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Bactericidal effect of 266 to 279 nm wavelength UVC-LEDs for inactivation of Gram positive and Gram negative foodborne pathogenic bacteria and yeasts

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ABSTRACT

Recently, UVC-LED technology has been validated as an alternative to irradiation with conventional mercury UV lamps. In this study, we sought to determine primary factors affecting reduction trends shown in several microorganisms. Four major foodborne pathogens (*Escherichia coli* O157:H7, *Salmonella* spp. *Listeria monocytogenes*, *Staphylococcus aureus*) and spoilage yeasts (*Saccharomyces pastorianus*, *Pichia membranaefaciens*), important to the brewing industry, were inoculated onto selective and non-selective media in order to investigate reduction tendencies at 4 different peak wavelengths (266 to 279 nm). As irradiation dose increased, inactivation levels for every microorganism were enhanced, but there were different UV-sensitivities in Gram positive bacteria (GP), Gram negative bacteria (GN), and yeasts (Y). Loss of membrane integrity measured by propidium iodide (PI) increased as peak wavelength increased for every microorganism studied. Similar results were observed in membrane potential measured by $DiBAC_4(3)$. However, there were contrasting results which showed that greater DNA damage occurred at a lower peak wavelength as measured by Hoechst 33,258. The level of DNA damage was strongly related to trends of microbial inactivation. This study showed that even though membrane damage was present in every microorganism studied, DNA damage was the primary factor for inactivating microorganisms through UVC-LED treatment.

1. Introduction

Minimally processed fresh produce has become popular due to consumers' increasing interest in wellness, a trend not unnoticed by the food industry (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). In this cultural context, non-thermal pasteurization technology, which can fulfill the goal of minimizing overall quality damage such as texture change and flavor deterioration, could be an alternative method in order to overcome some flaws inherent in conventional thermal treatment. As one of the non-thermal inactivation interventions, ultraviolet light irradiation is one of the methods that has potential to control foodborne pathogens (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). UV light falls within the electromagnetic wave spectrum ranging from 100 to 380 nm, and is classified as UV-A, UV-B, and UV-C regions according to wavelength range (Guerrero-Beltran & Barbosa-Canovas, 2004). Of these, UV-C has the maximum bactericidal effect and mercury lamps have been used to generate light in the UV-C region. Because UV lamps have some disadvantages such as leakage of mercury, necessity of warm-up time for maximum power and temperature dependency, light emitting diode (LED) type UV-C generating technology has been developed (Shin, Kim, Kim, & Kang, 2016). Temperature-independent irradiance output and prompt maximum power were observed in using UVC-LEDs while UVC lamps were affected by temperature and operating time. Also, small size UVC-LED modules compared to mercury lamps could be expeditious in applying into various device shapes. Above all, the peak wavelength of UV-LEDs can be manipulated to emit the target wavelength, so that maximum UV-light absorbance by DNA (ranging from 260 to 265 nm) (Kalisvaart, 2004) can be precisely adjusted with this new technology, whereas UV lamps can only emit a peak wavelength of 254 nm.

Formation of photoproducts by UV irradiation is generally known to be the key bactericidal effect of this treatment. Especially, when DNA absorbs UVC light, nucleic acid damage induced by pyrimidine dimer formation occurs, which leads to bacterial cell death provided irra-

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diated DNA molecules generate a sufficient level of dimers (Bintsis et al., 2000). Also, DNA destruction can occur by means of reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide generated by UV irradiation (Cadet & Wagner, 2013). The ROS stress inside bacterial cells causes damage to not only cellular components, but also nucleic acids so that changes in the pattern of gene expression arises (Scharffetter-Kochanek et al., 1997). Some research studies reported that UV treatment induced cell membrane damage which was assessed by the fluorescent dye propidium iodide (PI) (Ha & Kang, 2013, 2014; Schenk, Raffellini, Guerrero, Blanco, & Alzamora, 2011). Membrane damage induced by UV can be a potential mechanism of bactericidal inactivation.

Fluorescent dyes such as propidium iodide (PI), Bis-(1,3-Dibutylbarbituric acid) Trimethine oxonol ($DiBAC_4(3)$), and Hoechst staining are powerful techniques to investigate physiological properties during cellular changes. The fluorescent labelling dyes can be used in combination with flow cytometry, and the signals are analyzed and sorted in accordance with changes in membrane potential, membrane integrity, enzymatic activity and so forth.

In the current study, we evaluated not only the inactivation efficacy of UVC-LEDs but also the inactivating mechanism by using the fluorescent dyes in terms of peak wavelengths and types of micro-organisms: Gram positive bacteria (GP), Gram negative bacteria (GN), and yeasts (Y).

2. Materials and methods

2.1. Experimental setup

Electronic printed circuit boards (PCB) connected to UVC-LED modules (LG Innotek Co., Seoul, Korea) corresponding to four separate peak wavelengths (266, 270, 275, and 279 nm) were utilized (Fig. 1). Averaged UVC-LED voltages when appropriate currents (23 mA for 266 nm, 20 mA for 270, 275, 279 nm) were applied ranged from 6.36 V to 6.92 V and nominal power consumptions were 0.16 W (266 nm) or 0.13 W (270, 275, 279 nm). The four LEDs were arranged in the '4 corners' array which indicates 6 cm distance between each LED module and 4 cm distance between PCB and petri dish based on previous investigations conducted in our laboratory (Kim, Kim, & Kang, 2016; Shin et al., 2016). This arrangement and distances were optimized to accommodate a 90 mm diameter petri dish for equally distributed irradiance with high irradiance intensity given the petri factor. Because UVC-LEDs emit parallel and collimated ultraviolet light while radial light is generated by conventional UV lamps, gradient intensity distribution over the surface is developed. Therefore, the petri factor, which indicates averaged surface irradiance, should be taken into consideration in UVC-LEDs treatment. In this research the petri factor of the 4 corners array was over 0.9 (90%) which indicates nearly uniform distribution of light across the petri dish surface (Bolton & Linden, 2003). Constant electric current was provided by a DC power supply (TPM series, Toyotech; Incheon, Korea) which applied 23 mA for a 266 nm PCB, and 20 mA for 270, 275, and 279 nm PCBs.

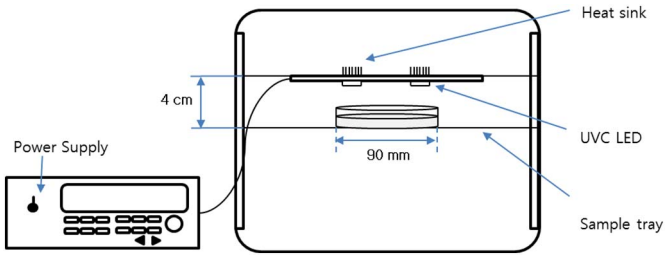

Fig. 1. Schematic diagram of the UVC-LEDs irradiation system in Seoul National University.

2.2. Irradiance measurements

Irradiance intensity of the UV-LED modules was measured with a spectrometer (AvaSpec-ULS2048-USB2-UA-50, Avantes; Apeldoorn, Netherlands) which was able to calibrate within a range of 200 to 400 nm which includes the entire UV wavelength spectrum. For sample treatment, the distance between PCB with LEDs and an optical probe was 4 cm and irradiance value at the peak wavelength was measured. Irradiance for every 5 mm of area corresponding to the dimensions of a petri dish was measured. Each measured intensity was divided by maximum irradiance value and averaged to obtain the petri factor. The final irradiance value was normalized by multiplying maximum intensity by the petri factor.

2.3. Bacterial and yeast strains

Three strains each of *Escherichia coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *Listeria monocytogenes* (ATCC 15313, ATCC 19111, and ATCC 19115), *Staphylococcus aureus* (ATCC 10390, ATCC 12598, and ATCC 27644), and *Salmonella* Enteritidis PT 30 (ATCC BAA1045) were obtained from the Bacterial Culture Collection at Seoul National University (Seoul, Korea). *Salmonella* Senftenberg (KVCC 0590) and *Salmonella* Tennessee (KVCC 0592) were obtained from the Korea Veterinary Culture Collection (Gimcheon, Korea). *Pichia membranaefaciens* (KCCM 12470) and *Saccharomyces pastorianus* (KCCM 11523) were obtained from the Korean Federation of Culture Collections (Seoul, Korea). Stock cultures were grown in Tryptic Soy Broth (TSB; Difco, Becton Dickinson and company; Sparks, MD, USA) at 37 °C for 24 h for bacteria and in Nutrient Yeast Dextrose (NYD) broth (1% nutrient broth + 0.6% yeast extract + 1.3% glucose) at 25 °C for 48 h for yeasts and stored at − 80 °C (0.7 ml of TSB culture with 0.3 ml of sterile 50% glycerol solution). To obtain working cultures, bacteria and yeasts were each streaked onto Tryptic Soy Agar (TSA; Difco) and Potato Dextrose Agar (PDA; Difco), respectively, and incubated at 37 °C for 24 h and 25 °C for 48 h, respectively, stored at 4 °C and used within 3 days.

2.4. Culture preparation

Each bacterial strain (*E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *S. aureus*) was cultured in 5 ml TSB at 37 °C for 24 h. Yeasts (*P. membranaefaciens* and *S. pastorianus*) were incubated in 5 ml NYD broth at 25 °C for 48 h. Each bacterial and yeast strain was cultured to stationary growth phase and harvested by centrifugation at 4000 × *g* for 20 min at 4 °C and the supernatant discarded. Obtained bacteria or yeast cell pellets were resuspended in sterile 0.2% Bacto peptone (Bacto, Becton, Dickinson and Company; Sparks, MD, USA) and centrifuged. This washing procedure was performed three times. Final pellets were resuspended in 9 ml peptone water (PW), corresponding to approximately 10^8 to 10^9 CFU/ml for bacteria and 10^6 to 10^7 for yeasts. Resuspended pellets of each strain of all bacterial pathogen species were combined to constitute a 3-pathogen mixed culture cocktail. A mixed culture cocktail comprised of the two yeast strains was similarly prepared.

2.5. Inoculation

In order to set a control solution, mixed bacteria culture cocktails were 10-fold serially diluted three times (10^{-3} dilution) and yeast suspensions were 10-fold (10^{-1}) diluted with 0.2% sterile PW resulting in a final concentration of approximately 5–6 log CFU/ml. For inoculation, the culture suspensions were further serially decimal diluted with 0.2% sterile PW to obtain countable colonies. One-tenth ml aliquots of selected diluents were spread-plated onto selective or nonselective media. Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), Oxford Agar Base with antimicrobial

supplement (OAB; MB Cell, Seoul, Korea), and Baird Parker Agar (BPA; Difco) were used as selective media to enumerate *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *S. aureus*, respectively. Phenol Red agar base (Difco) with 1% sorbitol (MB Cell) (SPRAB) was used to enumerate injured cells of *E. coli* O157:H7 and the overlay (OV) method was used to enumerate injured cells of *Salmonella* spp. and *L. monocytogenes* (Lee & Kang, 2001). TSA was used as non-selective medium for *S. aureus*. For yeasts, PDA with 2% sodium chloride (Daejung Chemicals & Metals; Gyeonggi-do, Korea) and PDA were used as selective and non-selective media, respectively. This level of sodium chloride was determined to be the maximum non-inhibitory concentration for yeast cells (data not shown). To obtain countable numbers of colonies on the tested media, two levels of sequential tenfold serial dilutions were spread-plated. After inoculation, the media were dried for approximately 30 min at room temperature prior to UV treatment.

2.6. UV treatments

Inoculated media were treated at dosages of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mJ/cm^2. Four different peak wavelengths of UV-LED PCBs were utilized and every treatment was implemented at room temperature (22 ± 1 °C) in the chamber. Treatment times were calculated by dividing UV doses by intensities. After treatments, to minimize photoreactivation, all UV-treated petri dishes were covered with aluminum foil and placed in the dark before incubating.

For damage assessment at the cellular level, each bacterial or yeast suspension was appropriately diluted with Phosphate-Buffered Saline (PBS, Corning Inc.; Corning, NY, USA) to adjust optical density (OD) to approximately 0.4, and 5 ml of suspensions were pipetted into empty petri dishes. Irradiation of 1 mJ/cm^2 by means of UV-LEDs was applied to the microbial suspension; this dose corresponds to media treatment of 0.6 mJ/cm^2 which induces a similar inactivation level (ca. 5 log reduction). After irradiation, 5 ml aliquots of suspensions were delivered to sterile conical tubes, and placed in the dark until assessments using fluorescence dye.

2.7. Bacterial and yeast enumeration

After treatment, plated media were incubated at 37 °C for 24 h for bacteria and at 25 °C for 48 h for yeasts. Typical colonies were counted following incubation.

To enumerate injured cells, the overlay (OV) method was used for *Salmonella* spp. and *L. monocytogenes*. For the first step of the overlay method, non-selective TSA medium was used, which enables injured cells to resuscitate. Plated and UV-treated TSA was incubated at 37 °C for 2 h to permit injured cells to recover. The plates were then overlaid with 7 ml of the selective medium XLD for *Salmonella* spp. or OAB for *L. monocytogenes*, respectively. Solidified plates were further incubated for an additional 22 h at 37 °C. After incubation, typical black colonies of both pathogens were enumerated. Enumeration of injured *E. coli* O157:H7 on SPRAB was implemented to count typical white colonies after incubation at 37 °C for 24 h with simultaneous serological confirmation of randomly selected characteristic white colonies representing 10% of the total (RIM, *E. coli* O157:H7 latex agglutination test; Remel, KS, USA). Injured cells of *S. aureus* were calculated after colony counts were taken on TSA following incubation at 37 °C for 24 h and compared with counts made on BPA plated simultaneously. Injury of *P. membranaefaciens* and *S. pastorianus* was assessed after incubation on non-selective PDA at 25 °C for 48 h and compared with simultaneous incubation on selective PDA + 2% NaCl.

2.8. Cellular membrane damage measurement

To investigate cellular membrane damage after UVC-LED irradiation, PI uptake and $DiBAC_4(3)$ accumulation values were measured. Propidium Iodide (PI; Sigma-Aldrich Corp; St. Louis, MO, USA) is a fluorescent dye used to quantitatively assess membrane destruction of bacteria or yeast cells induced by UV treatment. Treated bacteria and yeast cells were diluted in PBS to adjust optical density at 680 nm (OD680) to approximately 0.4 and then mixed with PI solution to a final concentration of 2.9 μM, followed by 10 min incubation in the dark at room temperature.

Membrane potential is the difference of electrical state between inner and outer sides of a cell membrane which can be an indicator of cellular damage (Rottenberg, 1979). To evaluate membrane potential of cells, bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$; Molecular Probes, Invitrogen, Thermo Fisher Scientific; Waltham, MA, USA) was used as a fluorescent dye. Suspensions of GP and Y were incubated in 0.5 μg $DiBAC_4(3)$/ml and 1 μg $DiBAC_4(3)$/ml in PBS, respectively. GN cells were incubated in 2.5 μg $DiBAC_4(3)$/ml in PBS with 4 mM ethylenediaminetetraacetic acid (EDTA). GN suspensions were incubated for 15 min at 37 °C, and GP and Y were incubated for 2 min and 30 min, respectively, at room temperature in a dark room. For positive controls, which were considered to receive complete cell membrane damage, cells were heat-shocked at 55 °C for 15 min. Also, untreated groups were assigned as negative controls.

After incubation, each dyed sample was centrifuged at 10,000 × *g* for 10 min and washed twice with PBS to remove excess dye. The cell pellet was resuspended in PBS, and fluorescence was measured with a spectrofluorophotometer (SpectraMax M2e; Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength (Ex) of 495 nm and an emission wavelength (Em) of 615 nm for PI uptake and Ex of 488 nm and Em of 525 nm for $DiBAC_4(3)$ accumulation. Fluorescence values from untreated cells were subtracted from those of treated cells, and the data were normalized against the OD680 of the cell suspensions (Lloyd, Salgado, Turner, Suller, & Murray, 2002; Park & Kang, 2013; Siva, Ferreira, Queiroz, & Domingues, 2011). Each value was expressed as a ratio to that of the positive control.

2.9. DNA damage measurement

It is well-known that the major inactivation mechanism of ultraviolet rays is DNA damage. We measured membrane damage of bacteria and yeasts after UV irradiation, and in order to compare the damage ratio between membranes and cellular DNA, Hoechst 33,258 (Molecular Probes, Invitrogen, Thermo Fisher Scientific; Waltham, MA, USA) was used to examine DNA damage after exposure to UVC-LED. Two μg/ml of Hoechst 33,258 dye was mixed with UVC-LED treated bacteria or yeast cells and incubated in the dark at room temperature for 10 min. For the positive control, which was considered to be a state of complete DNA damage, cells were irradiated at 250 mJ/cm^2 with a conventional 254 nm UV-lamp (G10T5/4P; 16W; 357 mm; Sankyo, Japan). Untreated groups were assigned as negative groups.

After incubation, each dyed sample was centrifuged at 10,000 × *g* for 10 min and washed twice with PBS to remove excess dye. The cell pellet was resuspended in PBS, and fluorescence was measured with a spectrofluorophotometer at an Ex of 352 nm and an Em of 461 nm. Fluorescence values from negative controls (untreated cells) were subtracted from those of treated cells, and the data were normalized against the OD680 of the cell suspensions. Measured values were expressed as a ratio to that of the positive control and the calculated ratio was subtracted from 100% to quantify DNA damage.

2.10. Statistical analysis

All experiments were duplicate-plated and replicated three times. All data were analyzed with ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and Duncan's multiple range test to determine if there were significant differences ($P < 0.05$) in mean values of log reduction of microorganism populations or fluorescence value ratios.

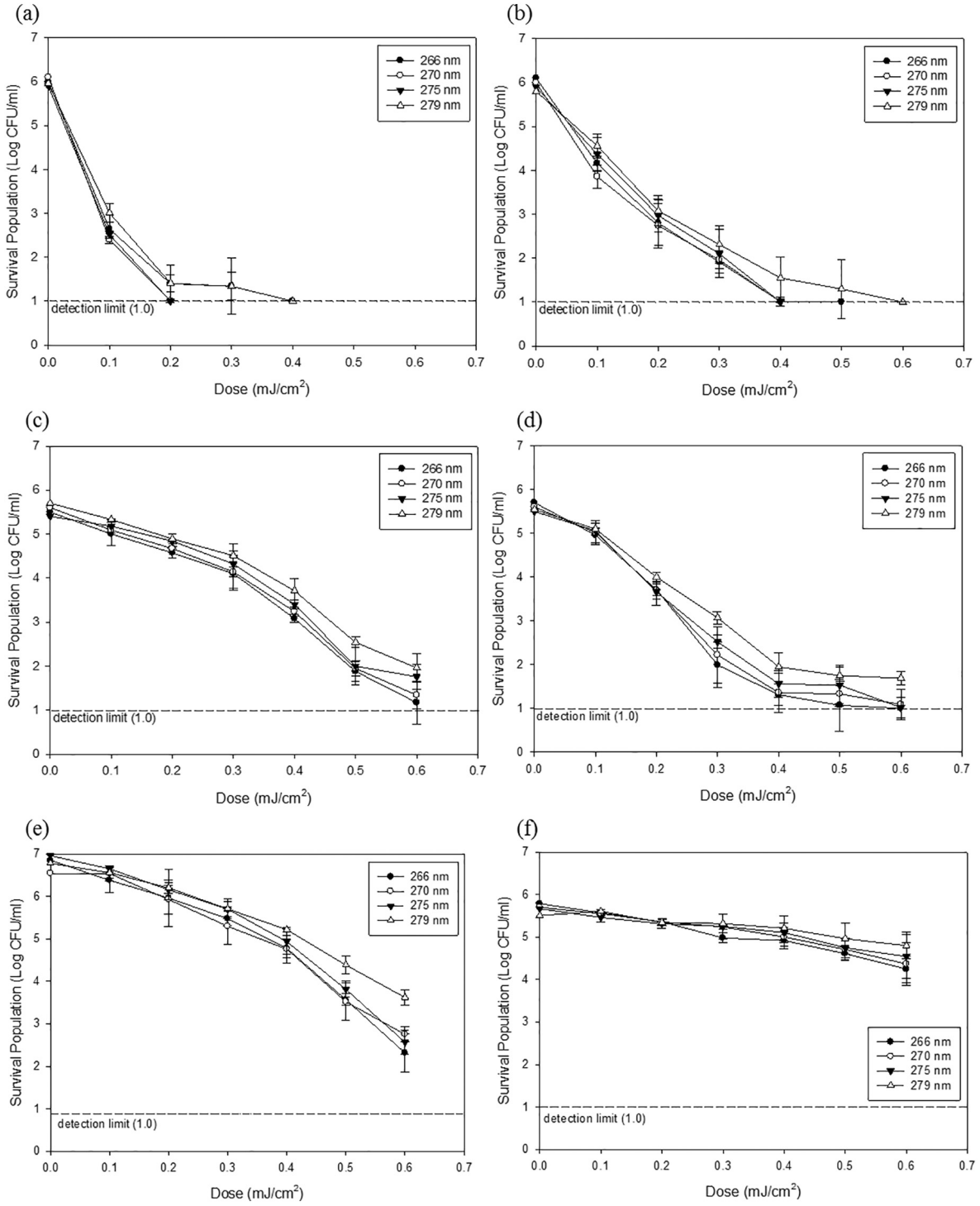

Fig. 2. Log reductions (CFU/ml) of Gram negative bacteria ((a) *E. coli* O157:H7, (b) *Salmonella* spp.), Gram positive bacteria ((c) *L. monocytogenes*, (d) *S. aureus*), and yeasts ((e) *P. membranaefaciens*, (f) *S. pastorianus*) after UVC-LED irradiation.

3. Results

3.1. Emission spectrum of UV-LED

All UVC-LEDs used in this study yielded a similar voltage at each rated current for an average of 6.55 ± 0.22 V. Spectral intensities of the UV-LEDs on PCBs was measured with a spectrometer; actual peak wavelengths of 266, 270, 275, and 279 nm UVC-LEDs were 266.25, 271.02, 275.80, and 279.37 nm, respectively. The average irradiance values at 266, 270, 275, and 279 nm were 4.24, 3.96, 3.76, and 3.77 μW/cm^2, respectively.

3.2. Inactivation effect of UV-LED on media

Bacteria or yeasts inoculated on solid media were treated with the four wavelength UVC-LEDs. Though low wavelength yielded higher microbial reduction of both bacteria and yeasts than did high wavelength UVCs, there was no significant difference ($P > 0.05$). Fig. 2 indicates log reductions of each bacterium or yeast. Increasing irradiation dose led to higher reductions of bacteria and yeasts. GN, as *E. coli*

O157:H7 and *Salmonella* spp., were easily inactivated by UVC-LED (Fig. 2a, b). At the low dose of 0.2 mJ/cm^2, *E. coli* O157:H7 was reduced by about 5 log and reduction of *Salmonella* spp. was close to 3 log. At same dose, GP, such as L. *monocytogenes*, achieved < 1 log reduction and *S. aureus* experienced slightly > 2 log reduction (Fig. 2c, d). Both yeasts, *P. membranaefaciens* and *S. pastorianus*, were inactivated by only < 1 log at 0.2 mJ/cm^2 (Fig. 2e, f). *E. coli* O157:H7 was not detected (> 5 log reduction) after 0.4 and 0.6 mJ/cm^2 of irradiance, while reduction of *Salmonella* spp. was over 5 log. GP were reduced by 3–5 log after 0.6 mJ/cm^2. As for yeasts, the inactivation tendency differed depending on species. *P. membranaefaciens* was inactivated by approximately 4 log after 0.6 mJ/cm^2 but *S. pastorianus* was inactivated only by about 1 log.

With respect to injured cell generation, *E. coli* O157:H7 and *Salmonella* spp. showed significant levels of resuscitated injured cells at 0.2 mJ/cm^2 and 0.4 mJ/cm^2 (data not shown). Injured cells were not detected at higher levels of irradiation. As for GP and yeasts, no injured cells were generated.

3.3. Cell membrane damage assessment

We measured PI uptake and DiBAC$_4$(3) accumulation values of each microorganism species after 1 mJ/cm^2 irradiation. PI values increased with increasing UV-LED peak wavelengths (Table 1). GP experienced higher PI values than GN, and Y showed the lowest PI uptake. DiBAC$_4$(3) values displayed the same tendency as PI values (Table 2). Because loss of membrane potential occurred prior to physical membrane destruction, DiBAC$_4$(3) values were higher than PI uptake values but the same trends were observed as already mentioned (Diaz, Herrero, Garcia, & Quiros, 2010).

3.4. DNA damage assessment

Hoechst 33,258 bonds with double stranded DNA at the AT-rich region (Disdale & Lloyd, 1995). Also, the binding affinity for dsDNA is 10^7-fold higher than that for ssDNA (Yuan, Ruina, Xiaomin, Meiping, & Yuanzong, 2007), and when Hoechst binds to Adenine-Thymine triplets in DNA, the fluorescence intensity increases approximately 100 times (Howard, 2000). Therefore, a low Hoechst 33,258 value indicates that many dsDNA strands were unwound or deformed by pyrimidine dimer, causing great DNA damage. As shown in Fig. 3, DNA damage was highest at the low wavelength, 266 nm, and with increasing wavelength, DNA damage diminished. GN showed over 90% DNA damage at 266 nm compared to the positive control but GP experienced < 90% DNA damage at the same wavelength. Yeasts received far less DNA damage than did bacteria (60–70%).

4. Discussion

Disinfection with light has been researched as a non-thermal inactivation technique and is one of the powerful surface sterilizing/pasteurizing technologies available to the food industry. Many researchers have studied light treatments including pulsed light, blue light and so forth for the purpose of inactivating foodborne pathogens and infectious viruses (Fornander, Wu, Billeter, Lincoln, & Norden, 2013; Uesugi, Hsu, Worobo, & Moraru, 2016; Wang et al., 2016). Of all these non-thermal control technologies, ultraviolet irradiation is the most widely used. UVC irradiation treatment was approved by the US-FDA in 2000 as a control method in order to assure food, water, and beverage safety (US-FDA, 2000). Far-UVC treatment at a dose of 75 mJ/cm^2 accomplished approximately 3 log CFU/ml reduction of *E. coli* O157:H7 in apple juice, with no photoreactivation observed following dark incubation (Yin, Zhu, Koutchma, & Gong, 2015). Also, 3 major foodborne pathogens (*E. coli* O157:H7, *S*. Typhimurium, and L. *monocytogenes*) on fresh cut lettuce were effectively inactivated by up to 4–5 log CFU/g reduction by using UVC irradiation (Kim et al., 2013). For water disinfection, UV treatment can effectively inactivate numerous pathogenic and non-pathogenic microorganisms (Hijnen, Beerendonk, & Medema, 2006).

Of special interest, UVC-LEDs, which are a potential alternative to low pressure mercury lamps, have been researched recently. Shin et al. (2016) reported on the fundamental characteristics of UVC-LEDs and revealed that effective foodborne pathogen inactivation had been validated by both media and water experiments. In addition, the inactivation efficacy at 4 peak wavelengths was evaluated and application to food samples was performed (Kim et al., 2016).

In the present study, we evaluated the bactericidal effect with regard to 4 wavelengths and different categories of microorganisms (GP, GN, and Y) and the major inactivation mechanism of UVC-LEDs was investigated by using fluorescence dyes.

Inactivation within microorganism groups showed a similar tendency, as higher doses of irradiation resulted in higher reduction levels. However, quite different levels of UVC-susceptibility were observed in that GN had the lowest level of resistance against UVC-LEDs and Y were the most resistant. These results are consistent with differences in UV-resistance by microorganism groups (Beauchamp & Lacroix, 2012; Guerrero-Beltran & Barbosa-Canovas, 2005). There are some factors that affect resistance, such as cell wall thickness, cell size, photoproducts generated by irradiation, and DNA repair ability (Lopez-Malo & Palou, 2005; Tran & Farid, 2004). Also, the physiological state of microorganisms determines the degree of UVC sensitivity (Bucheli-Witschel, Bassin, & Egli, 2010; Wassmann, Moeller, Reitz, & Rettberg, 2011).

Compared to conventional mercury UV lamps, UVC-LEDs can accomplish much higher levels of microorganism inactivation. According to Kim et al. (2016), 266 nm UVC-LEDs demonstrated over 5 log reductions in *E. coli* O157:H7 and *S*. Typhimurium while 1–2 log reductions were achieved using UV lamps at the same dosages with UV lamp intensity attenuated to the levels of UVC-LEDs. Moreover, the levels of microorganism inactivation when 1 mJ/cm^2 irradiation was

Table 1
Ratio of propidium iodide (PI) uptake values of bacteria or yeasts to that of the positive control after 1 mJ/cm^2 UVC-LED treatment.

Wavelength (nm)	PI uptake percentage[a](%)					
	Gram negative		Gram positive		Yeast	
	E. coli O157:H7	*Salmonella* spp.	*L. monocytogenes*	*S. aureus*	*P. membranaefaciens*	*S. pastorianus*
266	1.34 ± 1.07 A	1.45 ± 1.32 A	2.69 ± 1.10 A	3.39 ± 2.49 A	1.65 ± 0.19 A	0.29 ± 0.13 A
270	2.02 ± 0.74 A	2.03 ± 2.20 A	3.60 ± 1.15 A	4.66 ± 2.41 A	2.38 ± 0.07 B	1.48 ± 0.32 AB
275	2.36 ± 1.16 A	4.57 ± 3.49 AB	9.00 ± 5.62 A	14.66 ± 3.20 B	3.36 ± 0.19 BC	1.98 ± 0.20 C
279	5.52 ± 3.30 A	7.91 ± 1.04 B	16.98 ± 1.01 B	18.29 ± 4.64 B	5.81 ± 0.82 C	2.50 ± 0.25 D

[a] Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns are not significantly different ($P > 0.05$). Normalized data were obtained by subtracting OD680 fluorescence values of untreated cells from those of treated cells and dividing by the positive control value and expressing this value as a percentage (PI percentage = [fluorescence value after treatment − fluorescence value of untreated cells] / [OD680-fluorescence value of positive control]).

Table 2
Ratio of DiBAC4(3) (BOX) accumulation values of treated bacteria or yeasts to that of the positive control after 1 mJ/cm^2 UVC-LED treatment.

Wavelength (nm)	$DiBAC_4(3)$ percentage[a] (%)					
	Gram negative		Gram positive		Yeast	
	E. coli O157:H7	*Salmonella* spp.	*L. monocytogenes*	*S. aureus*	*P. membranaefaciens*	*S. pastorianus*
266	0.80 ± 0.21 A	3.76 ± 3.03 A	6.42 ± 1.87 A	7.65 ± 0.72 A	3.35 ± 0.43 A	0.28 ± 0.15 A
270	2.77 ± 0.24 AB	8.20 ± 2.70 AB	5.69 ± 2.36 A	10.72 ± 5.97 AB	7.97 ± 1.69 B	3.57 ± 0.55 B
275	5.04 ± 1.17 BC	13.04 ± 0.51 BC	9.01 ± 1.69 A	19.93 ± 6.39 B	10.74 ± 0.45C	4.29 ± 0.42 B
279	6.73 ± 2.12 C	15.35 ± 1.93 C	16.67 ± 3.87 B	35.11 ± 1.21C	12.55 ± 0.44C	5.36 ± 0.22C

[a] Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns are not significantly different ($P > 0.05$). Normalized data were obtained by subtracting OD680 fluorescence values of untreated cells from those of treated cells and dividing by the positive control value and expressing this value as a percentage ($DiBAC_4(3)$ percentage = [fluorescence value after treatment − fluorescence value of untreated cells] / [OD680·fluorescence value of positive control]).

applied, known as inactivation rate constant, k (cm^2/mJ), were significantly higher for UVC-LEDs than for conventional UV lamps, which indicated that UVC-LEDs showed more efficient bactericidal activity than did UV lamps (Kim, Kim, & Kang, 2017). Form an energy consumption aspect, a four-UVC-LED module, which consumes 0.16 W while UV lamp uses 16 W, conserves electric power while providing a better pathogen inactivation effect (Kim et al., 2016).

Since the fluorescent dyes can determine certain mechanisms of changes in cell physiology properties such as membrane integrity, membrane potential, enzyme activity, metabolic performance, DNA damage and so on (Diaz et al., 2010), they have been used in various research investigations (da Silveira, San Romao, Loureiro-Dias, Rombouts, & Abee, 2002; Sanchez, Garcia, & Heredia, 2010; Siva et al., 2011; Ha & Kang, 2013, 2014). By using propidium iodide (PI) and carboxyfluorescein diacetate (cFDA) with flow cytometry analysis, two bacterial cell properties - membrane integrity and activity of intracellular esterase - were determined after ultrasound treatment (Li et al., 2016). As the treatment progressed, cell properties shifted to greater PI and lower cFDA values which meant that not only cell integrity but also enzyme activity was degraded. Also, Schenk et al. (2011) reported that UV lamps had an effect on non-pathogenic bacteria relative to cell morphological change, membrane integrity, and enzyme activity by using PI and cFDA with flow cytometric analysis. Physiological changes in *E. coli* during orange juice clarification were investigated with PI and Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$) (Anvarian, Smith, & Overton, 2016).

In order to investigate the inactivation mechanism of this new technology, UVC-LED, fluorescence dyes were used in this study. Membrane integrity can be assessed by using PI which can enter the interior of cells and bind to nucleic acids only when the cell membrane is damaged. By using $DiBAC_4(3)$, membrane potential change, which is a prior symptom of membrane damage, can be assessed since depolarized cells cannot exclude cation/anion molecules such as $DiBAC_4(3)$ so that accumulation of charged particles proceeds (Diaz et al., 2010). Hoechst, which is able to permeate cell membranes, binds and stains minor grooves of the Adenine-Thymine rich region in double-stranded DNA (dsDNA) (Pjura, Grzeskowiak, & Dickerson, 1987) so that the dye can be used as a way to evaluate DNA damage because it prefers dsDNA.

In our study, there were significantly different PI uptake percentages between 266 nm and 279 nm peak wavelengths for all three microorganism types. PI uptake percentage values at 279 nm were 5–8 times greater than those at 266 nm, and the results were analogous through all types of microorganisms. It is well known that protein has a peak absorbance of approximately 280 nm light due to aromatic amino acids such as Tryptophan, Tyrosine, and by using this simple characteristic, the absorbance value at 280 nm is one of the methods that determines protein concentration (Pace, Vajdos, Fee, Grimsley, & Gray, 1995; Stoscheck, 1990). Light with an approximately 280 nm peak wavelength can be absorbed by proteins and transform them into an excitation state leading to their destruction. Therefore, higher peak wavelength irradiation induced a greater level of membrane protein vulnerability, so that membrane integrity deteriorated by means of DNA nick formation. Furthermore, reactive oxygen species (ROS) such as the superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl ion (OH^-) generated by UVC irradiation can easily cause heavy stress to aggravated membrane proteins (Scharffetter-Kochanek et al., 1997). Interestingly, GP showed a much higher PI uptake percentage than did GN or Y. GN and Y showed a maximum 8% PI uptake, while over 15% PI uptake was observed in GP. This indicates that there was twice the membrane damage in GP than in GN. Although ROS formation was not investigated in the current study, we could infer that these differences in PI uptake may be attributed to the degree of susceptibility of microorganisms' specific organelles to ROS from the fact that UV irradiation induces ROS (Anita & Miroslav, 2010; Scharffetter-Kochanek et al., 1997). ROS, except superoxide anion radicals ($\cdot O_2^-$), can penetrate the bacterial or yeast cell envelope and cause several types of damage to microorganism organelles. For different types of microorganisms, cellular components differ in their vulnerability to ROS, following this damage sequence: phospholipids > peptidoglycan in GP; lipopolysaccharides (LPS) > phospholipids > peptidoglycan in GN; and, glycoproteins > glucans > chitin in Y (Bogdan, Zaraynska, & Plawinska-Czarnak, 2015). Such different susceptibilities in cellular components were clearly related to membrane integrity and membrane potential of UVC-LED treated microorganisms. Because GP bacterial cell walls are composed of only peptidoglycan and phospholipid layers, membrane integrity was easily overcome by ROS so that PI could penetrate into cytosol and bind to DNA. However, because GN bacteria and Y have outer membrane obstacles including LPS (GN), glycoproteins, or glucans (Y), the degree of membrane damage in these two groups was reduced compared with GP, which induces lower fluorescence values.

Membrane potential forms a homeostasis activity which maintains different concentrations of ions inside and outside of the cell, and only active cells can generate differences in membrane potential (Disdale & Lloyd, 1995). In fact, reduced membrane potential does not indicate cell death, but is associated with decreasing cell activity; when cell activity declines, membrane depolarization occurs and charged ions can cross the membrane without hindrance. Therefore, membrane potential is strongly related to membrane damage, because membrane depolarization develops prior to structural membrane damage. That is, the membrane potential value, which is indicated by lipophilic dyes ($DiBAC_4(3)$ in the present study), has to be higher than the membrane damage value measured by PI uptake (Diaz et al., 2010). In this manner, Table 2 shows that $DiBAC_4(3)$ accumulation values show a similar trend to that of PI uptake; i.e., $DiBAC_4(3)$ values increase with higher peak wavelengths, and depolarization in GP was higher than for other microorganism groups.

DNA damage by UVC-LED treatment was measured by using Hoechst 33,258. This fluorescence dye binds to minor grooves of the

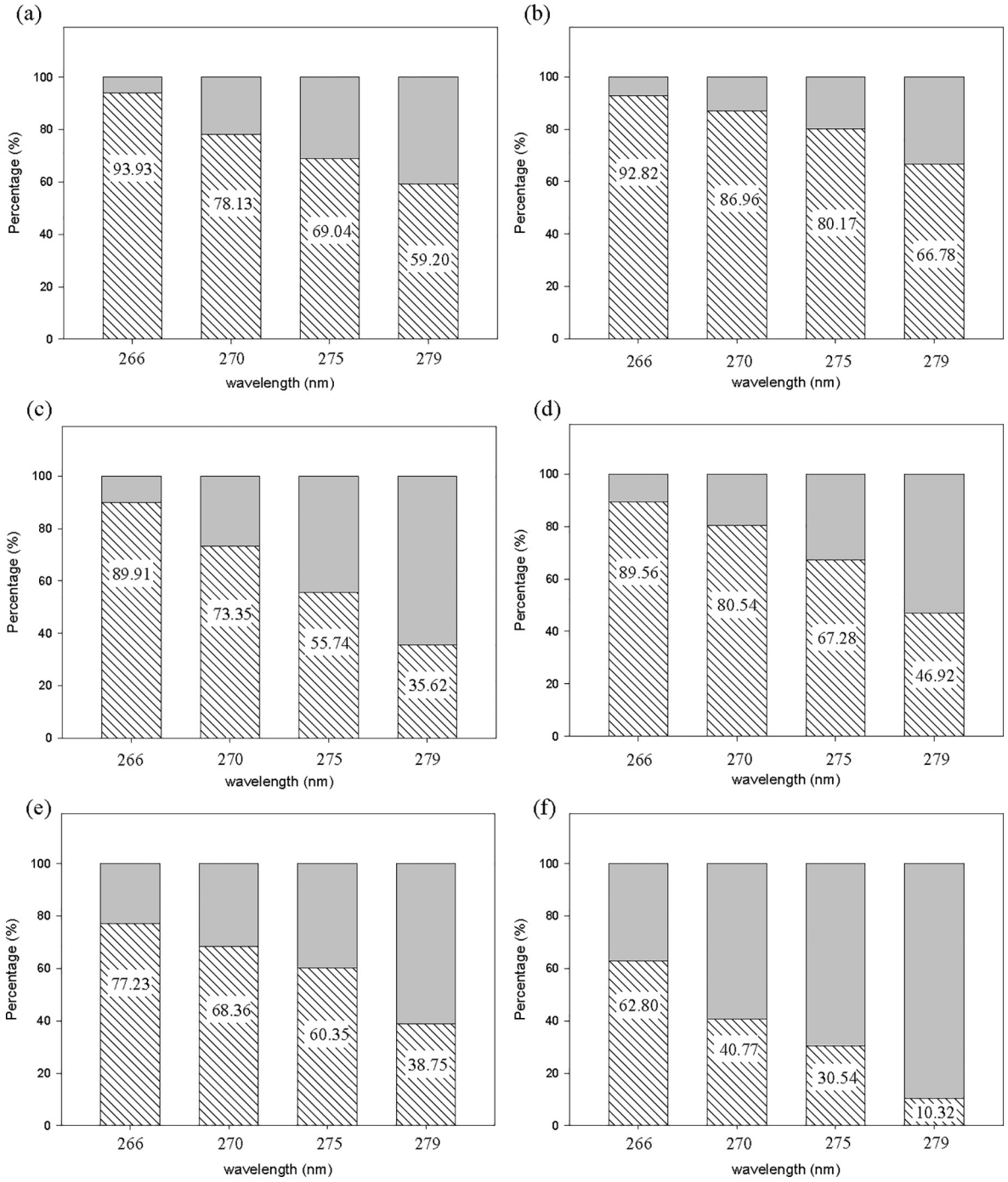

Fig. 3. Percent DNA damage following 1 mJ/cm^2 of UVC-LED irradiation. ▨ indicates Hoechst 33,258 uptake percentage of treated samples and ▭ represents DNA percent damage obtained by subtracting Hoechst 33,258 uptake percentage of treated samples from that of the positive control (100% value). (a) *E. coli* O157:H7, (b) *Salmonella* spp., (c) *L. monocytogenes*, (d) *S. aureus*, (e) *P. membranaefaciens*, and (f) *S. pastorianus*. The number expressed on the block indicates percent DNA damage (%) for each wavelength.

Adenine-Thymine rich region in double-stranded DNA (dsDNA) (Gavathiotis, Sharman, & Searle, 2000). Therefore, Hoechst 33,258 was selected to measure several DNA photo-damage mechanisms such as i) pyrimidine dimer formation which induces dsDNA conformation deterioration (kinking, twisting) (Park et al., 2002), ii) oxidation of DNA bases by ROS which induces DNA breakage (Cadet & Wagner, 2013), iii) dsDNA breakage into ssDNA (Santos et al., 2013). In many research studies, there has been a concomitant result that Y have more resistance to UV treatment than GP, followed by GN (Anderson, Rowan, MacGregor, Fouracre, & Faris, 2000; Koutchma, 2009; Lopez-Malo & Palou, 2005; Hijnen, Beerendonk and Medema, 2006). Our investigation has shown that different levels of reduction among microorganism groups were clearly attributed to DNA damage generated by UVC-LED treatment through quantitative analysis. GN showed high susceptibility to UV treatment (Fig. 2a, b) and the fluorescent value of Hoechst was determined to be less 10% at 266 nm (Fig. 3a, b); the DNA damage percentage was determined to be 90% that of the positive control when treated with a conventional 254 nm UVC lamp with a dose of approximately 250 mJ/cm^2. Y, which had the highest resistance to UV (Fig. 2e, f), showed 60–70% DNA damage compared to the positive control at 266 nm treatment(Fig. 3e, f). In addition, DNA damage to *S. pastorianus* treated with 279 nm UVC-LEDs was about 10%, which achieved only 1 log reduction. Even though there were changes in membrane integrity and membrane depolarization, tenden-

cies in microorganism reduction seemed to be highly related to DNA damage. A profound association between quantitative analysis of overall DNA destruction and microorganism resistance against UVC-LED treatment could be demonstrated by our research.

5. Conclusion

Through our study, the efficacy of UVC-LEDs for inactivating microorganisms was validated, and UVC-susceptibility followed the sequence of GN > GP > Y. By using quantitative methods for measuring membrane integrity, membrane potential, and DNA damage caused by UVC-LED treatment, the primary factors affecting inactivation levels of cells were evaluated in which the level of DNA damage was primarily responsible for the trend of microorganism death.

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