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Antimicrobial Effect of Photosensitized Rose Bengal on Bacteria and Viruses in Model Wash Water

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Abstract Peroxides and chlorite-based sanitizers are commonly used for washing procedures. The efficacy of these sanitizers is significantly reduced in the presence of organic content and may result in the formation of harmful secondary products. Therefore, new food-grade sanitizers with enhanced antimicrobial efficacy are needed. In this work, we tested a visible light-activated photosensitizer, Rose Bengal (RB), for the reduction of microbial load in simulated wash water. *Escherichia coli* BL21 and bacteriophage *T7* were selected as model bacterial and viral targets, respectively. Effects of duration of illumination, growth phase of the bacterium, and the presence of organic matter on efficacy of inactivation were evaluated. Photosensitized RB was able to achieve 6 log colony-forming units (CFU)/mL reduction in the exponential-phase bacteria in the presence of high organic content (2000 ppm LB broth) within 45 min of treatment. The results also demonstrated that the stationary-phase microbes were significantly more resistant to photoinactivation as compared to the exponential-phase microbes at both low and high organic loads (200 and 2000 ppm). The results indicate that RB dye can be internalized in bacteria and induces damage to the cell membrane upon photoactivation. Viral inactivation studies demonstrated that photoactivation of RB can achieve 5 log PFU/mL reduction in viral load in the presence of 2000 ppm LB after 30 min of treatment. Overall, these results highlight the efficacy of RB as an antimicrobial against bacteria and viruses in simulated wash water and demonstrate its potential as an alternative sanitizer for the food industry.

Keywords Rose Bengal · Photosensitization · Model wash water · *Escherichia coli* · *T7* bacteriophage

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Introduction

Produce washing is a critical process operation in the fresh produce industry that removes debris and soil and also provides an opportunity for reducing the microbial load (Palma-Salgado et al. 2014). Reduction in microbial load with produce washing may impact both the quality and the safety of fresh produce. To reduce the microbial load on fresh produce and to prevent cross contamination of produce from microbes in the wash water, various sanitizers such as sodium hypochlorite (SH) and peroxy acetic acid (PAA) are commonly used (Weller et al. 2013). The activity of these sanitizers is predominantly based on oxidative stress induced by interactions of these chemicals with microbial cells (Gomez-Lopez 2012). Despite their widespread use in the fresh produce industry, current sanitizers have significant limitations. These limitations include (a) production of harmful chemical derivatives upon reactions of SH with organic content in fresh produce (Fawell 2000); (b) limited efficacy of the selected sanitizer in reducing microbial load in high-organic-content environments (Shen et al. 2013; Sun. et al. 2012), and (c) reduced shelf life of the sanitizer as in the case of PAA when compared to

SH (Bachelli et al. 2014). Thus, there is a significant need to improve the selection of sanitizers that can reduce some of these concerns, particularly the limited efficacy of current sanitizers in the presence of organic content and their environmental and health concerns.

In recent years, significant research has been conducted in biomedical and environmental engineering to evaluate the role of photosensitization for the treatment of various diseases or oxidation of certain chemical compounds in water (Valkov et al. 2014). The reactive oxygen species (ROS) produced by photoactivation of the PS compounds can induce damage to the cellular components of the cells resulting in cell death or oxidation of chemicals (Cieplik et al. 2014; Kumar et al. 2004; Lopes et al. 2014). This approach has been evaluated to reduce proliferation of cancer cells (Agostinis et al. 2011), suppress growth of fungal infections and other skin diseases (Calzavara-Pinton et al. 2005), as well as inactivate residual chemicals in waste water (Kim et al. 2012).

To the best of our knowledge, the photoactivation approach has not been explored for the applications relevant to sanitation of wash water for the fresh produce or process water in food systems. Activity of the PS compounds is based on the generation of ROS upon exposure to light at a particular wavelength. The unique benefits of this photoactivation approach for sanitation application may include (a) the ability to activate compounds with light and the lack of residual chemical activity in the absence of excitation source and (b) some of the PS compounds may be selected from food-grade chemicals (both GRAS or food additives), thus reducing both the health risks and environmental concerns.

To demonstrate the potential of the photoactivation approach for sanitation in the fresh produce industry, several key questions need to be evaluated. In this study, we have focused on two key questions: (a) the ability of PS compounds to reduce microbial load (both viral and bacterial) in the presence of organic content and (b) the potential of PS compounds to inactivate microbes in both the exponential and stationary phases. These questions were selected because organic content is one of the significant factors that impact the efficacy of conventional sanitizers (Van Haute et al. 2013) and most of the prior studies with PS were conducted without organic content (Cherchi and Gu 2011). Similarly, most of the prior studies with PS compounds have been conducted using bacteria in the exponential growth phase. Prior studies with conventional sanitizers have indicated that bacteria in different growth phases can have significant differences in their resistance to sanitizers (Yousef and Juneja 2002). These differences in resistance have been attributed to varying expression patterns of stress resistance genes in various phases of the bacteria (Blom et al. 2011). In addition, it has been suggested that the specialized survivor cells, that neither grow nor die during antimicrobial processes, appear in the later phase of bacterial growth (Keren et al. 2004).

In this study, Rose Bengal (RB) was selected as a model PS. RB belongs to a class of xanthene-based dyes. Some of the xanthene-based dyes have been approved as food colorants; the examples include the use of RB dye as a food-grade color additive (Food Red No. 105) in Japan (Qi et al. 2010) and Erythrosine (E127) as a food color in the USA (FDA 2003), EU, and the UK (EFSA 2011). RB has also been previously used as a PS in diverse biomedical applications including photodynamic therapy to induce apoptosis and autophagy in Hela cells (Panzarini et al. 2014); to reduce proliferation of skin cancer (Gianotti et al. 2014), and for antifungal treatments (Cronin et al. 2014). In addition to biomedical applications, RB has been used for inactivation of RNA (Di Mascio et al. 1989) and DNA viruses in water (Schneider et al. 1993). However, to the best of our knowledge, RB has not been evaluated as a PS for wash water sanitation application. To simulate the organic content in wash water, lysogeny broth (LB) medium with different dilutions was used. To determine the influence of process conditions, i.e., PS concentration, organic content, and time of exposure on reduction of bacterial and viral contaminants, *Escherichia coli* and T7 bacteriophages were selected as the model bacterial and viral targets. To evaluate the influence of physiological states on sanitation efficacy, *E. coli* in two different phases of its growth (exponential- and stationary-phase growth) was selected for this study.

In summary, the results of this study indicated the potential of the PS approach for wash water sanitation and provided an alternative to chemical sanitizers that are currently being used.

Material and Methods

Photo-Illumination Device

A non-transparent taupe-colored plastic box (internal size 96.5 L × 43.1 W × 48.2 H cm) (Suncast Corporation, Batavia, IL, USA) with two dual-light 60.9-cm T8 fluorescent fixtures (120–277 V, 50/60 Hz) with an electronic ballast was used for the photo-illumination of samples. A total of four 15-W, 60.9-cm T8 Cool White 4100K Fluorescent Bulbs (Philips®) were set up on the fixtures, and a rigid white platform was placed under the lamps at 8 cm from the light source for the exposure of samples. The intensity of light at the platform was measured with a Dr. Meter SM206 digital power meter (Light In The Box, Seattle, WA, USA) and was found to be 57.8 ± 1.83 W/m^2 on the surface of the platform.

Chemical Oxygen Demand Measurements

In this study, LB was selected as a model system to simulate the influence of organic content on antimicrobial efficacy of the selected PS treatments. LB has already been used as a

model system for simulating the influence of organic content on conventional sanitizers (Le et al. 2015). Chemical oxygen demand (COD) measurements were performed using high-range COD digestion vials (Hach, Loveland, CO, USA). The measurements were conducted following the Hach Method 8000. The samples were digested in a DRB 200 reactor (Hach, Loveland, CO, USA), and the product was analyzed based on colorimetric analysis with a DR/800 colorimeter (Hach, Loveland, CO, USA). Three dilutions of LB medium (Fisher Scientific, Hampton, NH, USA) at 200, 2000, and 20,000 ppm were measured, and the organic contents were reported in milligrams O_2/liter COD.

Bacterial and Bacteriophages Cultures

E. coli BL21 (ATCC, BAA-1025, Manassas, VA, USA) was cultured in LB broth and LB agar (Fisher Scientific, Hampton, NH, USA). Ten milliliters of broth was inoculated with a colony of *E. coli* BL21 and grown at 37 °C at 150 rpm. The following day, 100 μL of the overnight culture was inoculated in 10 mL of pre-warmed LB broth and incubated until an absorbance of 0.4 was reached at 600 nm (approximately 2×10^8 colony-forming units (CFU) per mL, reflecting an exponential phase) or 1.5 (approximately 2×10^9 CFU/mL, reflecting a stationary phase). Bacteriophage *T7* (ATCC, BAA-1025-B2, Manassas, VA, USA) was cultured in stationary-phase *E. coli* BL21 cells in LB medium. Phage propagation was blocked with a chloroform treatment, and the recovery of lysate was performed as previously described (Han et al. 2014).

Diffusion and Release of Rose Bengal in Exponential- or Stationary-Phase *E. coli*

Exponential- or stationary-phase bacterial suspensions were centrifuged at 16,000g for 1 min and washed with 1 mL of sterile Milli-Q water. After a second step of centrifugation, pellets were re-suspended in sterile diluted LB (2000 ppm) to obtain a final bacteria concentration of approximately 2×10^8 CFU/mL. One milliliter of the bacterial suspension was immediately added to an equal volume of Rose Bengal (RB) sodium salt (Santa Cruz Biotechnology, Dallas, TX, USA) solution at a concentration of 0, 20, or 200 μM prepared in 2000 ppm LB solution to obtain a final suspension containing 1×10^8 CFU/mL bacterial load and 0, 10, or 100 μM of RB. This suspension was incubated in plastic sterile centrifuge tubes for 0, 30, or 60 min in the dark at room temperature. After selected time intervals, 1 mL of cell suspension was centrifuged and washed twice in sterile water to remove extracellular RB and the cell pellet was lysed using 1 mL of lysis solution (50 mM Tris-HCl, 25 mM EDTA, 3 % SDS). Cells in the lysis solution were subjected to three cycles of freezing and thawing at −30 °C for 5 min and +65 °C for 10 min.

Subsequently, the suspension was centrifuged at 16,000g for 10 min to pellet cellular debris. The supernatant was recovered for spectrophotometric analysis of recovered intracellular RB. Two hundred microliters of supernatant was pipetted in a well of a sterile 96-well flat-bottom transparent plate (Sarstedt, Inc., Newton, NC, USA), and the absorbance value at 549 nm was measured. Absorbance data were standardized on readings on 200 μL of lysis buffer and subtracted from readings at same lambda of 1×10^8 CFU/mL in LB 2000 ppm without RB. All the experiments were performed in triplicate and data were recorded as the average of three independent readings.

Fluorescence Microscope Imaging of RB Absorbed by *E. coli*

One milliliter of a bacterial culture containing 1×10^8 CFU was prepared in 2000 ppm of LB with 100 μM of RB and incubated in the dark at room temperature for 30 min. After incubation, the suspension was centrifuged and washed as described in the earlier section. The pellet was re-suspended in 1 mL of water, and 10 μL of the suspension was spotted on a microscope slide and a cover slip was placed on the top. The slide was visualized using an inverted optical microscope (Olympus IX-7). Fluorescence images were acquired using a ×60 objective, and the fluorescence excitation and emission were 541 and 572 nm, respectively. Control experiments with bacteria without RB were also conducted.

Photosensitization of *E. coli* BL21 in Rose Bengal Solution

For exponential-phase experiments, a culture of *E. coli* with an A600nm = 0.4 OD was diluted 1:100 in the appropriate dilution of LB broth to simulate the presence of various amounts of organic loads (200, 2000, and 20,000 ppm). One milliliter of this dilution (approximately 2×10^6 CFU/mL) was added to 1 mL of RB solution of identical organic load in order to incubate bacterial culture with 0, 10, or 100 μM of RB dye. The resulting 2 mL volume with 1×10^6 CFU/mL was placed within a well of a 12-well flat-bottom polystyrene plate and exposed to light for 0, 15, 30, 45, and 60 min. At each time point, an aliquot of the sample was withdrawn and serially diluted in phosphate buffer saline (PBS) (USB Corporation, Cleveland, OH, USA), and aliquots of 100 μL were plated on LB agar for the bacterial enumeration. The controls for this experiment consisted of incubation of bacteria at room temperature for up to 1 h either in the presence of RB but without light or in the absence of RB but in the presence of light. For the experiments involving stationary-phase bacteria, the stock culture was diluted 1000-fold to achieve the same level of initial microbial population as for the exponential phase. The protocol of photosensitization and enumeration of CFU for the stationary-phase bacteria was identical to that discussed for the exponential-phase bacteria.

Detection of Nucleic Acid and Protein Leakage

A stationary- or exponential-phase bacterial culture of 1×10^8 CFU/mL prepared in 2000 ppm of LB containing 100 μM RB was exposed to the light for 1 h. After the treatment, cells were centrifuged at 16,000g for 10 min and the absorbance of supernatant was measured at 260, 280, 549, and 600 nm with a GENESYS 10S UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) in UV-cuvettes (BRAND, Wertheim, Germany). The blank for this experiment consisted of bacterial culture that underwent the identical preparation and treatment except for the addition of RB. Nucleic acid and protein leakage was therefore standardized based on the absorbance values of supernatant extracted from cells in LB 2000 ppm without RB added at 549 and 600 nm.

Photosensitization of *T7* Bacteriophage from Light-Exposed Rose Bengal

E. coli BL21 lysate with *T7* was centrifuged and filtrated in a 0.22-μm sterile filter. The filtrated lysate was diluted in PBS until a titer of 2.0×10^9 plaque-forming units (PFU) per milliliter was obtained (confirmed by plaque count plating). Ten microliters of the dilution was injected in 2 mL of solutions with different LB organic contents (1.0×10^7 PFU/mL final concentration) containing the PS RB in different concentrations (0, 10, and 100 μM). Photosensitization was performed in a manner similar to the bacterial experiments above. At each time point, 10 μL of the sample was appropriately diluted in PBS and 10 μL of each dilution was injected in 100 μL of stationary-phase host *E. coli* BL21 (approximately A600nm = 1.5 OD), followed by incubation for 15 min at 23 °C. Afterwards, 4 mL of soft LB agar at 40 °C (LB broth 1×, 0.75 % agar, 10 mM MgSO$_4$·7H$_2$0 (Fisher Scientific, Hampton, NH, USA)) was added and mixed with the incubated bacteria plus phages and immediately poured on precast room temperature LB agar plates to be further incubated overnight at 37 °C after solidification. Plaques were counted and reported as PFU/mL.

Mathematical Modeling of Inactivation of T7 Bacteriophage and *E. coli* BL21

The Weibull model was used to describe the inactivation by visible light or during the treatments with photosensitized RB.

$$\log_{10} \frac{N}{N_0} = \frac{1}{2.303} \left(\frac{t}{\alpha} \right)^{\beta}$$

where N is the number of surviving population (CFU/mL or PFU/mL), N_0 is the initial number of population (CFU/mL or PFU/mL), t is the treatment time (min), α is the characteristic time (min), and β is the shape parameter (unitless). Then, α

and β were used to calculate the reliable life, t_R, which corresponds to the D-value for the first log CFU/milliliter reduction (van Boekel [2002]; Bialka et al. [2008]).

$$t_R = \alpha \times (2.303)^{\beta}$$

The models were constructed using averages from two experimental data sets. Non-linear least squares regression method was used to predict α and β. The third experimental data set was used for a comparison between experimental and predicted data. The goodness of fit was evaluated by root mean square error (RMSE) and mean absolute error (MAE) values between the calculated data from the model and experimental data.

Statistical Analysis

The results were plotted as the mean ± standard deviation, and the two-tailed t test was used to determine the statistical significance ($\alpha = 0.05$) of the inactivation of bacteria or viruses among the conditions tested or the significance of the leakage of nucleic acid or proteins from bacteria during the photosensitization assay. The Pearson correlation coefficient (PCC) was calculated to measure the strength of the linear relationship between survived population and organic content or between survived population and RB concentration ($\alpha = 0.05$) using the Minitab software.

Results and Discussion

COD Measurement of LB Medium Dilutions for Photosensitization Experiments

To characterize the correlation between organic content and COD (a standard approach used in industry to characterize organic content), serial dilutions of LB broth were prepared and the COD of the resulting dilutions was measured. A linear correlation between ppm of LB in Milli-Q water and COD of the solutions with a proportionality constant of $k = 0.913$ and R^2 of 0.998 was observed. In this study, 20,000, 2000, and 200 ppm of LB were selected to represent very high, high, and low COD levels, respectively. These selected levels correspond to 18,280, 1846, and 203 mg O$_2$/L COD, respectively. A COD value between 200 to 2000 mg/L represents the typical range of organic content associated with wash water from cut lettuce processing (Luo [2007]). These selected COD values represent a range between 2 kg/40 L and 18 kg/40 L for the ratio between the mass of produce to the mass of water for cut lettuce washing. Similarly, the COD of process water used for fresh-cut shredded carrot is approximately 3500 mg/L (Gonzalez et al. [2004]), while the COD for potato wash water can be in the range of 18,000 mg/L of organic content (Sayed

et al. 2005). Thus, the range of COD selected for this study encompasses a wide range of produce washing scenarios.

Inactivation of *E. coli* by Photosensitized RB: Effect of Bacterial Phase and Organic Load

We tested the efficacy of the visible light-sensitized RB against the Gram-negative bacterium *E. coli* BL21 in two different phases of its growth, i.e., stationary- and exponential-phase growths.

Figures 1 and 2 show inactivation of *E. coli* in the stationary and exponential phases upon exposure to photosensitized RB as a function of RB concentration and organic load. In the presence of RB or light alone, less than 1 log CFU/mL reduction in bacterial load was observed in all the cases, indicating
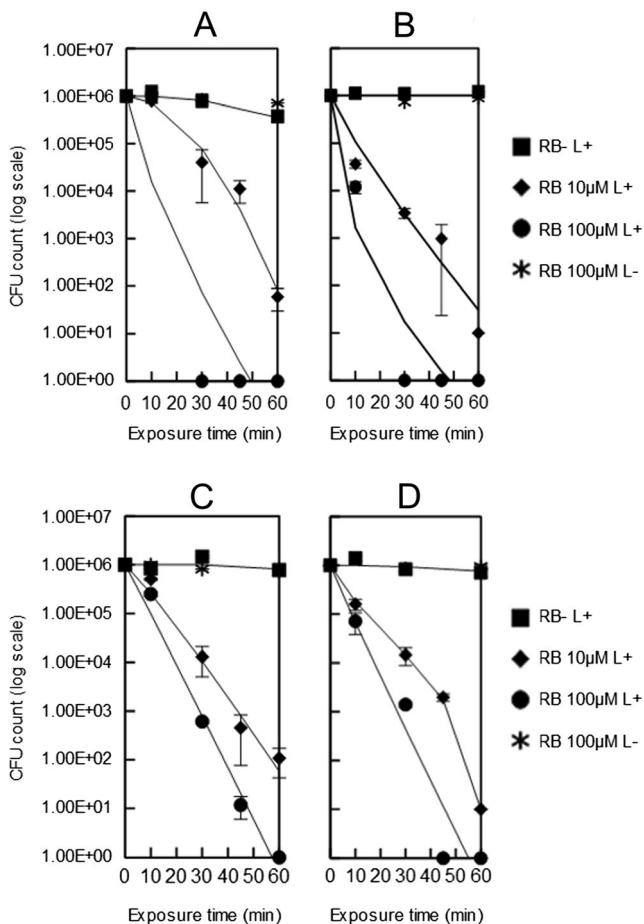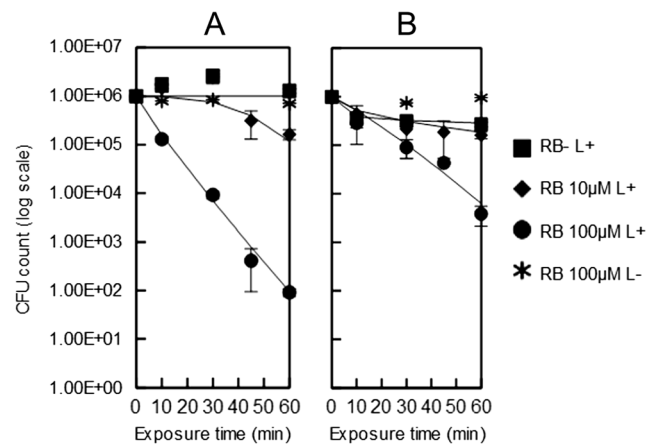
Fig. 2 Rose Bengal photosensitization antimicrobial assay against *E. coli* cells in very high organic content. Antimicrobial assays against stationary- (**a**) and exponential-phase (**b**) bacteria were conducted with 10 or 100 μM of RB in water solutions with 20,000 ppm of LB in the system as organic content. Standardized results on 1×10^6 CFU/mL were plotted in a log scale as average ± SD. The *icons* and the *curves* show the experimental values and the Weibull model's predicted values, respectively

little or no individual effect of the PS or light treatment on the bacterial population. However, significant microbial inactivation was observed when RB and light were both present. At the lowest organic load of 200 ppm and the highest RB concentration of 100 μM, a complete, 6 log CFU/mL inactivation for both the stationary- and exponential-phase bacteria was observed after 30 min of exposure to the light. At a lower RB concentration of 10 μM and 200 ppm organic content, only 1.39 ± 0.50 and 2.47 ± 0.10 log CFU/mL reductions after 30 min were observed for the stationary- and exponential-phase bacteria, respectively. These results indicate that the antimicrobial effect was dependent on the concentration range of RB. A qualitatively similar trend was observed at higher organic loads as well, further confirming that the antimicrobial effect of the PS in the presence of visible light excitation was concentration dependent.

Inactivation of the stationary-phase bacteria by 100 μM RB after 30 min of exposure was 6 log, 3.21 ± 0.03 log, and 2.03 ± 0.07 log CFU/mL in the presence of 200, 2000, and 20,000 ppm LB, respectively (Figs. 1 and 2). Thus, the extent of inactivation was significantly affected by the concentration of organic load ($p < 0.0001$).

Similarly, inactivation of exponential-phase bacteria by photosensitized RB at 100 μM was limited to a 2.41 ± 0.21 log CFU/mL reduction in 60 min in the presence of a very high organic content (20,000 ppm of LB) (Fig. 2). For an organic content of 2000 ppm, a logarithmic reduction of 5 and 6 log CFU/mL was achieved for the exponential-phase bacteria incubated with 10 and 100 μM concentrations of RB, respectively (Fig. 1).

Inactivation kinetics of the logarithmic and stationary-phase bacteria by visible light-irradiated RB was fitted using

Fig. 1 Rose Bengal photosensitization antimicrobial assay against *E. coli* cells in low and high organic content. Antimicrobial assays against stationary- (**a** and **c**) and exponential-phase (**b** and **d**) bacteria were conducted with 10 or 100 μM of RB in water solutions with 200 ppm (**a** and **b**) or 2000 ppm (**c** and **d**) of LB in the system as organic content. Experimental controls without illumination (RB 100 μM L−) or without RB (RB− L+) added were also performed. Standardized results on 1×10^6 CFU/mL were plotted in a log scale as average ± SD. The *icons* and the *curves* show the experimental values and the Weibull model's predicted values, respectively

the Weibull model (Table 1). Low MAE and RMSE based on the comparison of the experimental and fitted data indicated that the Weibull model described the inactivation kinetics. The reliable life (t_R) value, which explains the amount of time required for the first log CFU/milliliter reduction, was calculated from the Weibull model constants, α (characteristic time) and β (shape parameter) (van Boekel 2002). As the organic content increased from 200 ppm to 2000 and 20,000 ppm in the presence of 10 μM RB under visible light, the t_R for exponential-phase bacteria increased by 1.99- and 10.25-fold. An increase in the RB concentration to 100 μM resulted in a decrease in t_R values by 3.55-, 2.41-, and 8.22-fold at 200, 2000, and 20,000 ppm, respectively, as compared to t_R values for bacteria incubated with 10 μM RB. Similarly, the t_R increased as the organic content increased for the stationary-phase bacteria.

Comparison between the t_R values for the exponential- and stationary-phase bacteria shows that at low organic content, the t_R values were lower for exponential-phase bacteria than stationary-phase bacteria, indicating a lower resistance of exponential-phase bacteria. At a higher organic content of 2000 ppm, the t_R values for the exponential- and stationary-phase bacteria were similar, while at the highest levels of organic content (20,000 ppm), the t_R values for the stationary-phase bacteria were significantly lower than those for the exponential-phase bacteria. These results also indicate that the stationary-phase microbes were more resistant to PS treatment as compared to the exponential-phase microbes except in the case of the highest organic content (LB 20,

000 ppm) (Fig. 2). In this specific case, we observed that the reduction in microbial count for the exponential phase was significantly lower than for the stationary phase. This difference in resistance between the stationary and exponential phases for the case of very high organic content can be attributed to the potential growth of exponential-phase bacteria during the treatment interval of 1 h in a nutrient-rich broth.

The PCCs reported in Table 2 show that the number of surviving E. coli after PS treatment at both the selected levels of RB concentration was positively and strongly correlated (correlation factor > 0.9; p value < 0.05) with the concentration of organic content. This implies that organic content has a strong protective effect during PS treatment. On the other hand, Table 3 shows that the survival population of E. coli was inversely and moderately correlated (absolute magnitude of correlation factor < 0.7 and p value > 0.05) with the concentration of RB in the presence of organic content at the levels of 200 and 2000 ppm of LB. However, when the organic content was increased to 20,000 ppm, the number of surviving E. coli was inversely and strongly correlated with the RB concentration for the exponential-phase bacteria, but the same correlation factor strength was not significantly influenced for the stationary-phase bacteria. These results indicate that increase in RB concentration can counter the influence of organic content at high levels for the exponential-phase bacteria, but the influence of RB concentration is moderate for the stationary-phase microbes. These results further highlight the significance of bacterial resistance to oxidative stress as a function of physiological state in influencing the outcome of PS treatment.

Table 1 The Weibull model constants and goodness of fit for the inactivation experiments of E. coli and T7 bacteriophage

	Organic content	200 ppm		2000 ppm		20,000 ppm	
	Rose Bengal	10 μm	100 μM	10 μm	100 μM	10 μm	100 μM
E. coli BL21 exponential phase	α	3.9	0.22	11.56	3.52	22.43	14.94
	β	0.86	0.48	1.5	1	0.5	1.2
	t_R	10.28	1.25	20.47	8.47	108.24	30.47
	MAE	0.2	0.01	0.1	0.1	0.3	0.2
	RMSE	0.32	0.72	0.36	0.57	0.4	0.37
E. coli BL21 stationary phase	α	18.27	1.5	7.56	4.38	46.24	5.05
	β	1.88	0.75	1.1	1.02	2.93	0.89
	t_R	28.47	4.56	16.13	9.92	61.47	12.89
	MAE	0.04	0.11	0.02	0.07	0.23	0.14
	RMSE	0.19	1.26	0.21	0.22	0.33	0.29
T7 bacteriophage	α	0.28	0.07	0.6	2.24	32.82	17.68
	β	0.52	0.4	0.55	0.94	1.88	0.59
	t_R	1.39	0.56	2.73	5.44	51.15	72.7
	MAE	0.01	0.09	0.11	0.06	0.05	0.18
	RMSE	0.01	0.21	0.19	0.14	0.12	0.31

The constants and goodness of fit are shown for exponential and stationary phase E. coli BL21 and T7 bacteriophage antimicrobial studies. Parameters shown are α = characteristic time (min), β = shape parameter, t_R = reliable life, RMSE = root mean square error, and MAE = mean absolute error

Table 2 The Pearson correlation factors between the survived population of *E. coli* or *T7* bacteriophage and organic content

	10 μM Rose Bengal	100 μM Rose Bengal
E. coli BL21 exponential phase	0.984*	0.909*
E. coli BL21 stationary phase	0.971*	0.971*
T7 bacteriophage	0.935*	0.834*

**p* value 0.05

Overall, the bacterial photosensitization data showed how the highest concentration of RB tested in this study (100 μM) resulted in up to 6 log CFU/mL reduction at COD ~1900 mg/L both for the exponential- and stationary-phase bacteria after 1 h of photosensitization treatment. However, the 6 log CFU/mL reduction was achieved in the exponential-phase bacteria within 45 min of light exposure. These results indicate that the stationary-phase bacteria have higher resistance to photosensitization than exponential-phase bacteria at high (2000 ppm) and low (200 ppm) concentrations of LB as organic content. With a RB concentration of 100 μM, approximately 3 log CFU/mL reduction in bacteria can be achieved in 30 min for both exponential- and stationary-phase bacteria in the presence of organic content ~1900 mg/L COD. Reduction in microbial load in the presence of organic content achieved with light activation of RB is similar to the microbial reductions achieved using SH and H_2O_2 with similar order of magnitude incubation periods (Florkowski et al. 2014).

Diffusion, Recovery, and Detection of Internalized Rose Bengal in *E. coli*

Experiments involving absorption and internalization of RB by *E. coli* were performed with either 10 or 100 μM RB for 0, 30, and 60 min in the presence of 2000 ppm of LB. Figure 3 shows the corrected absorbance from RB internalized by the bacterial cells in the stationary (Fig. 3a) and exponential phases (Fig. 3b), respectively. A gradual increase in absorbance as a function of RB concentration and incubation time suggested that RB was internalized by bacterial cells. Although there was an increase in both the rate of uptake of RB dye and the total amount of RB in bacterial cells (both the

Table 3 The Pearson correlation factors between the survived population of *E. coli* or *T7* bacteriophage and Rose Bengal concentration

	200 ppm LB	2000 ppm LB	20,000 ppm LB
E. coli BL21 exponential phase	−0.577	−0.570	−0.931*
E. coli BL21 stationary phase	−0.560	−0.574	−0.664
T7 bacteriophage	−0.576	−0.573	−0.695*

**p* value 0.05

exponential and stationary phases) with increasing concentration of RB in the extracellular phase, the increase in the total amount of RB dye uptaken within the cells was limited. It was observed that with a tenfold increase in RB concentration, approximately threefold increase in the RB concentration was observed in both the exponential- and stationary-phase bacteria. This result indicates that the passive diffusion of RB dye in bacteria was constrained.

The results demonstrate the total intracellular uptake of the RB dye after 60 min of incubation was higher in the case of the stationary-phase microbes compared to the exponential-phase ones. Additionally, the rates of uptake in both the stationary-phase and exponential-phase bacteria were a function of RB concentration present in the extracellular medium. In the stationary-phase bacteria incubated with 10 μM RB, the difference in the intracellular concentration of RB dye compared to the control was only detected after 60 min of incubation. In the case of the exponential phase under an identical set of conditions, this difference in concentration could be detected after 30 min of incubation. Upon incubation of bacteria (stationary or exponential phase) with 100 μM RB concentration, statistically significant differences in intracellular concentration of RB could be detected at 30 min of incubation compared to the control.

The higher accumulated levels of RB in cells in the stationary-phase bacteria can be explained based on a combination of altered osmotic pressure (Koch 1985) and increased thickness of the cell envelope in the stationary-phase bacteria compared to the exponential-phase bacteria (Leduc et al. 1989). This combination of factors may increase the permeation and/or reduce the leaching of RB during the subsequent washing step. To complement spectroscopic measurements and provide a direct evidence of internalization of RB within bacterial cells, we also performed fluorescence imaging of bacteria (Fig. 3c, d). Consistent with spectroscopic measurements, an increase in fluorescence within bacteria can be observed upon incubation with RB in both stationary and exponential phases. These results further confirm that at least a fraction of RB was internalized within bacteria.

Previous experiments based on spectroscopic studies using quenching of RB have indicated localization of RB to the outer membrane of bacterial cells, although no direct imaging observation was reported (Dahl et al. 1989). Fluorescence imaging results from this study (Fig. 3) indicate an increased internalization of RB in the stationary-phase bacteria compared to the exponential-phase bacteria. This trend is similar to the differences in RB uptake between stationary and exponential bacteria measured using spectroscopy. Since the cells were washed prior to imaging, it is possible that the RB bound to the outer membrane of the cell was removed during the washing and re-suspension steps.

Surprisingly, an increased amount of internalized RB in the stationary-phase bacteria during the diffusion test did not

Fig. 3 Rose Bengal absorption by *E. coli* in stationary- or exponential-phase growth bacteria. Graphs show the absorbance at 549 nm of clarified lysates of *E. coli* after diffusion of 10 or 100 μM of RB in stationary- (**a**) or exponential-phase (**b**) bacteria. The amounts of RB (μg) recovered at each time point from 1×10^8 CFU are shown on the *top* of each *time point* analyzed. Microscope fluorescence images show stationary-phase bacteria (**c**) and exponential-phase bacteria (**d**) after 30 min of incubation with or without 100 μL of RB. Images were captured in brightfield (BF) or with rhodamine isothiocyanate (TRITC) filter
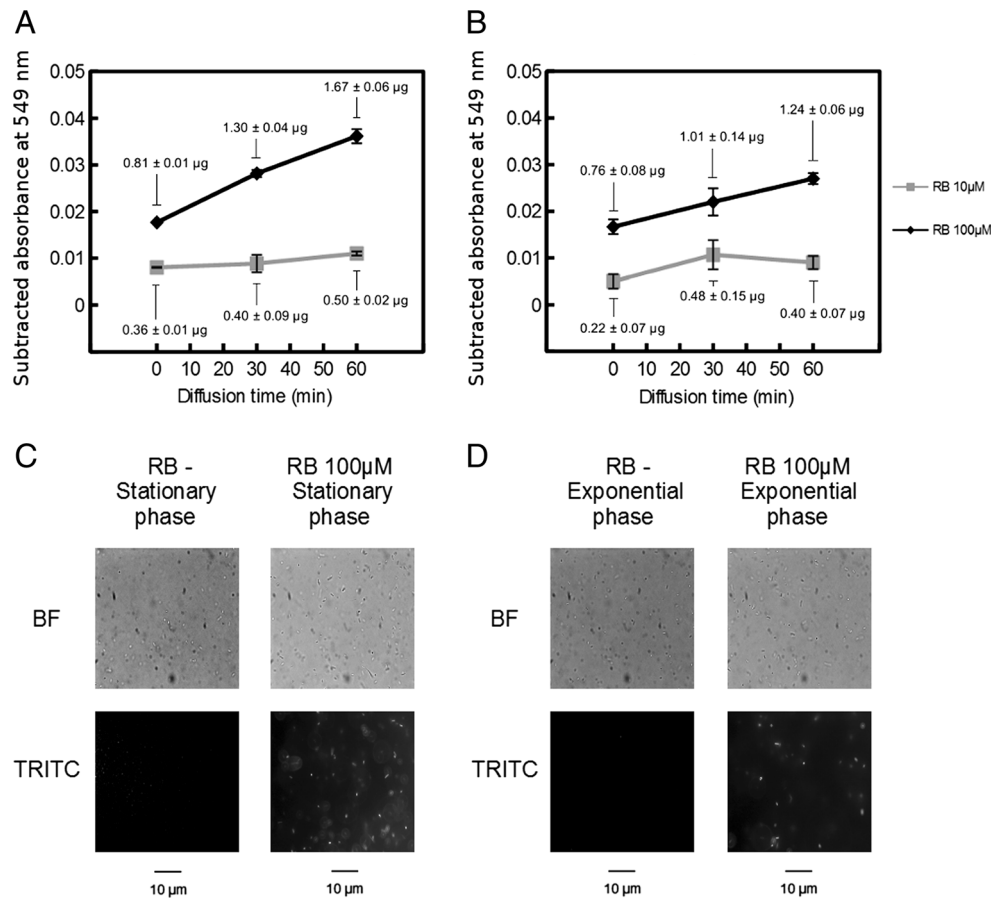
correlate with the enhanced antimicrobial activity. This may be due to the fact the stationary-phase bacteria are more resistant to PS compared to the exponential-phase bacteria. Also, it is possible that a significant antimicrobial activity of RB is the result of generation of ROS in the extracellular space including the increased absorption of the dye in the outer membrane, especially since a relatively large concentration of RB is located in the extracellular space as compared to the intracellular fraction. Based on this, we hypothesize that in addition to the ROS that may be generated by internalized RB molecules, ROS generated in the extracellular medium may exert antimicrobial effect by damaging cell membranes and intracellular content.

Measurement of Nucleic Acid and Protein Leakage upon Rose Bengal Photosensitization

To assess the damage induced on bacterial cell membranes by photosensitized RB, leakage of nucleic acids and proteins was detected after 1 h of treatment. Supernatants of pelleted culture were analyzed for spectrophotometric absorbance readings at 260 and 280 nm. Figure 4 shows the results of leaked nucleic acid and protein molecules from the stationary- and exponential-phase bacteria. After 1 h of photosensitization treatment using RB at 100 μM concentration, no differences in the concentration of leaked nucleic acids and proteins were

detected between the stationary- and exponential-phase bacteria samples. This is expected as complete reduction in microbial load was achieved at higher concentration levels of RB. At a lower concentration of 10 μM, the exponential-phase bacteria showed a slightly higher but statistically significant relative concentration of leaked nucleic acid ($t = 5.6822$; df = 4; $p = 0.0047$) and proteins ($t = 4.3198$; df = 4; $p = 0.0124$) compared to the stationary-phase bacteria. These results support the observations reported in Fig. 1, indicating higher susceptibility of the exponential-phase microbes to PS treatment.

This antimicrobial mechanism can be explained by the formation of singlet oxygen species upon photoactivation of RB (Yesuthangam et al. 2011). Singlet oxygen species are known to induce damage to cell membrane (Schäfer et al. 1998) and also induce DNA damage (Di Mascio et al. 1989). Based on this understanding, we can conclude that the antimicrobial activity of RB results from both damage to the cell wall as well as nucleic acids. This is supported by the experimental evidence in this study that demonstrates increased resistance of the stationary-phase bacteria compared to the exponential-phase bacteria. Thicker cell wall and membrane (Makinoshima et al. 2003) and condensed DNA structure can provide a higher protection against the ROS-induced damage in the stationary-phase bacteria (Martinez and Kolter 1997) and may result in increased resistance to photosensitization.
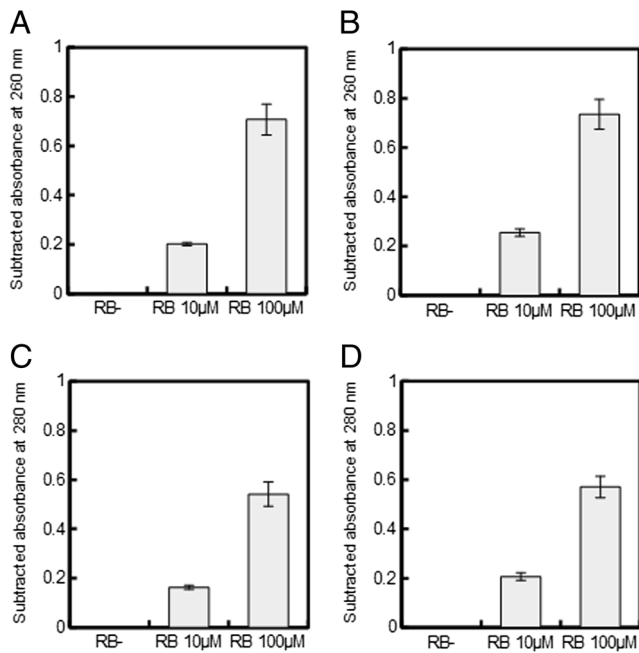
Fig. 4 Membrane damage and leakage of nucleic acids and proteins from *E. coli* upon photosensitization with Rose Bengal. Experiments of photosensitization were carried out on stationary- (*left*) or exponential-phase (*right*) bacteria in a system with an organic content given by 2000 ppm of LB. Leakage experiments involved the analyses of supernatant recovered from stationary (**a** and **c**) or exponential (**b** and **d**) bacteria photosensitized for 60 min. Absorbances at 260 nm (**a** and **b**) and 280 nm (**c** and **d**) were subtracted by the same wavelength readings of supernatant derived by 1×10^8 CFU/mL without RB added

Antiviral Effect of Rose Bengal Photosensitization

Antiviral efficacy of photosensitized RB was evaluated using *T7* bacteriophage as a model DNA phage. We observed that in 30 min, a 3.58 ± 0.29 and 5 log PFU/mL reduction was achieved, respectively, with 10 and 100 μM of RB in the presence of 2000 ppm of simulated organic content (Fig. 5). Comparison of results in Figs. 5 and 1 shows that viral

particles are more susceptible to photosensitization compared to bacteria.

The viral inactivation by visible light-irradiated RB was described by the Weibull model (Table 1). The MAE and RMSE calculated by comparing the fitted data and experimental data were close to 0. The t_R, which represents the time for first log PFU/mL reduction, was calculated from the Weibull model constants. The t_R increased by 1.96- and 36.79-fold as the organic content increased from 200 to 2000 and 20, 000 ppm of LB, respectively. The increase in the RB concentration from 10 to 100 μM caused a 2.48-fold decrease in the t_R in the presence of 200 ppm of LB. However, at higher concentrations of LB, increase in the RB concentration did not lower the t_R.

Table 2 shows that at both selected levels of RB concentration, the organic content concentration was positively and strongly correlated with the survival population of T7 bacteriophage (*p* value   0.05). On the other hand, the results in Table 3 show that increase in RB concentration was only moderately and inversely correlated with the survival population of T7 phage after PS treatment at all three levels of organic content selected in this study.

An analysis of the results reported in Figs. 5 and 1 indicates that viral particles are more susceptible to photosensitization compared to bacteria. A concentration of 100 μM RB in the presence of a high organic content (2000 ppm LB) resulted in 5 log PFU/mL reduction of viral particles after 30 min of visible light exposure, while in the same organic load, both exponential- and stationary-phase bacteria were reduced only by 3 log CFU/mL after 30 min of treatment. Following the same trend, after 30 min of exposure in the presence of a low organic content of 200 ppm LB, bacteria were inactivated by less than 3 log CFU/mL in both growth phases at the lowest concentration of RB used (10 μM). On the other hand, with the same organic content and RB concentration, viral particles were inactivated by at least 5 log PFU/mL.
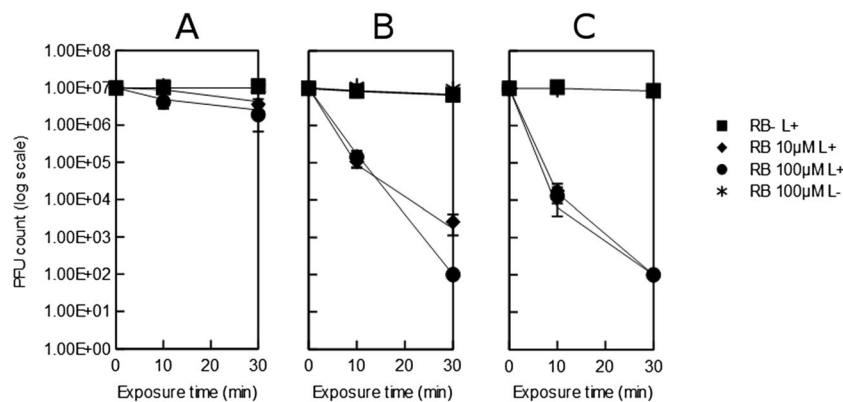

Fig. 5 Rose Bengal photosensitization antimicrobial assay against *T7* phage in water solution. The antiviral assay was conducted with 10 or 100 μM of RB in water solutions with 20,000 (**a**), 2000 (**b**), or 200 ppm (**c**) of LB in the system. Controls without illumination or without RB added were also performed. The *icons* and the *curves* show the experimental values and the Weibull model's predicted values, respectively

Viral particles have already been shown as more susceptible to photosensitization treatments than bacteria (Wagner et al. 2005). Hotze and colleagues suggested that singlet oxygen may induce crosslinking of capsid proteins in bacteriophages, resulting in a direct impairment of the phages' ability to bind to the host surface (Hotze et al. 2009). This study shows that RB is a powerful PS for the inactivation of viral particles. Since singlet oxygen has been indicated as the ROS produced upon photosensitization of RB (Yadav et al. 2010), the results of this work may strengthen the role of singlet oxygen as the leading source of oxidative stress during photodynamic treatment of viral particles.

Conclusions

This study was aimed at evaluating the potential of photosensitized Rose Bengal (RB) dye on the inactivation of bacteria and viruses in the presence of organic content. The effect of light exposure duration, the concentration of the RB dye, and the physiological state of the cells on the inactivation rate of both bacterial and viral model systems was determined. The study also evaluated the potential mechanisms for the antimicrobial activity of photosensitized RB based on its internalization within the bacteria and its ability to cause membrane damage. The results of this study demonstrate that photosensitized RB can be an effective approach to reduce bacterial and viral loads in the presence of wash water. The study also highlights the differences in resistance between the stationary- and exponential-phase microbes and delineates a significant role of membrane damage in PS-induced inactivation of the bacteria. The results also reflect upon the challenges introduced by organic content in reducing the efficacy of PS treatment. These challenges may result by the scavenging of the dye molecule or the radical species generated by the dye.

References

Agostinis, P., Berg, K., Cengel, K. A., Foster, T. H., Girotti, A. W., Gollnick, S. O., et al. (2011). Photodynamic therapy of cancer: an update. *CA: a Cancer Journal for Clinicians, 61*(4), 250–281.

Bachelli, M. L. B., Amaral, R. D. Á., & Benedetti, B. C. (2014). Alternative sanitization methods for minimally processed lettuce in comparison to sodium hypochlorite. *Brazilian Journal of Microbiology, 44*(3), 673–678.

Bialka, K. L., Demirci, A., & Puri, V. M. (2008). Modeling the inactivation of *Escherichia coli* O157:H7 and *Salmonella enterica* on raspberries and strawberries resulting from exposure to ozone or pulsed UV-light. *Journal of Food Engineering, 85*(3), 444–449.

Blom, E.-J., Ridder, A. N. J. A., Lulko, A. T., Roerdink, J. B. T. M., & Kuipers, O. P. (2011). Time-resolved transcriptomics and bioinformatic analyses reveal intrinsic stress responses during batch culture of *Bacillus subtilis*. *PloS One, 6*(11), e27160.

Calzavara-Pinton, P. G., Venturini, M., & Sala, R. (2005). A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin. *Journal of Photochemistry and Photobiology, B, Biology, 78*(1), 1–6.

Cherchi, C., & Gu, A. Z. (2011). Effect of bacterial growth stage on resistance to chlorine disinfection. *Water Science and Technology, 64*(1), 7–13.

Cieplik, F., Tabenski, L., Buchalla, W., & Maisch, T. (2014). Antimicrobial photodynamic therapy for inactivation of biofilms formed by oral key pathogens. *Frontiers in Microbiology.* doi:10.3389/fmicb.2014.00405.

Cronin, L., Moffitt, M., Mawad, D., Morton, O. C., Lauto, A., & Stack, C. (2014). An in vitro study of the photodynamic effect of rose bengal on *Trichophyton rubrum*. *Journal of Biophotonics, 7*(6), 410–417.

Dahl, T. A., Valdes-Aguilera, O., Midden, W. R., & Neckers, D. C. (1989). Partition of rose bengal anion from aqueous medium into a lipophilic environment in the cell envelope of *Salmonella typhimurium*: implications for cell-type targeting in photodynamic therapy. *Journal of Photochemistry and Photobiology B, Biology, 4*(2), 171–184.

Di Mascio, P., Wefers, H., Do-Thi, H. P., Lafleur, M. V., & Sies, H. (1989). Singlet molecular oxygen causes loss of biological activity in plasmid and bacteriophage DNA and induces single-strand breaks. *Biochimica et Biophysica Acta, 1007*(2), 151–157.

EFSA Panel on Food Additives and Nutrient Sources added to Food (2011). Scientific opinion on the re-evaluation of erythrosine (E 127) as a food additive. *EFSA Journal, 9*(1), 1854.

Fawell, J. (2000). Risk assessment case study—chloroform and related substances. *Food and Chemical Toxicology.* doi:10.1016/S0278-6915(99)00129-5.

FDA (2003). Color additives: FDA's regulatory process and historical perspectives. WebContent. http://www.fda.gov/ForIndustry/ColorAdditives/RegulatoryProcessHistoricalPerspectives/. Accessed 24 July 2015

Florkowski, W. J., Shewfelt, R. L., Brueckner, B., & Prussia, S. E. (2014). *Postharvest handling: a systems approach*. New York: Academic Press.

Gianotti, E., Martins Estevão, B., Cucinotta, F., Hioka, N., Rizzi, M., Renò, F., & Marchese, L. (2014). An efficient rose bengal based nanoplatform for photodynamic therapy. *Chemistry (Weinheim an Der Bergstrasse, Germany), 20*(35), 10921–10925.

Gomez-Lopez, V. M. (2012). *Decontamination of fresh and minimally processed produce*. Oxford, UK: John Wiley & Sons.

Gonzalez, R. J., Luo, Y., Ruiz-Cruz, S., & McEvoy, J. L. (2004). Efficacy of sanitizers to inactivate *Escherichia coli* O157:H7 on fresh-cut carrot shreds under simulated process water conditions. *Journal of Food Protection, 67*(11), 2375–2380.

Han, J.-H., Wang, M. S., Das, J., Sudheendra, L., Vonasek, E., Nitin, N., & Kennedy, I. M. (2014). Capture and detection of *T7* bacteriophages on a nanostructured interface. *ACS Applied Materials & Interfaces, 6*(7), 4758–4765.

Hotze, E. M., Badireddy, A. R., Chellam, S., & Wiesner, M. R. (2009). Mechanisms of bacteriophage inactivation via singlet oxygen generation in UV illuminated fullerol suspensions. *Environmental Science & Technology, 43*(17), 6639–6645.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., & Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters, 230*(1), 13–18.

Kim, H., Kim, W., Mackeyev, Y., Lee, G.-S., Kim, H.-J., Tachikawa, T., et al. (2012). Selective oxidative degradation of organic pollutants by singlet oxygen-mediated photosensitization: tin porphyrin versus C60 aminofullerene systems. *Environmental Science & Technology*, *46*(17), 9606–9613.

Koch, A. L. (1985). How bacteria grow and divide in spite of internal hydrostatic pressure. *Canadian Journal of Microbiology*, *31*(12), 1071–1084.

Kumar, V., Lockerbie, O., Keil, S. D., Ruane, P. H., Platz, M. S., Martin, C. B., et al. (2004). Riboflavin and UV-light based pathogen reduction: extent and consequence of DNA damage at the molecular level. *Photochemistry and Photobiology*, *80*, 15–21.

Le, P., Zhang, L., Lim, V., McCarthy, M. J., & Nitin, N. (2015). A novel approach for measuring resistance of Escherichia coli and Listeria monocytogenes to hydrogen peroxide using label-free magnetic resonance imaging and relaxometry. *Food Control*, *50*, 560–567.

Leduc, M., Fréhel, C., Siegel, E., & Van Heijenoort, J. (1989). Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli*. *Journal of General Microbiology*, *135*(5), 1243–1254.

Lopes, D., Melo, T., Santos, N., Rosa, L., Alves, E., Clara Gomes, M., et al. (2014). Evaluation of the interplay among the charge of porphyrinic photosensitizers, lipid oxidation and photoinactivation efficiency in *Escherichia coli*. *Journal of Photochemistry and Photobiology B: Biology*, *141*, 145–153.

Luo, Y. (2007). Fresh-cut produce wash water reuse affects water quality and packaged product quality and microbial growth in romaine lettuce. *Hortscience*, *42*(6), 1413–1419.

Makinoshima, H., Aizawa, S.-I., Hayashi, H., Miki, T., Nishimura, A., & Ishihama, A. (2003). Growth phase-coupled alterations in cell structure and function of *Escherichia coli*. *Journal of Bacteriology*, *185*(4), 1338–1345.

Martinez, A., & Kolter, R. (1997). Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. *Journal of Bacteriology*, *179*(16), 5188–5194.

Palma-Salgado, S., Pearlstein, A. J., Luo, Y., Park, H. K., & Feng, H. (2014). Whole-head washing, prior to cutting, provides sanitization advantages for fresh-cut iceberg lettuce (*Latuca sativa* L.). *International Journal of Food Microbiology*, *179*, 18–23.

Panzarini, E., Inguscio, V., Fimia, G. M., & Dini, L. (2014). Rose Bengal acetate photodynamic therapy (RBAc-PDT) induces exposure and release of damage-associated molecular patterns (DAMPs) in human HeLa cells. *PloS One*, *9*(8), e105778.

Qi, H., Kawagishi, M., Yoshimoto, M., Takano, H., Zhu, B., Shimoishi, Y., et al. (2010). Artificial food colorants inhibit superoxide production in differentiated *HL-60* cells. *Bioscience, Biotechnology, and Biochemistry*, *74*(8), 1725–1728.

Sayed, S. K., El-Ezaby, K. H., & Groendijk, L. (2005). Treatment of potato processing wastewater using a membrane bioreactor. In *Proceedings of the 9 Th international water technology conference, IWTC9-2005*. Sharm El-Sheikh: Egypt.

Schäfer, M., Schmitz, C., & Horneck, G. (1998). High sensitivity of *Deinococcus radiodurans* to photodynamically-produced singlet oxygen. *International Journal of Radiation Biology*, *74*(2), 249–253.

Schneider, J. E., Phillips, J. R., Pye, Q., Maidt, M. L., Price, S., & Floyd, R. A. (1993). Methylene blue and rose bengal photoinactivation of RNA bacteriophages: comparative studies of 8-oxoguanine formation in isolated RNA. *Archives of Biochemistry and Biophysics*, *301*(1), 91–97.

Shen, C., Luo, Y., Nou, X., Wang, Q., & Millner, P. (2013). Dynamic effects of free chlorine concentration, organic load, and exposure time on the inactivation of *Salmonella*, *Escherichia coli* O157:H7, and Non-O157 Shiga toxin-producing *E. coli*. *Journal of Food Protection*, *76*(3), 386–393.

Sun, S. H., Kim, S. J., Kwak, S. J., & Yoon, K. S. (2012). Efficacy of sodium hypochlorite and acidified sodium chlorite in preventing browning and microbial growth on fresh-cut produce. *Preventive Nutrition and Food Science*, *17*(3), 210–216.

Valkov, A., Nakonechny, F., & Nisnevitch, M. (2014). Polymer-immobilized photosensitizers for continuous eradication of bacteria. *International Journal of Molecular Sciences*, *15*(9), 14984–14996.

Van Boekel, M. A. J. S. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology*, *74*(1–2), 139–159.

Van Haute, S., Sampers, I., Holvoet, K., & Uyttendaele, M. (2013). Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Applied and Environmental Microbiology*, *79*(9), 2850–2861.

Wagner, S. J., Skripchenko, A., Donnelly, D. J., Ramaswamy, K., & Detty, M. R. (2005). Chalcogenoxanthylium photosensitizers for the photodynamic purging of blood-borne viral and bacterial pathogens. *Bioorganic & Medicinal Chemistry*, *13*(21), 5927–5935.

Weller, L. D., Daeschel, M. A., Durham, C. A., & Morrissey, M. T. (2013). Effects of water, sodium hypochlorite, peroxyacetic acid, and acidified sodium chlorite on in-shell hazelnuts inoculated with *Salmonella enterica* serovar *Panama*. *Journal of Food Science*, *78*(12), M1885–M1891.

Yadav, D. K., Kruk, J., Sinha, R. K., & Pospíšil, P. (2010). Singlet oxygen scavenging activity of plastoquinol in photosystem II of higher plants: electron paramagnetic resonance spin-trapping study. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1797*(11), 1807–1811.

Yesuthangam, Y., Pandian, S., Venkatesan, K., Gandhidasan, R., & Murugesan, R. (2011). Photogeneration of reactive oxygen species and photoinduced plasmid DNA cleavage by novel synthetic chalcones. *Journal of Photochemistry and Photobiology B: Biology*, *102*(3), 200–208.

Yousef, A. E., & Juneja, V. K. (2002). *Microbial stress adaptation and food safety*. Boca Raton, FL: CRC Press.