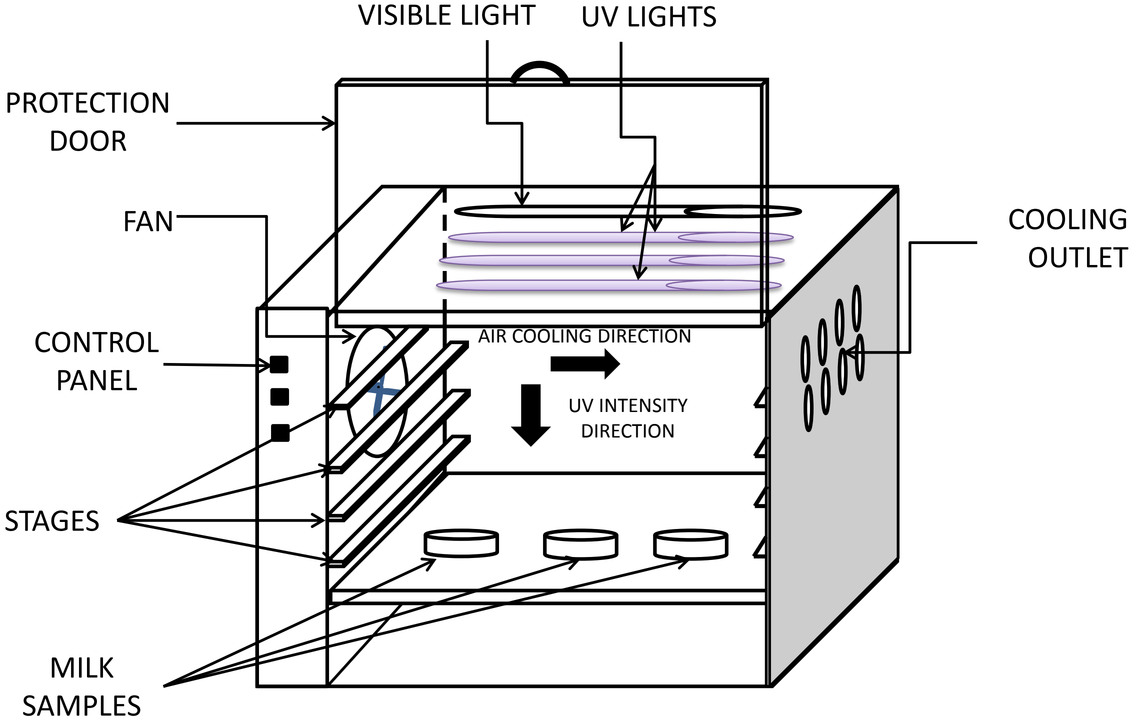

Fig 1: Schematic diagram of the UV treatment chamber

Statistical analysis

Regression analyses were conducted using Curve expert software (Curve expert, Version 1.4, 2009, Microsoft Corporation, Mississippi, USA). Analysis of variance (ANOVA) was performed using the General Linear Models procedures (GLM) of the Statistical Package for the Social Sciences (SPSS, Version® 14, ESI, 2013, Statistical

Packages, Chicago, IL). Microsoft Excel software (Microsoft 1 Excel 2010) was used to plot the curve of inactivation. Experiments were triplicated and the means of the three data sets are presented. In all cases, significantdifference was based on the 5% level (P ≤ 0.05).

Results and discussions

The treatment was consisted of to expose strains of *S.aureus, E. coli* and *P. aeruginosa* inoculated in skimmed milk to UV-C radiation for 5, 10 and 15 min and to vary the distance between the product and UV light to 5, 15, 25, and 45 cm. Survival fraction of all bacteria decreased while treatment time was increased and the distance between product and UV light was decreased as shown in Figs 2, 3 and 4.

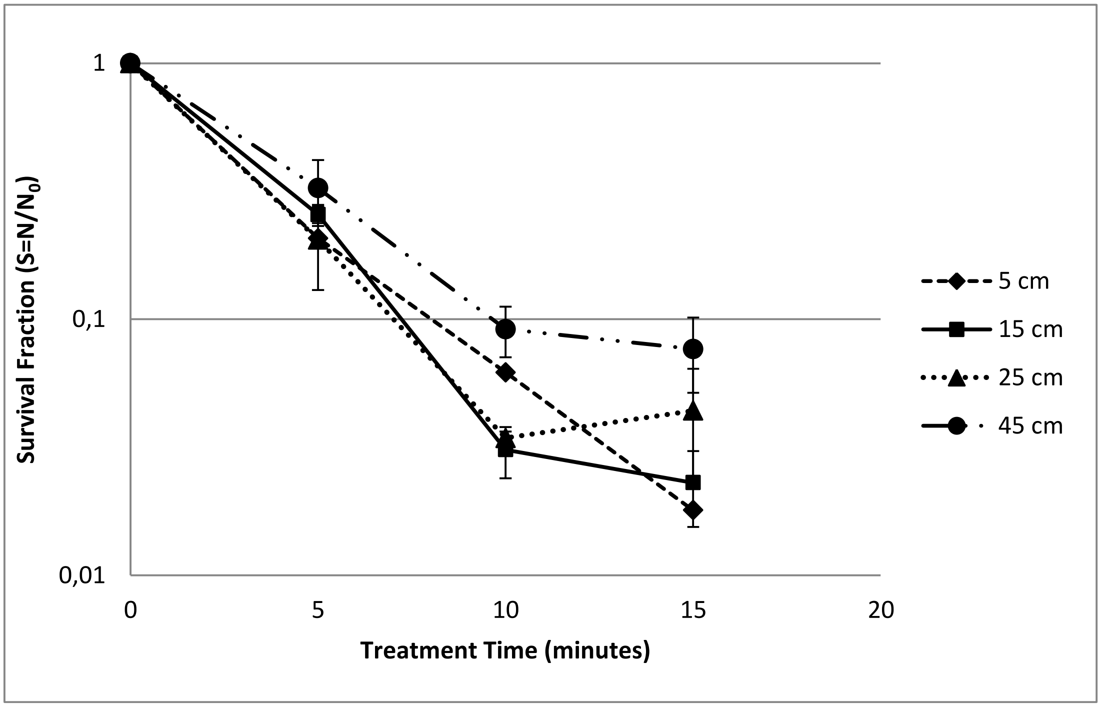

Fig 2: Survival fraction of *Staphylococcus aureus* (N/No) in function of treatment time and distance from UV source

A maximum of about 1.8, 1.95 and 2.05 log reductions were obtained after 15 min of treatment at a distance of 5 cm from the UV light, *for S. aureus, E. coli* and *P. aeruginosa*, respectively. An average temperature of 25.6±2°C was maintained using a fan to aerate the inside of the treatment chamber. The maximum dose required to achieve the highest inactivation rate was 338.53 J/cm^2. Lu et al. (2011) obtained 2.1, 2.5 and 2.2 Log cycle reduction of *S. aureus, E. coli* and *P.aeruginosa*, respectively, inoculated in skimmed milk using lower energy density of 21.7J/cm^2. Their study consisted of continuous UV apparatus with shorter residence treatment time resulting therefore in lower energy density consumption.

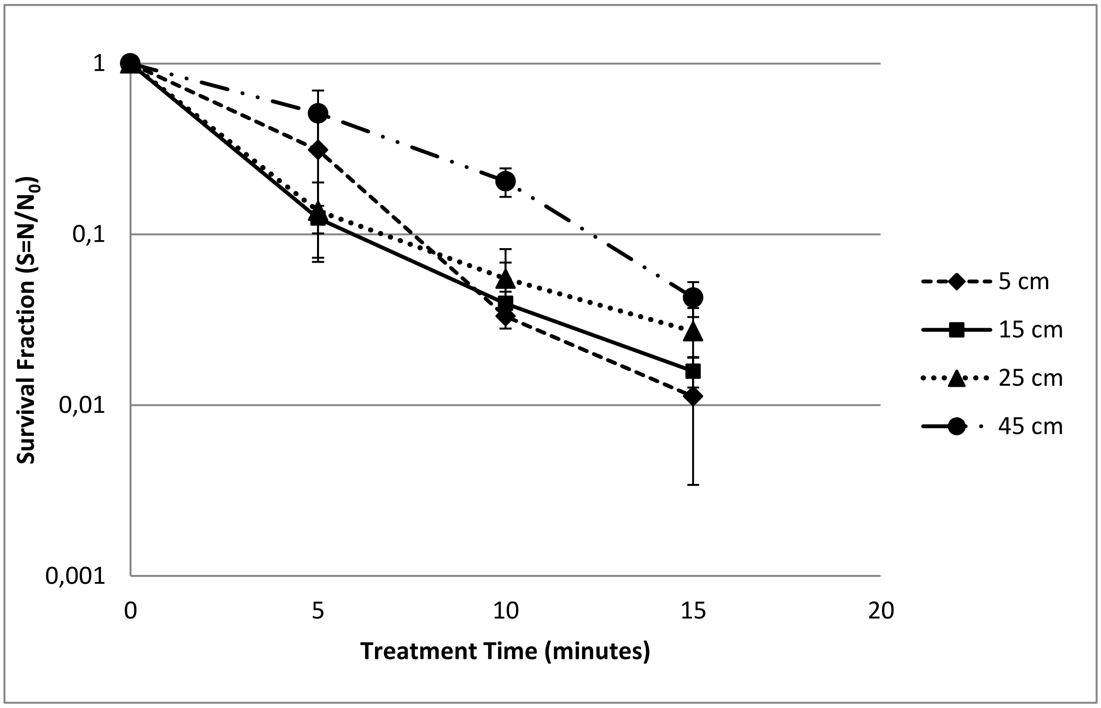

Fig 3: Survival fraction of *Esherichia coli* in function of treatment time and distance from UV source

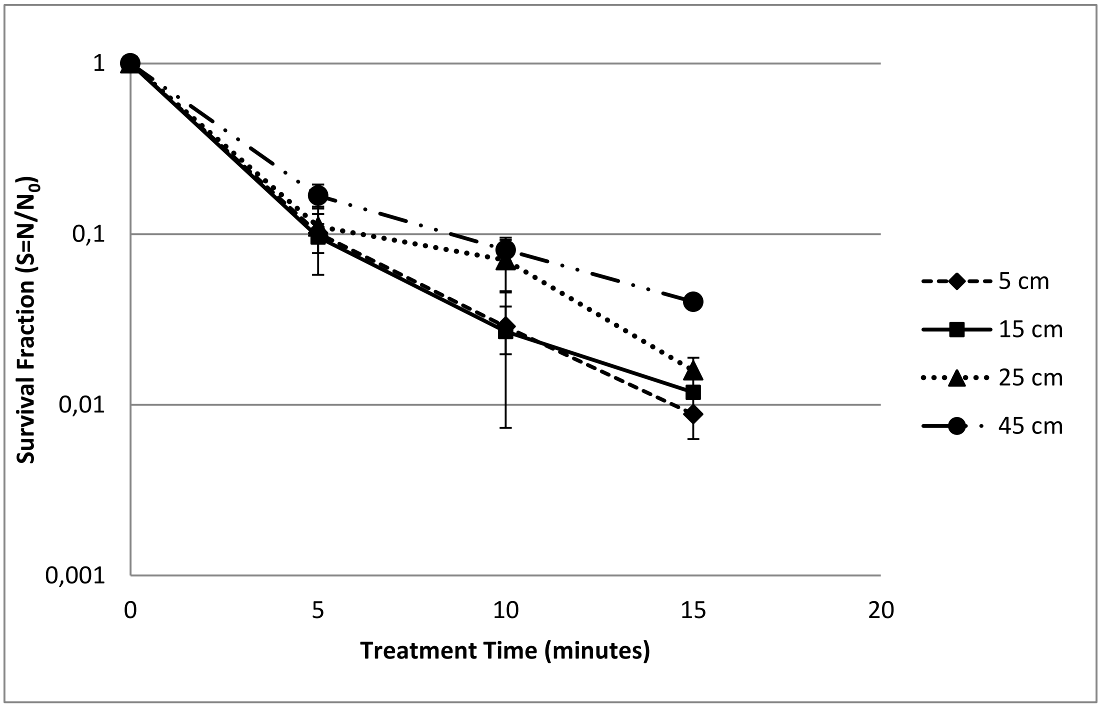

Fig 4: Survival fraction of *Pseudomonas aeruginosa* in function of treatment time and distance from UV source

Krishnamurthy et al. (2007) reported the same log reduction for *S.aureus* strain (2.07 log cycles) inoculated in milk using pulsed UV treatment at a flow rate of 20 mL/min at 8 cm distance from the light source. Choudhary et al. (2011) obtained up to 2.5 log reduction of *E. coli* W1485 inoculated in raw milk in a continuous system at a higher Reynolds number (Re=1064). Matak (2004) reported 2.27 and 1.43 log cycles of E. coli O157:H7 inoculated in skimmed and whole milk, respectively, after an UV-treatment time of 1.5 seconds at a temperature of 20°C. The authors suggested that the decreasing in turbidity of skim milk samples may have contributed to the greater reduction of pathogens in the skim milk samples. In addition, it was theorized that the differences in bacterial reduction are due to the lower transmittance of milk and the higher solid content in milk (such as fats, proteins, etc.) that may protect organisms from UV penetration, therefore as the amount of fat and other solids increase, the average bacterial reduction would decrease (Mataket al., 2004). In addition, the continuous and batch UV-C system showed a great influence on the effectiveness of the treatment and the inactivation rate.Our study demonstrated that UV resistance of microorganisms varied from species to species (Figures 2, 3 and 4). The results were in agreement with the work of Lu et al. (2011) which reported that gram-positive bacteria were more resistant to the effects of UV light than gram-negative bacteria. Therefore, in relation to the susceptibility of Gram-positive (*S. aureus*) vs.Gram-negative (*E. coli* and *P. aeruginosa*) to static UV-C light treatment, the results indicated significant differences ($p < 0.05$) in viable counts. This variation in light sensitivity may be due to the structural/compositional differences in the cell walls and membranes, in particular the thicker peptidoglycan cell wall (20-80 nm) of Gram-positive compared to Gram-negative organisms (1-2 nm) (Alcamo, 1997).

Accurate mathematical models describing the kinetics of inactivation of microbial population in real food systems are needed to establish appropriate UV-C process conditions to obtain known levels of microbial inactivation, which is necessary to achieve stable and safe products without over-processing (Wouteret al., 2001; Amiali et al., 2004). Different kinetic models have been used to describe microbial inactivation kinetics during UV-C treatment. These models include Weibull distribution, exponential decays with or without tailing effect and exponential decay with critical survival fraction (Table 1).

Table 1: Kinetic models for microbial inactivation by UV-C

Model	Equations
Weibull distribution	$S = e^{-\left(1/\delta \cdot t\right)^{n}}$
Exponential Decay	$S = e^{-K_1 . t}$
Exponential Decay with Critical Survival Fraction	$S = S_c e^{-K_3} + (1 - S_c) e^{-K_2 t}$
Exponential Decay with Tailing effect	$S = S_t + (1 - S_t)\, e^{-K_4 . t}$
	where, $S = N/N_0$, Survival fraction N, Bacterial Cells at treatment time t N_0, Bacterial Cells at treatment time $t = 0$ S_t, Tailing Survival fraction S_c, Tailing Survival fraction δ, Caracteristic time in minute n, Shape parameter describing concavity K_1, constant 1* K_2, constant 2* K_3, constant 3* K_4, constant 4* t, Treatment time (min)

*$K_i = k_i \cdot E$, where k_i (cm^2/mJ) is the independent kinetic constant and, E (mJ/cm^2), the fluence of UV light.

The monophasic traditional exponential decay accurately fit the experimental data inactivations (Table 2). However, this model may not be suitable to describe bacterial inactivation kinetics in real food system such as milk. Several studies observed biphasic curves with tailing effect when treated microorganisms in food system. (Murakami et al., 2006; Schenk et al., 2008). In addition, others researcher have reported a rapid inactivation in the first phase followed by a notorious decrease in the survival fraction rate (Schenk et al., 2008; 2011; Koutchma, 2009). However, in our study we practically did not have any tailing effect for all bacteria (Table 3). In this case we can assume that the microbial UV-C inactivation is an all or nothing event. This was also confirmed by the non-significant injured cells (data not shown).

The model represented in Table 4 indicated that there is a critical survival fraction with two inactivation phases except for *P. aeruginosa* that showed one phase equation since the critical survival fraction is almost equal to 1. Therefore, microbial inactivation during UV-C treatment appears to follow one phase kinetic for all models tested in this study.

Table 2: Parameters for Exponential Decay model at 5 cm distance from UV source light

Model $S = e^{-K_1 .t}$	Parameter K_1	Standard error of estimate	Coefficient of determination r^2
S. aureus	0.25	0.014750	0.9989
E. coli	0.25	0.032400	0.9950
P. aeruginosa	0.86	0.008683	0,9996

Table 3: Parameters for Exponential Decay with tailing effect model at 5 cm distance from UV-C source

Model $S = S_t + (1 - S_t)\, e^{-K_4.t}$	Parameter S_t	Parameter K_4	Standard error of estimate	Coefficient of determination r^2
S. aureus	-0.0214	0.23	0.01294	0.9994
E. coli	-0.0453	0.22	0.02948	0.9972
P. aeruginosa	0.0117	0.52	0.00066	0.9999

Table 4: Parameters for Exponential Decay with critical survival fraction model at 5 cm distance from UV-C source

Model $S = S_c e^{-K_3} + (1 - S_c)\, e^{-K_2.t}$	Parameter S_c	Parameter K_2	Parameter K_3	Standard error of estimate	Coefficient of determination r^2
S. aureus	0.624	0.25	0.25	0.02555	0.9989
E. coli	0.614	0.25	0.25	0.05612	0.9950
P. aeruginosa	0.978	0.53	0.048	0.00067	0.9999

Table 5: Parameters for Weibullian model at 5 cm distance from UV source light

Model $S = e^{-\left(1/\delta \cdot t\right)^n}$	Parameter $\delta\,(min)$	Parameter n	Standard error of estimate	Coefficient of determination r^2
S. aureus	2.453	0.61	0.018065	0.9989
E. coli	1.410	0.36	0.039685	0,9951
P. aeruginosa	1.009	0.49	0.010635	0,9996

The Weibullian equation is a flexible nonlinear model which considers that there is heterogeneity between microbial cells of population (Mytilinakiet al., 2011). The Weibullian model curves (see Tables 5) exhibited in all cases upward concavity, being almost biphasic. These are verified by the shape parameters (n) which were < 1 for all inactivation curves. Hence, the experimental curves were highly correlated to the predicted data, obtaining therefore significant determination coefficients ($r^2 > 0.9$). The inactivation achieved was almost similar in all microbial species. However, characteristic time was higher for *S. aureus*, followed by *E. coli* and *P. aeruginosa*, indicating that the most resistant bacteria were the Gram + cells. This may be due to the thick peptidoglucan membrane that can efficiently protect the cells against the UV-C radiation compared to Gram - bacteria cells. Studies carried out by Uesugiet al. (2007) demonstrated that Pulsed UV light is capable to deliver the same level of microbial reduction in clear liquids, regardless of the level of contamination. According to these authors, the Weibullian model is adequate to accurately predict microbial inactivation in clear liquids, but it fails for products where the influence of various substrate properties on inactivation is significant such opaque fluids. In our case, skimmed milk (0.2% fat content) has a nearly clear appearance; for that reason, Weibullian model fit adequately and with a high accuracy the results obtained in the present study.

Conclusion

From the results of this study, it can be concluded that UV-C treatment can be successfully applied to obtain reasonable levels of destruction with respect to the selected foodborne spoilage and/or pathogenic bacteria suspended in skimmed milks. The bacterial inactivation was a function of time and distance from UV-C source. UV-C treatment with energy of 339.53 J/cm^2, distance from the light of 5 cm and 15 min treatment time, inactivated the bacteria up to 2 log cycle reduction withno risk of milk spoiling. The kinetic models used in this study showed that microbial inactivation with respect to treatment time, followed an exponential decay behavior without any tailing effect or critical survival fraction. The inactivation curves were well represented by the weibullian model.

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