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Combined treatment with a 222-nm krypton-chlorine excilamp and a 280-nm LED-UVC for inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes*

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ABSTRACT

The purpose of this study was to identify the combined effect of a 222-nm krypton-chlorine (KrCl) excilamp and a 280-nm light-emitting-diode (LED-UVC) on inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes*. Distilled water samples inoculated with pathogens were subjected to KrCl excilamp, LED-UVC, and combined treatment. The combined treatment exhibited an additive and synergistic effect on the inactivation of *S.* Typhimurium and L. *monocytogenes*. When recovery of pathogens after treatment was observed, the recovery level was reduced by combining the two treatments. Cell membrane damage was identified, and 75 s combined treatment resulted in significantly higher levels of inactivation than individual treatment in *L. monocytogenes*. These results indicate that combined treatment with a KrCl excilamp and LED-UVC can be used effectively for the inactivation of foodborne pathogens with reduced cell recovery, and increased cell membrane damage contributes to the additive effect.

1. Introduction

According to the Centers for Disease Control and Prevention (CDC), 5760 outbreaks of foodborne pathogens were reported resulting in 100,939 infections, 5699 hospitalizations and 145 deaths in the United States from 2009 to 2015 (Dewey-Mattia, Manikonda, Hall, Wise, & Crowe, 2018). These reports indicate the significance of food safety in the food industry. Therefore, various processing techniques have been developed to control foodborne pathogens. Irradiation with ultraviolet-C (UVC), a nonthermal germicidal technology, has germicidal advantages for water, air, liquid food, and fresh products (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Choudhary & Bandla, 2012). UVC disinfection is mainly performed with lamps with a mercury source; however, the Minamata Convention requires that mercury lamps be replaced because of the risks of mercury exposure in humans (Selin, 2014). As an alternative, novel technologies such as excilamps and light emitting diodes (LED)-UVC were developed. The excilamp emits light using excimer molecules, whereas a diode is used to irradiate light for LED-UVC (Kang & Kang, 2019a). observed that the 280 nm krypton-chlorine (KrCl) excilamp inactivates *Escherichia coli* O157:H7 and *Salmonella* Typhimurium effectively in apple juice. LED-UVC at 280 nm also has been proven to have pathogenic microbial inactivation effects on air, water, and food contact surfaces through previous studies (D.-Kim & Kang, 2018a, 2018b; D.-K. Kim, Kim, & Kang, 2017b). The inactivation mechanism of the excilamp is via cell membrane damage (Ha, Lee, & Kang, 2017), and LED-UVC causes DNA damage (D.-K. Kim, Kim, & Kang, 2017a). Because of the different mechanisms of action, we expected a synergistic effect by combining these two UVC treatments.

S. Typhimurium and *Listeria monocytogenes* were used for this study. Outbreaks of *Salmonella* are rampant and was the second most common pathogen causing foodborne disease outbreak from 2009 to 2015 in the United States (Dewey-Mattia et al., 2018). *L. monocytogenes* is a psychrotrophic bacteria that causes safety problems in fresh products (Stephan et al., 2015) and can cause premature birth, miscarriage, and stillbirth in infected pregnant women (Radoshevich & Cossart, 2018).

Therefore, in this study, we investigated the combined effect of a 222-nm KrCl excilamp and a 280-nm LED-UVC on inactivation of

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Salmonella Typhimurium and *Listeria monocytogenes* in-vitro. The recovery level of the pathogens and bactericidal mechanism were also compared between the individual and combined treatments.

2. Materials and methods

2.1. Bacterial cultures and cell suspension, sample preparation and inoculation

Three strains each of *S.* Typhimurium (ATCC 19585, ATCC 43971 and DT 104) and *L. monocytogenes* (ATCC 19111, ATCC 19115 and ATCC 15313) were obtained from Seoul National University (Seoul, South Korea). Single colonies from a working culture were inoculated into 5 mL of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD) and incubated at 37 °C for 24 h with shaking at 200 rpm, and the cells were collected by centrifugation at 4000 × *g* for 20 min at 4 °C. The pellets of *S.* Typhimurium and L. *monocytogenes* were resuspended with 0.2% peptone water (PW; Bactp, Becton, Dickinson, Sparks, MD) and combined to make a cocktail culture. The final concentrations of *S.* Typhimurium and L. *monocytogenes* were 10^8 and 10^9 CFU/mL, respectively. For the UVC treatment, 10 mL of the distilled water sample was prepared in a 60 × 15 mm petri dish. The cocktail culture (250 μl) of *S.* Typhimurium and L. *monocytogenes* was inoculated in a petri dish.

2.2. Experimental setup and UVC treatment

In this study, a 222-nm krypton-chlorine excilamp and 280-nm LED UVC were used as the UVC sources (S.-S. Kim, Shin, Kang, Kim, & Kang, 2020). For the combination treatment, the excilamp and LED-UVC were placed on the top of the frame, and the water sample was placed 22 cm away (Fig. 1). The intensity was measured by a previously reported method (Shin, Kim, Kim, & Kang, 2016). The modified intensities of this condition were 422.15 and 104.31 μW/cm^2 for the excilamp and LED-UVC, respectively. Treatment times were 0, 15, 30, 45, 60 and 75 s for all inactivation treatments. The treatment times were chosen based on the preliminary experiments.

2.3. Bacterial enumeration

One milliliter of UVC-treated or untreated water sample was serially ten-fold diluted with 9 mL of sterile 0.2% PW, and 0.1 mL of diluted water was plated onto each selective medium. Xylose lysine deoxycholate (XLD) agar (Difco) and Oxford agar base (OAB; Difco) with an antimicrobial supplement (Bacto Oxford antimicrobial supplement; Difco) were used as the selective medium for *S.* Typhimurium and L. *monocytogenes*, respectively. For injured cell enumeration, overlay methods were used (D. H. Kang & Siragusa, 1999). Briefly, 0.1 mL of diluted water sample was spread onto tryptic soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) and incubated at 37 °C for 2 h, and the plates were overlaid using XLD and OAB with the antimicrobial supplement for *S.* Typhimurium and L. *monocytogenes*, respectively. All plates were incubated at 37 °C for 24–48 h before counting the colonies. The recovery level of sublethally injured cells was calculated by subtracting the pathogen population on the selective media from that on the resuscitation media (overlay method). Detection limit of two pathogens in this study was 1 log CFU/ml.

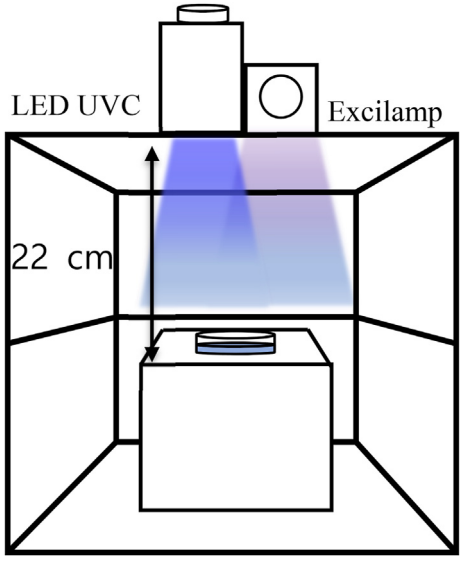

Fig. 1. Schematic diagram of the LED-UVC and excilamp treatment system at Seoul National University.

2.4. Propidium iodide uptake test

To confirm cell membrane damage, the fluorescent dye propidium iodide (PI; Sigma-Aldrich, P4170) was used. The PI uptake test was conducted according to the method described previously (Kim & Kang, 2017). For the PI test, 1 mL of each pathogen suspension was inoculated into 5 mL of DW and optical density at 600 nm (OD_{600}) was adjusted to approximately 0.2–0.3. PI was added to untreated and treated samples to a final concentration of 2.9 μM and incubated for 10 min at 37 °C. After incubation, the dyed samples were centrifuged at 10,000 × *g* for 10 min. The obtained pellets were resuspended with 1 mL of PBS and centrifuged in the same conditions, and this process was repeated twice. The final cell pellets were resuspended in 1 mL of PBS, and the fluorescence was measured with a spectrofluorophotometer (Spectramax M2e; Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 493 nm and an emission wavelength of 630 nm. The fluorescence data obtained for untreated cells were subtracted from those obtained for all treated cells and divided by the value of OD_{600} to normalizing PI uptake value against the cell density (OD_{600}). The treatment times for the PI uptake values were determined as 60 a and 75 s, respectively, by considering the pathogen inactivation results.

PI value = (fluorescence value of treated cells/OD_{600}) – (fluorescence value of untreated cells/OD_{600})

2.5. Statistical analysis

All experiments were conducted with three replicates, and the data were analyzed by analysis of variance (ANOVA) using Statistical Analysis System (version 9.3, SAS Institute, Cary, NC). Duncan's multiple-rage test was used for the post hoc analysis of the mean values. Significant differences were determined at a significance level of $p = 0.05$.

3. Results and discussion

3.1. Inactivation of S. Typhimurium and L. monocytogenes subjected to LED-UVC, the KrCl excilamp, and combined treatment

UV-C irradiation has long been used for pathogen inactivation. Minimizing the deterioration of quality is a representative advantage of UV-C irradiation compared to that of thermal treatments, but the levels of pathogen inactivation are marginal compared to those of thermal treatments. Several studies attempted to combine UV-C irradiation with other thermal or nonthermal technologies to increase antibacterial efficacy. Many researchers found a synergistic bactericidal effect of UV-C irradiation and mild heat (Gayán, García-Gonzalo, Álvarez, & Condón, 2014; Gayán, Serrano, Raso, Álvarez, & Condón, 2012; Gouma, Álvarez, Condón, & Gayán, 2015) including (Cheon, Shin, Park, Chung, & Kang, 2015) who a reported synergistic effect of UV-C irradiation and mild

Table 1
Population (log CFU/mL) of *S*. Typhimurium and L. *monocytogenes* in distilled water following excilamp, LED-UVC, and combination treatments and grown on selective media[a,b].

Treatment time (s)	*S*. Typhimurium			*L. monocytogenes*		
	LED-UVC	Excilamp	Combination	LED-UVC	Excilamp	Combination
0	7.03 ± 0.40Aa	7.03 ± 0.40Aa	7.03 ± 0.40Aa	8.14 ± 0.10Aa	8.14 ± 0.10Aa	8.14 ± 0.10 Aa
15	7.36 ± 0.19Aa	6.17 ± 0.45Bab	5.92 ± 0.69Bb	8.14 ± 0.07Aa	7.99 ± 0.03ABa	7.81 ± 0.16 Ba
30	7.01 ± 0.15Aa	5.69 ± 0.67Bbc	4.33 ± 0.56Cc	7.97 ± 0.13Aa	7.58 ± 0.11Ab	6.46 ± 0.36 Bb
45	6.66 ± 0.42Aa	4.32 ± 0.86Bd	3.14 ± 0.49Bd	7.96 ± 0.04Aa	6.90 ± 0.18Bc	5.39 ± 0.15Cc
60	5.96 ± 0.68Ab	3.33 ± 0.31Bde	2.88 ± 0.23Bd	7.68 ± 0.06Ab	6.53 ± 0.10Bd	4.51 ± 0.15Cd
75	5.53 ± 0.26Ab	3.22 ± 0.62Be	2.43 ± 0.26Bd	7.27 ± 0.20Ac	5.77 ± 0.22Be	3.51 ± 0.18Ce

Mean values ± standard deviation.
Selective media: XLD and OAB for *S*. Typhimurium and L. *monocytogenes*, respectively.
[a] Values in the same row followed by the same upper-case letter are not significantly different for each pathogen ($p > 0.05$).
[b] Values in the same column followed by the same lower-case letter are not significantly different ($p > 0.05$).

heat combination treatment for inactivation of *E.coli* O157:H7 and *S*. Typhimurium inoculated on powdered red pepper. UV-C irradiation was also combined with other nonthermal treatments or chemicals such as hydrogen peroxide (Hadjok, Mittal, & Warriner, 2008), fumaric acid (Y. Kim, Kim, & Song, 2009), ozone (Rodriguez-Romo & Yousef, 2005), and chlorine dioxide gas (Park, Kang, & Kang, 2018). Moreover (Kang & Kang, 2019b), recently reported the synergistic bactericidal mechanisms of simultaneous 222-nm KrCl excilamp and 254-nm low-pressure mercury lamp treatment, which indicated the possibility of combination treatment with two or more UV-C irradiation methods with different wavelengths.

In the present study, two alternative UV-C technologies (280 nm LED-UVC and a 222 nm KrCl excilamp) were combined. They exhibited an accelerated effect on the inactivation of *S*. Typhimurium and L. *monocytogenes* (Table 1). The combined treatment showed an additive effect for inactivation of *S*. Typhimurium while a synergistic effect was observed for inactivation of *L. monocytogenes*. For example, reduction levels (log CFU/mL) of *S*. Typhimurium after 60 s treatment were 1.74, 3.33, and 4.30 for LED-UVC, excilamp, and combination treatment, respectively. Meanwhile, reduction levels (log CFU/mL) of *L. monocytogenes* after 60 s treatment were 0.40, 1.54, and 3.63 for LED-UVC, excilamp, and combination treatment, respectively. The resistance of *L. monocytogenes* was higher than that of *S*. Typhimurium for every treatment. Other studies also indicated that the resistance of Gram-positive pathogens was higher than those of the Gram-negative pathogens when 222 nm KrCl excilamp (J.-W. Kang, Kim, & Kang, 2018) or LED-UVC (Shin et al., 2016) were used for disinfection. About these results (McKinney & Pruden, 2012), explains that Gram-positive microorganisms are protected against LED-UVC by their thicker peptidoglycan layer. Even though the additive bactericidal impact of the combined treatment was dependent on the treatment time and type of pathogen, the applicability of the combined treatment to increase the bactericidal effect was shown.

3.2. Recovery of S. Typhimurium and L. monocytogenes subjected to LED-UVC, KrCl excilamp, and combined treatment

Recovery of foodborne pathogens has been reported continuously; this is a potential microbiological hazard after processing (Han, Song, & Kang, 2019; Wesche, Gurtler, Marks, & Ryser, 2009; Wu, 2008). Some portion of pathogens that are damaged but not fully inactivated will not grow in selective media because the selectivity of media is too strong. Optimization of processing conditions based on the inactivation level determined on selective media is problematic because damaged pathogens recover and grow in favorable conditions. Therefore, the resuscitation level should be checked after thermal or nonthermal treatments. There are various cell recovery methods such as pour-overlay, surface-overlay plating method, thin agar layer, and agar underlay as reviewed by (Wu, 2008), but the surface-overlay plating method has been widely used to determine cell resuscitation after bactericidal treatments including UV-C irradiation (Ha & Kang, 2018). determined the resuscitation level of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* after 222-nm KrCl excilamp treatment in water and reported that significant resuscitation was observed only for *S*. Typhimurium (S.-J. Kim, Kim, & Kang, 2016). also reported that significant cell recovery was observed for *S*. Typhimurium, but not for *E. coli* O157:H7 and *L. monocytogenes*, when 266–279 nm LED-UVC was used for pathogen inactivation. The same trend was observed in the present study, indicating that the overall resuscitation levels of *S*. Typhimurium were higher than those of *L. monocytogenes* even though the resuscitation level was dependent on the type of pathogen and bactericidal treatment (Table 2). For *S*. Typhimurium, the resuscitation level was the greatest after treatment with the KrCl excilamp followed by combined treatment and LED-UVC irradiation. The resuscitation level was

Table 2
Population (log CFU/mL) of *S*. Typhimurium and L. *monocytogenes* in distilled water following excilamp, LED-UVC, and combination treatments and enumerated with resuscitation media[a,b].

Treatment time (s)	*S*. Typhimurium			*L. monocytogenes*		
	LED-UVC	Excilamp	Combination	LED-UVC	Excilamp	Combination
0	7.83 ± 0.11Aa	7.83 ± 0.11Aa	7.83 ± 0.11Aa	8.24 ± 0.04Aa	8.24 ± 0.04Aa	8.24 ± 0.04Aa
15	7.83 ± 0.06Aa	7.46 ± 0.04Bb	7.15 ± 0.13Cb	8.10 ± 0.06Aa	8.13 ± 0.09Aa	7.86 ± 0.07Bb
30	7.37 ± 0.03Ab	6.77 ± 0.08Bc	5.60 ± 0.39Cc	8.07 ± 0.02Aa	7.66 ± 0.13Bb	6.58 ± 0.26Cc
45	7.12 ± 0.05Ac	6.09 ± 0.26Bd	4.25 ± 0.12Cd	7.82 ± 0.20Ab	6.89 ± 0.09Bc	5.35 ± 0.11Cd
60	6.70 ± 0.12Ad	5.42 ± 0.24Be	3.71 ± 0.59Cd	7.70 ± 0.02Ab	6.21 ± 0.12Bd	4.58 ± 0.09Ce
75	6.14 ± 0.04Ae	3.70 ± 0.20Bf	2.80 ± 0.26Ce	7.17 ± 0.13Ac	5.73 ± 0.29Be	3.02 ± 0.18Cf

Mean values ± standard deviation.
Resuscitation media: OV-XLD and OV-OAB for *S*. Typhimurium and L. *monocytogenes*, respectively.
[a] Values in the same row followed by the same upper-case letter are not significantly different for each pathogen ($p > 0.05$).
[b] Values in the same column followed by the same lower-case letter are not significantly different ($p > 0.05$).

Table 3
PI uptake values of *S*. Typhimurium and L. *monocytogenes* by LED-UVC, excilamp, and combined treatments.

	Time	LED-UVC	Excilamp	Combination
S. Typhimurium	60	5.24 ± 2.71A	14.88 ± 1.48B	13.75 ± 3.45B
	75	9.35 ± 3.32A	22.45 ± 1.62B	27.17 ± 2.08B
L. monocytogenes	60	11.51 ± 4.29A	16.17 ± 3.34A	16.79 ± 3.16A
	75	9.70 ± 1.40A	17.88 ± 3.78B	23.94 ± 3.36C

Mean values ± standard deviation.
[1]Values in the same row followed by the same letter are not significantly different ($p > 0.05$).

the highest after 60 s KrCl excilamp, indicating the 2.09 log CFU/mL while the highest resuscitation level after combined treatment and LED-UVC irradiation were 1.27 and 0.08 log CFU/mL, respectively. From these results, we suggest that combined treatment of KrCl excilamp with LED-UVC can reduce the cell resuscitation level compared to KrCl excilamp treatment alone.

3.3. Cell membrane damage of S. Typhimurium and L. monocytogenes subjected to LED-UVC, KrCl excilamp, and combined treatment

The bactericidal mechanism of an individual or combined treatment has long been of interest (Liao et al., 2017; Maness et al., 1999). The inactivation mechanism of UV-C irradiation is known to be dependent on the wavelength and irradiation source. The conventional 254-nm Hg UV-C lamp is known to damage DNA, primarily by forming 6-4 pyrimidine dimers (Ha, Back, Kim, & Kang, 2016). (D.-K. Kim et al., 2017a) reported that DNA damage is the primary factor affecting pathogen inactivation by 266- to 279-nm LED UV-C treatment. However (J.-W. Kang et al., 2018), indicated that the 222-nm KrCl excilamp would affect not only the DNA but also cellular enzymes or membrane lipids. When DNA damage was measured in the present study, a distinct trend was not observed (data not shown). Therefore, cell membrane damage after 60 or 75 s LED-UVC, KrCl excilamp, and combined treatment was detected by measuring PI uptake (Table 3). For *S*. Typhimurium, PI uptake values of the combined treatment were significantly higher than those of LED-UVC, but not for excilamp, regardless of treatment time. For L. *monocytogenes*, PI uptake values after 60 s were not significantly different among the treatments, while PI uptake value after 75 s combined treatment was significantly higher than that of the LED-UVC and excilamp treatments individually. These results indicated that increased cell membrane damage contributes to the accelerated pathogen inactivation caused by combination treatment.

4. Conclusion

Combined treatment with a KrCl excilamp and LED-UVC exhibited an additive or synergistic bactericidal effect in the present study. Even though some pathogen recovery after bactericidal treatment was observed, the recovery level was reduced by combining the two bactericidal treatments. The bactericidal mechanism of the combined treatment was suggested to be increased cell membrane damage. The results of the present study could be used as fundamental data for the application of the combined treatment to ensure the microbiological safety of drinking water or liquid food products.

CRediT authorship contribution statement

Minjung Shin: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Software. **Sang-Soon Kim:** Conceptualization, Data curation, Writing - original draft, Visualization. **Dong-Hyun Kang:** Resources, Supervision, Project administration.

Declaration of competing interest

No conflicts of interest to declare.

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