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Inactivation by 405 ± 5 nm light emitting diode on *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Shigella sonnei* under refrigerated condition might be due to the loss of membrane integrity

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ABSTRACT

The objective of this study was to evaluate the antibacterial effect of 405 ± 5 nm light emitting diode (LED) on *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Shigella sonnei*. Its antibacterial mechanism was also investigated by determining the permeability of bacterial membrane and DNA degradation. Bacterial strains in phosphate-buffered saline were exposed to 405 ± 5 nm LED to a final dose of 486 J/cm^2 (7.5 h) at 4 °C. The inactivation curves were fitted by Weibull model to compare the sensitivities of pathogens to the LED illumination by calculating the decimal reduction times (t_R). The bacterial sensitivity to bile salts and NaCl by LED illumination was also determined. LIVE/DEAD® *Bac*Light™ staining as well as comet assay and DNA ladder analysis were carried out to determine the bacterial membrane integrity and DNA degradation, respectively. Results showed that LED illumination inactivated 1.0, 2.0, and 0.8 log CFU/ml for *E. coli* O157:H7, *S.* Typhimurium, and *S. sonnei* for 7.5 h, respectively. The comparison of t_R values demonstrated that *S.* Typhimurium was found to be the most ($P < 0.05$) susceptible strain to LED illumination. Regardless of the bacterial strain, the sensitivity of illuminated bacterial cells to bile salts and NaCl considerably increased compared to non-illuminated controls. Furthermore, LIVE/DEAD® assay clearly showed that LED illumination resulted in loss of bacterial membrane permeability. On the other hand, no DNA degradation was observed by both comet assay and DNA ladder analysis. Therefore, these results suggest that the antibacterial effect of 405 ± 5 nm LED might be partly attributed to the physical damage to bacterial cell membrane. This study proposes that 405 ± 5 nm LED under refrigerated conditions may be effective to control the pathogens on foods.

1. Introduction

Consumption of foods contaminated with infectious levels of bacterial pathogens cause serious illness in humans. According to the data estimated by the United States Centers for Disease Control and Prevention (US CDC), a total of 48 million cases of infections, 127,839 hospitalization 3,037 deaths were caused by major known pathogens and unspecified agents transmitted via food every year in US. The number of infections caused by three major Gram-negative pathogens was as follows: 1 million by *Salmonella*, 131,254 by *Shigella*, 112,752 by non-O157 Shiga toxin-producing *Escherichia coli* (STEC), and 63,153 by O157 STEC (Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011; Scallan, Hoekstra, et al., 2011). It was also estimated that annual health related costs due to foodborne disease in US range from $51 billion to $77.7 billion (Scharff, 2012). In Singapore, the Ministry of Health (MOH) estimates that about 0.1 million people per annum seek medical care due to acute diarrheal illnesses (MOH, 2010). Among causative agents, non-typhoidal *Salmonella* spp. have been identified as major pathogenic bacteria to cause foodborne illness, followed by *Campylobacter*, Hepatitis A and E viruses, and *Shigella* spp. during the last decade (Kondakci & Yuk, 2012).

To inhibit or inactivate these pathogenic bacteria on food products during storage, food processors and handlers have

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manipulated intrinsic or extrinsic factors such as temperature, pH, water activity, and antimicrobial agents (Lim, Kim, Lee, & Yuk, 2013; Yuk & Geveke, 2011). Among these, the most widely used preservation technique is cold storage; however, some pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella* spp. are able to survive at refrigeration temperature during transportation and storage. For this reason, refrigeration should not be used as the sole preservation method. Therefore, to ensure food safety and to extend the shelf-life of perishable foods such as ready-to-eat foods, an additional hurdle with refrigeration should be developed and employed for better food preservation without a loss of the food quality (Ghate et al., 2013; Lim et al., 2013).

Ultraviolet (UV) light is able to inactivate microorganisms on the surfaces of foods during storage. However, UV light leads to decolourization in certain products at high dose as well as has harmful effects on the skin tissue and eyes of the operator, resulting in the limitation of UV light as a preservation technology in food industry (Maclean, MacGregor, Anderson, & Woolsey, 2009; Murdoch, Maclean, MacGregor, & Anderson, 2010). To overcome these shortcomings of UV light, light emitting diodes (LED) of visible wavelengths have been investigated as an alternative. A LED, as a semiconductor device, has the capability of emitting visible light within a very narrow wavelength spectrum, resulting in nearly monochromatic light. LEDs have several advantages such as lower energy consumption, high durability, reduced heat output, and long life compared to traditional visible light sources (Ghate et al., 2013; Mori et al., 2007). Also, LEDs can be fabricated in small sizes and various shapes, which could be applied to most designs (Ghate et al., 2013; Mori et al., 2007).

Previous studies have shown that bacteria can be photodynamically inactivated by visible light illumination, especially in the wavelength range of 400–420 nm (Endarko, Maclean, Timoshkin, MacGregor, & Anderson, 2012; Maclean et al., 2009). During photodynamic inactivation (PDI), bacterial cells are exposed to the energy of light, which leads to the excitation of photosensitizers such as either exogenous or endogenous porphyrin molecules. Reactive oxygen species (ROS) are produced, once these porphyrin compounds absorb visible light of 400–420 nm in the presence of oxygen (Lukšiene, 2003, 2005; Lukšiene & Zukauskas, 2009). The ROS such as singlet oxygen, superoxide anion, and the hydroxyl radical may damage membrane lipids, enzymes, proteins, or DNA, consequently inducing bacterial death (Ghate et al., 2013; Lukšiene, 2005; Lukšiene & Zukauskas, 2009).

Recent studies with 405 nm LED have reported its antibacterial effect on many bacterial species, including *Staphylococcus, Streptococcus, Bacillus, Escherichia,* and *Acinetobacter* by the addition of δ-aminolevulinic acid (ALA) as an exogenous photosensitizer (Nitzan, Salmon-Divon, Shporen, & Malik, 2004). For example, methicillin resistant *Staphylococcus aureus* (MRSA) was inactivated by 405 nm LED in clinical environments such as the Burns Unit and the wards (Maclean et al., 2009, 2010). Guffey and Wilborn (2006) reported that the populations of *Pseudmonas aeruginosa* and *Staphylococcus aureus* after 405 nm LED illumination were reduced by 95 and 88%, respectively. Similarly, the study conducted by Murdoch et al. (2010) has shown that *Campylobacter jejuni* and *Salmonella* Enteritidis were inactivated by 405 nm LED illumination without any exogenous photosensitizer. Based on these studies, LED technology has recently received increasing attention in its potential as light therapy for medical purposes, perhaps preventing antibiotic abuse (Maclean et al., 2009). Besides the field of medicine, LEDs have also attracted the attention of researchers in an agriculture area for the control of plant pathogens and to assist in the growth of the plant, and flowering. In particular, blue (430–450 nm) and/or red (650–670 nm) LEDs contribute to the photosynthesis in plants, resulting in the improvement of nutrition quality of vegetables (Olle & Viršile, 2013). However, little information is available on the effectiveness of 405 ± 5 nm LED on the inactivation of various foodborne pathogens and its antibacterial mechanism by endogenous photosensitizer. Therefore, the objective of this study was to investigate the antibacterial effect of 405 ± 5 nm LED on *E. coli* O157:H7, *S.* Typhimurium and *Shigella sonnei*. Its antibacterial mechanism was also elucidated by determining the bacterial sensitivity to bile salts and NaCl as well as by examining loss of bacterial membrane permeability and DNA degradation. Gram-negative pathogens were used in this study since they have similar membrane structure.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli O157:H7 (EDL 933) used in this study was provided by Dr. Henry Mok from the Department of Biological Sciences at National University of Singapore. *Salmonella* Typhimurium (ATCC 14028) and *S. sonnei* (ATCC 29031) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and stored at −70 °C. Frozen stock cultures were activated in 10 ml of sterile tryptic soy broth (TBS, Oxoid, Basingstoke, UK) for 18–24 h at 37 °C. After two consecutive transfers for 18–24 h at 37 °C, working culture at stationary phase was used for experiments.

2.2. Light emitting diode (LED) source

High intensity 405 ± 5 nm LED was purchased from Shenzhen Getian Opto-Electronics Co., Ltd. (Shenzhen, Guangdong, China). The lamp had a square 8 × 8 mm shape and the irradiance (W/cm^2) of the light emitted from the 405 ± 5 nm LED unit was measured at the surface of bacterial suspension using a 405 ± 5 nm radiometer (UHC405, UVATA Ltd., Hong Kong). The irradiance of the 405 ± 5 nm LED was 18 ± 2 mW/cm^2. The dosage received by each bacterial suspension was calculated using the following equation (Maclean et al., 2009):

$$E = Pt$$

where E = dose (energy density) in J/cm^2, P = Irradiance (power density) in W/cm^2, and t = time in sec.

2.3. LED illumination system

A LED illumination system has been described elsewhere (Ghate et al., 2013). Briefly, the 405 ± 5 nm LED was attached to a cooling fan and a heat sink to dissipate the heat generated from the LED. A resistance of 5 Ω was used in the circuit in order to protect the LEDs from excessive current. Each LED system was set up in an acrylonitrile butadiene styrene (ABS) housing for the illumination to prevent the entry of external light. The distance between the LED source and the bacterial suspension in a sterile glass Petri dish (60 mm diameter) was adjusted to 4.5 cm to illuminate the entire Petri dish. Fluke 5.4 thermocouple (Everett, WA, USA) was used to monitor the temperature of the bacterial suspension during LED illumination.

2.4. Bacterial inactivation by 405 ± 5 nm LED illumination

One ml of the working culture was centrifuged at 6000 × *g* for 10 min at 4 °C. The obtained pellet was washed with 1 ml of phosphate-buffered saline (PBS; Vivantis Inc., Oceanside, CA, USA) and centrifuged again. The resultant pellet was resuspended in 1 ml of PBS and diluted to a final concentration of approximately

10^8 CFU/ml in PBS. Ten ml of the bacterial suspension in a glass Petri dish was placed under the LED illumination system and illuminated at 4 ± 1 °C for 7.5 h (a total dose of 486 J/cm^2) in a temperature controlled incubator (MIR-154, Panasonic Healthcare Co., Ltd., Osaka, Japan). The illumination time was arbitrarily chosen based on a previous study (Ghate et al., 2013). The non-illuminated bacterial suspension was also set up in dark conditions as a control in the same incubator. An aliquot of 0.5 ml was withdrawn after every 1.5 h (97.2 J/cm^2). LED-illuminated and non-illuminated cells were serially diluted with PBS, if necessary, and the diluents were plated onto tryptic soy agar (TSA, Oxoid) by spiral plating (WASP 2, Don Whitley Scientific Ltd., West Yorkshire, UK), followed by incubation at 37 °C for 24–48 h. The number of viable cells was enumerated with an automated colony counting system (Acolyte, Synbiosis, Frederick, MD, USA) and expressed as log CFU/ml.

2.5. Weibull model for bacterial inactivation kinetics

The modified Weibull model was used to describe the bacterial inactivation and to compare the sensitivity of the bacterial strains to LED illumination since it can describe many types of bacterial inactivation such as concave, convex, and linear shapes due to its flexibility (Ferrario, Alzamora, & Guerrero, 2013; Marc, Buchovec, George, Baranyi, & Lukšiene, 2009). The parameters of Weibull distribution consist of α and β (Bialka, Demirci, & Puri, 2008; van Boekel, 2002; Unluturk, Atılgan, Baysal, & Unluturk, 2010):

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

where t is the exposure time to LED in hours, N is the microbial population after LED exposure (CFU/ml), N_0 is initial inoculation level (CFU/ml), α and β are the scale and shape parameters of the Weibull model. The reliable life (t_R) was calculated using the following equation (Bialka et al., 2008):

$$t_R = \alpha(2.303)^{\frac{1}{\beta}}$$

The values were analyzed with Origin 9.0 software (OriginLab Co., Northampton, MA, USA). The reliable life (t_R) indicates the time (h) needed for 90% reduction in bacterial population based on the scale (α) and shape (β) parameters of Weibull distribution, which is the same concept with the D-value for the first-order inactivation kinetics (Bialka et al., 2008).

2.6. Bacterial sensitivity to bile salts and NaCl

To investigate a loss of bacterial cytoplasmic or outer membrane functionality by LED illumination, the sensitivities of illuminated and non-illuminated cells to bile salts and NaCl were compared. The increased bacterial sensitivity to bile salts and NaCl implies the losses of outer membrane function as a permeability barrier to (A) hydrophobic compounds and to (B) osmotic functionality of cytoplasmic membrane, respectively (Ait-Ouazzou, Mañas, Condón, Pagán, & García-Gonzalo, 2012; Jasson, Uyttendaele, Rajkovic, & Debevere, 2007; Somolinos, García, Pagán, & Mackey, 2008).

The sensitivity was measured by comparing the difference in the number of colonies grown on TSA (non-selective agar) and TSA supplemented with NaCl (Goodrich Chemical Enterprise, Singapore) or ox-bile (Sigma–Aldrich, St. Louis, MO, USA) (selective agar). LED illuminated or non-illuminated cells were plated onto TSA with 2% (w/v) NaCl for *S. sonnei* or 3% (w/v) NaCl for *E. coli* O157:H7 and *S.* Typhimurium and onto TSA containing 1% (w/v) bile salts, respectively. After incubation at 37 °C for 24–48 h, the number of colonies was enumerated and the sensitivity was calculated with the following equation (Ghate et al., 2013):

$$\text{Sensitivity } (\%) = \left[\left(1 - \frac{\text{Colonies on TSA} + \text{NaCl or bile salts}}{\text{Colonies on TSA}}\right) \times 100\right]$$

The levels of NaCl and bile salts used for each bacterial strain were determined as the maximum non-inhibitory concentration for stationary-phase cells. The maximum non-inhibitory concentration is defined as the maximum concentration of NaCl or bile salts that does not inhibit the growth of healthy and intact cells. To determine these concentrations, stationary-phase cells grown at 37 °C for 24 h were plated onto both TSA and TSA containing various concentrations of NaCl (1–4%) or bile salts (1–3%) and the sensitivity was compared as described above.

2.7. Determination of cell membrane permeability

Bacterial membrane permeability after LED illumination was observed using the LIVE/DEAD® *Bac*Light Viability Kit L-7007 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. The kit consists of two dyes, SYTO®9 (green fluorescence) and propidium iodide (PI) (red fluorescence). Briefly, 3 μl of the dye mixture was added into 1 ml of non-illuminated control or LED-illuminated bacterial suspension exposed to 486 J/cm^2. The mixture was incubated in the dark for 15 min at room temperature. Five μl of the stained bacterial suspension was placed on a slide and covered with a square coverslip. The slides were immediately examined under oil immersion in Olympus BX51 epifluorescent microscope (Melville, NY, USA) equipped with an Olympus DP71 camera, an U-RFL-T mercury lamp and set of fluorochrome filters: SYTO®9 (WB, 450–480 nm) and PI (WG, 510–550 nm).

2.8. Comet assay

The alkaline version of comet assay was performed with the OxiSelect™ Comet Assay Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions with a slight modification. A10-μl aliquot of non-illuminated or LED-illuminated cell suspension for 7.5 h was mixed with 90 μl Comet Agarose, 5 μg/ml RNase A solution (Sigma–Aldrich), 0.25% N-Lauroylsarcosine sodium salt solution (Sigma–Aldrich) and 0.5 mg/ml lysozyme (Sigma–Aldrich). Thereafter, 75 μl of the mixture was placed on Comet slides and refrigerated for 15 min at 4 °C in the dark to solidify the agarose. The slides were incubated at 37 °C for 20 min, followed by immersing in 1 × lysis buffer (pH 10) for 1 h at 4 °C in the dark. After then, the slides were immersed in alkaline solution for 30 min at 4 °C in the dark and electrophoresed in alkaline electrophoresis buffer for 20 min at 12 V and 100 mA. After single cell gel electrophoresis, the slides were rinsed three times with distilled water for 2 min, dehydrated with cold 70% (v/v) ethanol for 10 min and air-dried. The slides were stained by adding 100 μl of Vista Green DNA Dye per well and visualized by an Olympus BX51 epifluorescent microscope equipped with an Olympus DP71 camera, an U-RFL-T mercury lamp and fluorochrome filter: Vista Green DNA Dye (WB, 450–480 nm).

2.9. DNA ladder analysis

Genomic DNA from non-illuminated or LED-illuminated cells for 7.5 h was extracted and purified by GenElute™ Bacterial Genomic

DNA Kit (Sigma–Aldrich) according to manufacturer's direction. The purified DNA was dissolved in 100 μl of Tris–EDTA (TE) buffer. The DNA solution was treated with RNase. Tris-acetate-EDTA (TAE; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) was used as buffer for electrophoresis. Two μl of purified DNA extract was solubilized in Tri-Color 6x DNA Loading Dye (1st BASE, Singapore) and was electrophoresed by 1% (w/v) agarose gel containing FloroSafe DNA Stain (1st BASE, Singapore) at 100 V for the analysis of DNA fragmentation. The gel was visualized with G:Box EF^2 Fluorescence Imaging System (Syngene, Frederick, MD, USA).

2.10. Statistical analysis

All experiments were carried out in independent triplicate with duplicate sampling (n = 6). Data were expressed by mean ± standard deviation. Significant differences in the mean values were calculated at the 95% confidence interval ($P < 0.05$) using one-way analysis of variance (ANOVA) and means were separated by least significant difference (LSD) with the IBM SPSS statistical software (version 17.0; SPSS Inc., IBM Co., Armonk, NY, USA).

3. Results

3.1. Change in temperature during 405 ± 5 nm LED illumination

To determine the heat transfer from the LED to bacterial suspension, the temperature of the bacterial suspension was monitored for 300 min with 1-min interval during 405 ± 5 nm LED illumination (data not shown). It was noticed that the temperature of the bacterial suspension increased by 6–7 °C during LED illumination and the increased temperature was maintained until the end of measurement, when the set temperature of incubator was 4 °C. Based on this observation, non-illuminated controls were held at the incubator temperature of 10 °C to compensate for the effect of the elevated temperature during the illumination.

3.2. Antibacterial effect of 405 ± 5 nm LED

In order to evaluate the antibacterial effect of 405 ± 5 nm LED, *E. coli* O157:H7, *S.* Typhimurium and *S. sonnei* were treated with 405 ± 5 nm LED for 7.5 h up to a final dose of 486 J/cm^2 at intensity of 18 mW/cm^2 (Fig. 1). Regardless of the bacterial strain, no significant ($P > 0.05$) change in the number of non-illuminated cells was observed after 7.5 h at set temperature of 10 °C. Compared to the control cells, the populations of *E. coli* O157:H7, *S.* Typhimurium and *S. sonnei* were significantly ($P < 0.05$) reduced by 1.0, 2.0, and 0.8 log CFU/ml, respectively, at the end of 405 ± 5 nm LED illumination.

The Weibull survival model was used to compare the bacterial sensitivity to LED illumination with the reliable life (t_R). The scale (α) parameter indicates the mean of the distribution describing inactivation time (h) of bacterial population, whereas the parameter β determines the shape of Weibull distribution and represents an effect on the predicted inactivation rate (Couvert, Gaillard, Savy, Mafart, & Leguérinel, 2005; Ferrario et al., 2013). A value of $\beta > 1$ indicates an increase in accumulated damaging and killing rates of 405 ± 5 nm LED with an increase light dose (Couvert et al., 2005). On the other hand, when $\beta < 1$, it means that higher rates of microbial inactivation are observed at lower LED light dose (McKenzie et al., 2013). Among three bacterial pathogens, *S.* Typhimurium had the lowest α value during LED illumination, while those of *E. coli* O157:H7 and *S. sonnei* were not significantly ($P > 0.05$) different (Table 1). The t_R-values predicted by Weibull model were significantly ($P < 0.05$) different for each pathogen studied, achieving

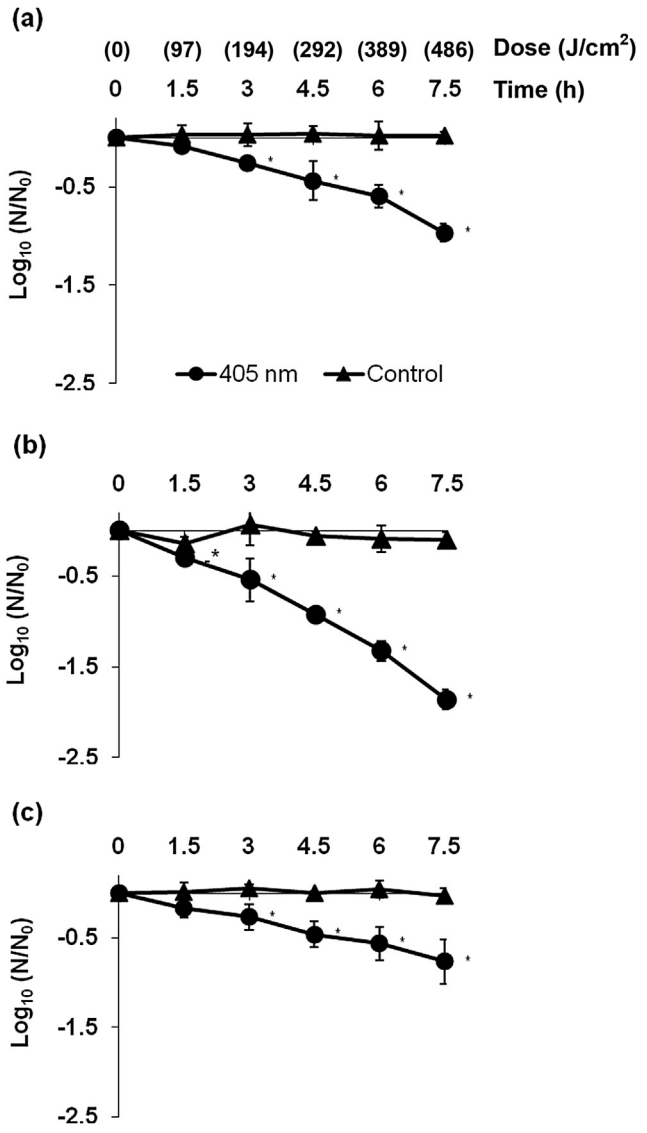

Fig. 1. Inactivation of *E. coli* O157:H7 (a), *S.* Typhimurium (b) and *S. sonnei* (c) during the illumination with 405 ± 5 nm LED at 4 °C. Asterisk (*) indicates significant ($P < 0.05$) difference between LED-illuminated and non-illuminated bacterial cell counts.

10.36, 7.64 and 4.78 h for *S. sonnei*, *E. coli* O157:H7 and *S.* Typhimurium, respectively. These results indicate that *S.* Typhimurium was the most sensitive pathogen to 405 ± 5 nm LED illumination followed by *E. coli* O157:H7 and *S. sonnei*. These results correspond with the highest inactivation observed for *S.* Typhimurium in Fig. 1.

3.3. Bacterial sensitivity to bile salts and NaCl by 405 ± 5 nm LED illumination

Changes in the bacterial sensitivity to bile salts and NaCl were used to determine the damage to bacterial cell membranes caused by LED illumination. The percentage of bacterial sensitivity to bile salts increased up to approximately 60% for *E. coli* 0157:H7 for 6 h, while the same degree of sensitivity for *S.* Typhimurium and *S. sonnei* was achieved after 3 h illumination (Fig. 2). For NaCl, the maximum percentages were 87% for *E. coli* 0157:H7, 92% for *S.* Typhimurium, and 99% for *S. sonnei* after LED illumination for 4.5–6 h. Although there was no significant ($P > 0.05$) increase in the

Table 1
Weibull model parameters for the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *S. sonnei* by 405 ± 5 nm LED illumination.

Bacterial strain	α (h)			β			t_R (h)	R^2
	95% confidence intervals			95% confidence intervals				
	Average	Lower	Upper	Average	Lower	Upper		
E. coli O157:H7	4.58 ± 0.14^{b}	4.36	4.98	1.68 ± 0.34^{a}	0.83	2.52	7.64 ± 0.48^{b}	0.98 ± 0.07
S. Typhimurium	2.63 ± 0.302^{a}	1.89	3.36	1.39 ± 0.12^{a}	1.09	1.70	4.78 ± 0.32^{a}	1.00 ± 0.01
S. sonnei	4.87 ± 1.11^{b}	2.11	7.62	1.11 ± 0.19^{a}	0.63	1.59	10.36 ± 1.10^{c}	0.99 ± 0.03

All measurements were done in triplicate with replication, and all values are means ± standard deviation. Different letters within the same column are significantly ($P < 0.05$) different.

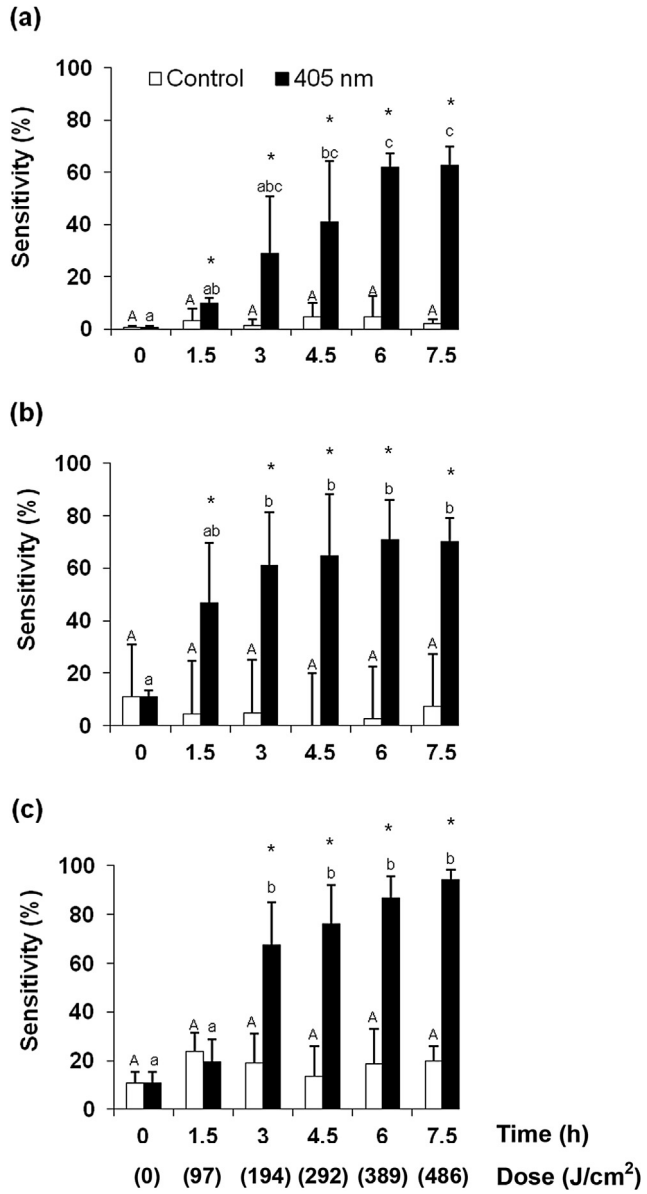

Fig. 2. Changes in the sensitivity of *E. coli* O157:H7 (a), *S.* Typhimurium (b) and *S. sonnei* (c) to 1% bile salts during 405 ± 5 nm LED illumination. Different letters (A-B or a-b) within the same bar indicate that the mean values are significantly ($P < 0.05$) different from each other. Asterisk (*) indicates significant ($P < 0.05$) difference between LED-illuminated and non-illuminated bacterial cell counts.

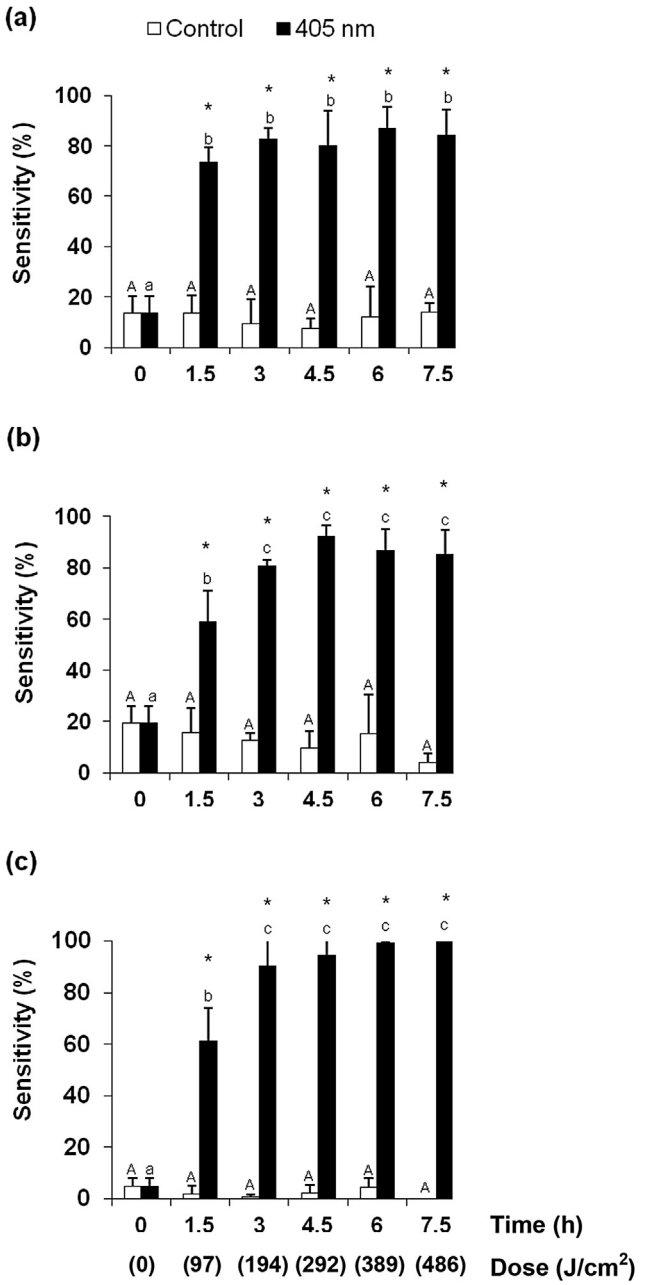

Fig. 3. Changes in the sensitivity of *E. coli* O157:H7 (a), *S.* Typhimurium (b) and *S. sonnei* (c) to NaCl (2% for *S. sonnei*, and 3% for *E. coli* O157:H7 and *S.* Typhimurium) during 405 ± 5 nm LED illumination.

sensitivity with the increase in exposure time (Fig. 3), LED-illuminated cells were significantly ($P < 0.05$) more sensitive to bile salts and NaCl than non-illuminated control cells regardless of the bacterial strain. These data exhibit that LED illumination included the malfunction of bacterial outer and cytoplasmic membranes.

3.4. Loss of cell membrane permeability by 405 ± 5 nm LED illumination

LIVE/DEAD® *Bac*Light™ assay was carried out to determine the cell membrane permeability and the microscopic images of stained bacterial populations are shown in Fig. 4. LIVE/DEAD® *Bac*Light™ is dual nucleic acid staining method used to evaluate cellular viability based on the changes in their membrane permeability. Green fluorochrome SYTO®9 (480/500 nm) of low molecular weight (~10 Da) is able to penetrate both intact and damaged cell membranes, whereas red fluorochrome PI (490/635 nm) of higher molecular weight (668 Da) can penetrate only damaged membranes, displacing SYTO®9 with red fluorescence (Bleichert, Santo, Hanczaruk, Meyer, & Grass, 2014; Joux & Lebaron, 2000; Lacombe, Tadepalli, Hwang, & Wu, 2013). In this study, non-illuminated cells showed green fluorescence of SYTO®9 stain, indicating that they maintained intact cell membrane over the exposure time (7.5 h) at 10 °C. On the other hand, some LED-illuminated cells revealed red fluorescence, indicating that these cells underwent a loss in the physical membrane integrity due to exposure to 405 ± 5 nm LED treatment.

3.5. Effect of 405 ± 5 nm LED illumination on DNA damage

Comet assay was performed to determine whether ROS produced by 405 ± 5 nm LED illumination cause DNA degradation by rupturing the phosphodiester bonds in its primary structure of base sequence. As shown in Fig. 5, regardless of the bacterial strain, no tails (comets) were observed in both LED-illuminated and non-illuminated cells, indicating that LED treatment did not caused DNA fragmentation. All microscopic photographs of single cell electrophoresis showed clear zones of nucleus within the cells without presence of DNA tailing. In parallel, DNA ladder analysis was performed in order to confirm the results obtained with the comet assay. There was only one band present in all profiles (1–9) of agarose gel electropherograms, indicating no difference observed in total genomic DNA among healthy, non-illuminated and LED-illuminated cells (Fig. 6). No shorter DNA fragments were observed in the gel in the DNA ladder profile, and migration of bands was similar for all samples. These results indicate that 405 ± 5 nm LED illumination did not induce DNA fragmentation.

4. Discussion

The present study determined the bacterial membrane and DNA damage to provide insights into the antibacterial mechanism of 405 ± 5 nm LED and evaluated the effectiveness of 405 ± 5 nm LED in inactivating major Gram-negative foodborne pathogens to see if the LED technology has a potential of application for food preservation application.

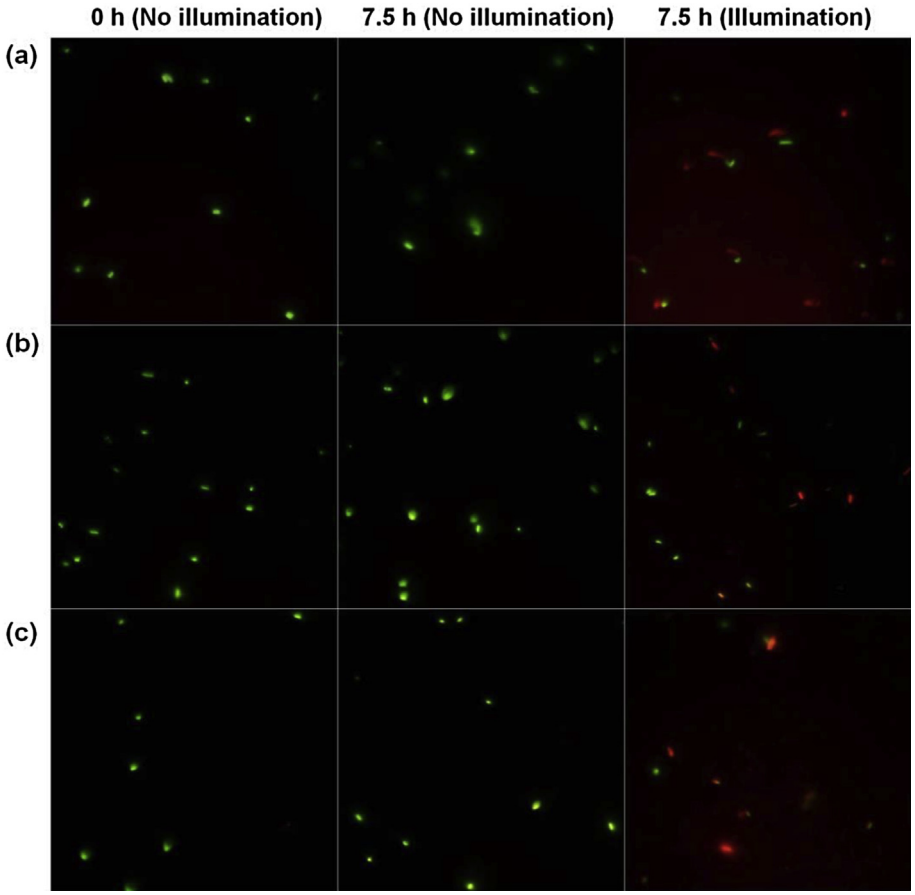

Fig. 4. The microscopic images of *E. coli* O157:H7 (a), *S.* Typhimurium (b) and *S. sonnei* (c) cells stained with LIVE/DEAD® *Bac*Light™ before and after LED illumination at the dose of 486 J/cm^2.

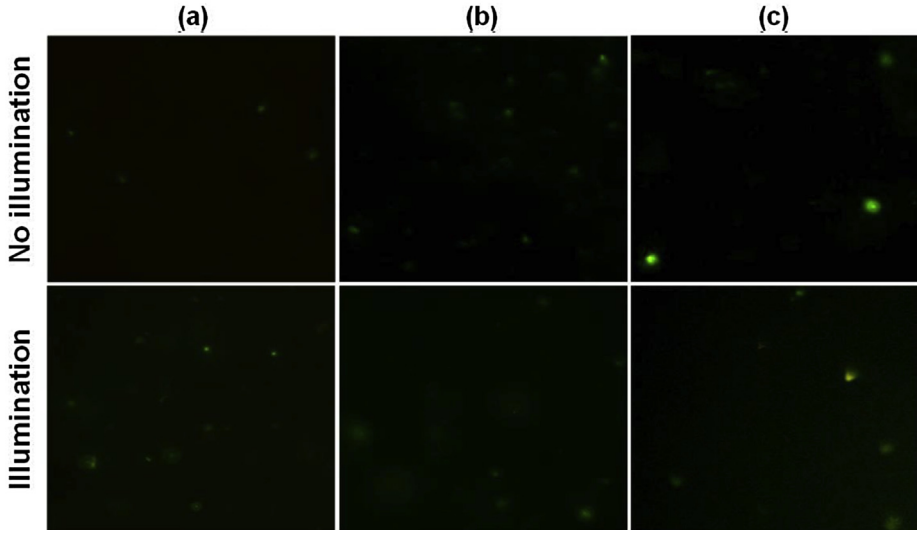

Fig. 5. Comet assay of DNA extracted from healthy, non-illuminated and LED-illuminated *E. coli* O157:H7 (a), *S.* Typhimurium (b) and *S. sonnei* (c) at the dose of 486 J/cm^2.

The antibacterial effect of 405 ± 5 nm LED in this study was evaluated at a set temperature of 4 °C, which simulated refrigeration conditions. In addition, a previous study has shown that the antibacterial effect of 460 nm LED was enhanced at lower temperature, exhibiting two to three times more susceptibility at 10 °C than 15 °C, while no antibacterial effects were revealed at 20 °C (Ghate et al., 2013). Approximately 0.5–1 ml of bacterial suspension was naturally evaporated due to the temperature increase during the LED illumination for 7.5 h. In addition, the thickness of the bacterial suspension decreased by taking samples (0.5 ml/1.5 h), and thus the depth of the suspension was reduced by 0.2 cm for 7.5 h. However, these changes were negligible compared to the total sample volume (10 ml) and depth (1.2 cm), and thus not considered in this study.

The results showed that 405 ± 5 nm LED illumination inactivated 0.8–2 log CFU/ml of the populations of *E. coli* O157:H7, *S.* Typhimurium, and *S. sonnei* during storage at 4 °C for 7.5 h (a total dose of 486 J/cm^2), and the comparison of inactivation rate revealed that *S.* Typhimurium was the most susceptible to LED illumination, followed by *E. coli* O157:H7 and *S. sonnei*. A possible explanation for the different bacterial sensitivity to LED treatment could be the variations in the amounts and the types of endogenous porphyrin compounds present in bacterial cells, which play a major role in photodynamic inactivation (PDI). The study conducted by Hamblin et al. (2005) showed that *Propionibacterium acnes* and *Helicobacter pylori* could produce and accumulate porphyrins inside the cells, which might make the cells more susceptible to visible light. Nitzan et al. (2004) also suggested that different types of porphyrin compounds might contribute to the difference in bacterial inactivation rates when they were exposed to 405 nm blue light. This might be also due to the differences in bacterial repair mechanisms under the oxidative stress (Demidova & Hamblin, 2004).

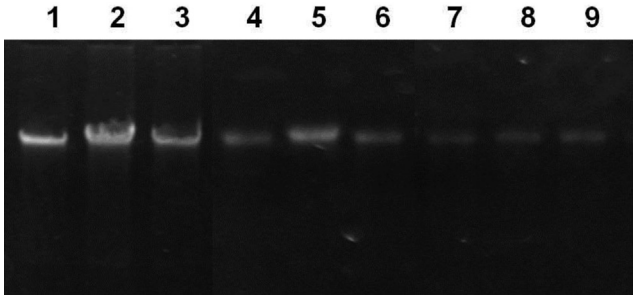

Fig. 6. Agarose gel electrophoresis profiles of DNA extracted from healthy, non-illuminated and LED-illuminated cells at the dose of 486 J/cm^2. Lane: 1, healthy *S.* Typhimurium; 2, *S.* Typhimurium without LED illumination for 7.5 h; 3, LED-illuminated *S.* Typhimurium for 7.5 h; 4, healthy *E. coli* O157:H7; 5, *E. coli* O157:H7 without LED illumination for 7.5 h; 6, LED-illuminated *E. coli* O157:H7 for 7.5 h; 7, healthy *S. sonnei*; 8, *S. sonnei* without LED illumination for 7.5 h; 9, LED-illuminated *S. sonnei* for 7.5 h.

Unlike the present study, Endarko et al. (2012) reported that 405 nm LED inactivated the population of *E. coli* O157:H7 by 4.52 log and *S. sonnei* by 3.9 log at a total dose of 554.7 J/cm^2 (for 1.8 h at the irradiance of 85.6 mW/cm^2). Another study carried by Murdoch, Maclean, Endarko, MacGregor, and Anderson (2012) with 405 nm LED at the irradiance of 10 mW/cm^2 demonstrated that 5-log reductions of *S. sonnei* and *E. coli* O157:H7 were achieved at 180 and 288 J/cm^2(5 and 8 h), respectively. These differences in the effectiveness of 405 nm LED illumination might be due to the different experimental design such as the distance between LED and bacterial suspension, total volume and depth of bacterial suspension, initial population and set temperature (Endarko et al., 2012; Murdoch et al., 2010). In particular, the previous studies used magnetic stirring plate to agitate bacterial suspension during the illumination, probably maximizing its antibacterial effect (Endarko et al., 2012; Murdoch et al., 2010). However, the bacterial suspension was not agitated during illumination in the present study to simulate a food matrix. Moreover, it seems that the antibacterial efficacy of 405 nm LED might be strain dependent. For example, a 2-log reduction of *S.* Typhimurium was achieved at a total dose of 486 J/cm^2 in this study, whereas in the study conducted by Endarko et al. (2012) the population of *S.* Enteritidis was reduced by 1.36 log at a total dose of 739 J/cm^2, while the number of *S.* Enteritidis decreased by 3–5 log at 288 J/cm^2 in a study carried out by Murdoch et al. (2010).

Bacterial sensitivity to bile salts and NaCl was studied to evaluate whether LED illumination causes damage to outer and cytoplasmic cell membranes. The results showed that 405 ± 5 nm LED illumination significantly enhanced the bacterial sensitivity to bile salts and NaCl compared to non-illuminated control cells. It is known that Gram-negative bacteria have a lipopolysaccharide (LPS) in the outer membrane, which provides a permeability barrier to hydrophobic molecules such as bile salts in the external

environment. Moreover, the cytoplasmic membrane is responsible for the regulation of osmotic pressure (Ait-Ouazzou et al., 2012; Jasson et al., 2007; Somolinos et al., 2008). Thus, these results indicate that both outer and cytoplasmic membranes of bacteria might become damaged or suffer loss of their functionality during 405 ± 5 nm LED illumination. Similarly, Ait-Ouazzou et al. (2012) reported that the combined treatment of heat and acid also made *E. coli* cells more sensitive to bile salts and NaCl and demonstrated that outer and cytoplasmic membranes were sublethally injured by the treatment.

The loss of cell membrane integrity due to LED illumination was also observed by LIVE/DEAD® *Bac*Light™ assay because some cells illuminated with 405 ± 5 nm LED showed red fluorescence. A significant damage on cell membrane may be due to the loss of their physiological functions such as permeability barrier, membrane potential, and efflux pump activity. However, the present results directly indicate that that LED illumination altered the permeability of cell membranes since PI (red fluorescence) with high molecular weight can only enter into the cells with the loss of membrane function as a permeability barrier (Bleichert et al., 2014; Joux & Lebaron, 2000; Lacombe et al., 2013). A similar result of cytoplasmic membrane damage was observed in *E. coli* cells exposed to the oxidizing effects of copper alloy, UV light, and TiO_2 due to lipid peroxidation (Hong, Kang, Michels, & Gadura, 2012). Thus, free radicals generated by 405 ± 5 nm LED illumination could physically or chemically damage the components of the bacterial membrane (Lukšiene, 2003).

Genomic DNA is also one of the major targets of ROS generated from oxidization, UV light and ionizing radiation. The ROS can attack both the sugar moieties and the base, causing DNA breakage, formation of oxidized base derivatives such as 8-OHdG (8-hydroxydeoxyguanosine) and cross-linking of DNA-protein (Nitzan & Ashkenazi, 2001; Prasad, Patel, Patel, Patel, & Selvaraj, 2013). The superoxide anion (O_2^-) can bind to DNA and hydrogen peroxide (H_2O_2) directly oxidizes free ferrous iron, forming the hydroxyl radical ($OH^{\cdot}$) as a strong oxidant that attacks the adjacent DNA (Henle et al., 1999; Keyer & Imlay, 1996; Park & Imlay, 2003). The damaged DNA may also result in DNA breakage by oxidative destruction of deoxyribose residues or replication forks (Kumari, Rastogi, Singh, Singh, & Sinha, 2008). Thus, we hypothesized that bacterial DNA could be fragmented by the attack of ROS generated during LED illumination. To prove this hypothesis, the comet assay and DNA ladder analysis were carried out. The comet assay is a simple and fast technique for assessment of DNA damage in all cell types and it is used to detect the breakage of single- or double-stranded DNA. Single bacterial cell electrophoresis results in a microscopic image, showing a clear head composed of intact DNA within nucleus and a tail including broken or damaged DNA (comet). The comet assay is based on quantification of the DNA strand fragmentation by migrating out of the nucleus in individual cells during electrophoresis under alkaline conditions (Liao, McNutt, & Zhu, 2009). In the present study, no DNA tailing and no difference in total genomic DNAs were observed, indicating that bacterial DNA was not fragmented by ROS. Probably, the concentration of ROS generated during LED illumination was not high enough to break down bacterial DNA. Thus, the low concentration of ROS might only oxidize DNA as well as other intracellular components such as proteins and lipids (Imlay, 2008). Sies and Menck (1992) reported that 8-OHdG, oxidized base derivative, was formed as a product of deoxyguanosine oxidation by methylene blue plus light illumination. This might be due to the fact that guanine residues have been reported to be the most easily oxidized (Hamblin & Hasan, 2004). Similar to the present results, Nitzan and Ashkenazi (2001) reported that no DNA breakage in *E. coli* and *Acinetobacter baumannii* was observed by visible light of various wavelengths (400–450, 480–550, and 600–700 nm) with the addition of a photosensitizer, whereas cytoplasmic membrane damage was observed.

Since 405 ± 5 nm LED illumination gave only 1–2 log reductions in an aqueous system in this study, its effectiveness may be very limited if the pathogens on foods are illuminated. In addition, long-term exposure of bacteria to LED was required for significant changes in bacterial populations, and thus this technology may be applicable to the food storage conditions during displaying. However, it would be still worthy to evaluate the bacteriostatic effect of the LED for food industry applications because LED technology can easily applied to the food showcase by replacing the fluorescent lights and saving energy. For its application for food preservation, further research on the effect of LED on physiochemical and sensory quality of foods is also necessary.

5. Conclusions

This study investigated the antibacterial effect and mechanism of 405 ± 5 nm LED on *E. coli* O157:H7, *S.* Typhimurium and *S. sonnei*. Results showed that 405 ± 5 nm LED illumination inactivated 0.8–2.0 log CFU/ml of bacterial populations under refrigerated conditions. Among bacterial strains, *S.* Typhimurium was identified as the most susceptible bacterial pathogen to 405 ± 5 nm LED illumination. In addition, LED illumination increased bacterial sensitivity to bile salts and NaCl. Due to LED illumination, the loss of bacterial membrane integrity was confirmed, whereas no DNA fragmentation was observed. Thus, the present results proved that the bacterial inactivation by 405 ± 5 nm LED illumination might be partly due to physical damage to the bacterial membranes. This study also suggests that 405 ± 5 nm LED under refrigerated conditions may be a useful technology to control foodborne pathogens in foods during storage.

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