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in Agricultural Biotechnology

Application of a 222-nm Krypton-Chlorine excilamp to control *Alicyclobacillus acidoterrestris* spores

알리사이클로바실러스 에시도테레스트리스 포자를
제어하기 위한 222-nm KrCl 엑시램프의 적용

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The Graduate School
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Department of Agricultural Biotechnology

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ABSTRACT

Alicyclobacillus acidoterrestris spores, which have high resistance to thermal treatment and can germinate even at low pH, are very troublesome in the juice industry. UV technology, a nonthermal treatment, can be an excellent means to control heat-resistant *A. acidoterrestris* spores in place of thermal treatment. However, the traditionally applied UV sources are lamps that contain mercury (Hg), which is harmful to humans and the environment; thus, there is a need to apply novel UV technology without the use of Hg. In response to this issue, excilamps, an Hg-free UV source, have been actively studied. However, no studies have been conducted applying this technique to control *A. acidoterrestris* spores. The aim of this study was to investigate the sporicidal effect of a krypton-chlorine (KrCl) excilamp against *A. acidoterrestris* spores and to compare its inactivation mechanism to that of a conventional UV lamp containing mercury (Hg). The inactivation effect of the KrCl excilamp was not significantly different from that of the Hg UV lamp for *A. acidoterrestris* spores in apple juice despite the 222-nm

wavelength of the KrCl excilamp having a higher absorption coefficient in apple juice than the 254-nm wavelength of the Hg UV lamp; this is because KrCl excilamps have a fundamentally greater inactivation effect than Hg UV lamps, which is confirmed under ideal conditions (phosphate-buffered saline). The inactivation mechanism analysis revealed that the DNA damage induced by the KrCl excilamp was not significantly different ($P > 0.05$) from that induced by the Hg UV lamp, while the KrCl excilamp caused significantly higher ($P < 0.05$) lipid peroxidation incidence and permeability change in the inner membrane of *A. acidoterrestris* spores than did the Hg UV lamp. Meanwhile, the KrCl excilamp did not generate significant ($P > 0.05$) intracellular reactive oxygen species, indicating that the KrCl excilamp causes damage only through the direct absorption of UV light. In addition, after KrCl excilamp treatment with a dose of 2,011 mJ/cm^2 to reduce *A. acidoterrestris* spores in apple juice by 5 logs, there were no significant ($P > 0.05$) changes in quality parameters such as color (L*, a*, and b*), total phenolic compounds, and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical

scavenging activity. Therefore, the results of this study, which applied a KrCl excilamp for the control of *A. acidoterrestris* spores and elucidated the inactivation principle, are expected to be utilized as important basic data for application to actual industry or conducting further studies.

Keywords: 222-nm KrCl excilamp, *Alicyclobacillus acidoterrestris*, spore, inactivation mechanism, apple juice

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I. INTRODUCTION

Traditionally, a thermal pasteurization process that maintains products at 86 to 96°C for about 2 min has been applied in the fruit juice industry, and the processed products are stored in a refrigerator or on shelves (Smit et al., 2011; Steyn et al., 2011). These thermal process conditions can inactivate non-spore-forming microorganisms that have the potential to spoil the product. Furthermore, fruit juice products are usually acidic (pH < 3.8), which prevents germination and growth after thermal processes, and the acidic conditions of these products make them microbiologically stable (Huertas et al., 2014; Silva et al., 2015).

In 1982, however, a new type of bacterium was reported that caused spoilage in aseptically packed apple juice (Cerny et al., 1984); subsequently, this spoilage bacterium was classified as a new genus, *Alicyclobacillus*, through 16S RNA sequencing analyses and the unique characteristic that this bacterium has the major lipid component of its cellular membrane called ω-alicylic fatty acid (Wisotzkey et al., 1992). Among various *Alicyclobacillus* species, *Alicyclobacillus acidoterrestris* is regarded as the most important spoilage microorganisms because it has frequently been observed in commercially processed fruit juice products and has been associated with spoilage problems (Chang and Kang, 2004; Groenewald et al., 2009; Kim et

al., 2017; Silva et al., 1999). *A. acidoterrestris*, which is a thermophilic, spore-forming, Gram-positive, nonpathogenic spoilage microorganism, and acidophilic, has ability to grow at a wide range of temperature (26 – 60°C), and its spores have a high thermal resistance, with a D-value of 16 – 23 min at 90°C, and can germinate in acidic products pH 3.0 – 4.5 (Spinelli et al., 2009; Splttstoesser et al., 1994). Therefore, *A. acidoterrestris* can survive even after the conventional pasteurization process, and the surviving spores can cause spoilage problems by germinating and proliferating in fruit juices (Baysal et al., 2013; Chang, 2008; Spinelli et al., 2009).

A. acidoterrestris uses vanillin and tyrosine as precursors to form guaiacol; this organic compound exhibits a distinct phenolic, medical, or antiseptic odor, and off-flavor by this compound is recognized when cell growth reaches 10^4 – 10^5 colony-forming units (CFU) (Bahçeci et al., 2005; Pettipher et al., 1997; Shi et al., 2018). However, since spoilage caused by *A. acidoterrestris* has no apparent signs such as increased turbidity, sedimentation, or swelling, it is difficult for processors to recognize the occurrence of spoilage, so the spoilage of juice products caused by this bacterium is noticed after the products have been delivered to consumers (Chang and Kang, 2004; Pettipher et al., 1997). Therefore, it is very

important to inactivate *A. acidoterrestris* before final juice products are shipped (Kim et al., 2017).

As a solution to the spoilage problem caused by *A. acidoterrestris*, an approach of increasing the thermal treatment intensity may be proposed, but this method is rarely feasible due to the increased temperature and processing time, which can incur undesired nutritional and sensory properties of the product (Bevilacqua et al., 2009; Huertas et al., 2014). Therefore, the application of nonthermal technologies can be an alternative; among them, ultraviolet (UV) irradiation is regarded to be a great substitution of thermal treatment because of its control effect on a wide range of pathogens, as well as advantages such as its easy application to existing processes, the lack of residual disinfectant, and high energy efficiency (Chen et al., 2017; Guerrero-Beltr· n and Barbosa-C· novas, 2004). The UV disinfection system is conventionally based on lamps containing mercury (Hg) because Hg UV lamps are electrically efficient and inexpensive compared to other UV sources (Naunovic et al., 2008). However, there is always a concern that using conventional UV lamps is a potential hazard since Hg can harm humans and pollute the environment (Chevremont et al., 2013; Close et al., 2006). In response, over the last few decades, many researchers have introduced dielectric barrier discharge (DBD)-driven Hg-free excimer lamps

(excilamps) and confirmed their potential as a UV disinfection system to replace conventional Hg lamps. Compared with conventional Hg UV lamps, excilamps have several merits, including being mercury-free, as well as having wavelength selectivity, lamp geometry freedom, high UV irradiation intensity, and intensity stability over a wide range of temperatures (Ha et al., 2017; Kang et al., 2018; Matafonova and Batoev, 2012; Sosnin, 2007; Sosnin et al., 2006). Excilamps emit UV radiation that is almost monochromatic from 172 – 345 nm, and several researchers have conducted studies applying various types of excilamp such as Xe_2, KrCl, KrF or XeBr excilamp, with peak emissions of 172, 222, 248 or 282 nm, respectively, to spore control (Clauß, 2006; Pennell et al., 2008; Wang et al., 2010; Warriner et al., 2002; Warriner et al., 2000). In particular, the results of researches by Pennell et al (Pennell et al., 2008) and Wang et al (Wang et al., 2010) comparing the control effect of a conventional Hg UV lamp (254 nm) and Xe_2 (172 nm), krypton-chlorine (222 nm) and XeBr excilamps (282 nm) on *Bacillus subtilis* spores indicated that the KrCl excilamp exhibits the greatest inactivation effect; thus, it can be considered that the KrCl excilamp is a highly promising candidate to effectively control *A. acidoterrestris* spores in fruit juices.

Despite the high applicability of the KrCl excilamp, no study to date has been conducted to control *A. acidoterrestris* spores using excilamps, including the KrCl excilamp. Therefore, in this study, I investigated the control effect of a KrCl excilamp on *A. acidoterrestris* spores in apple juice compared to that of a conventional Hg UV lamp. In addition, since the understanding of the control principle can be used as an important basis for further research and to establish an effective application strategy, the inactivation mechanism of the KrCl excilamp against *A. acidoterrestris* spores was elucidated by various approaches.

II. MATERIALS AND METHODS

2.1. Bacterial strains and culture preparation

A. acidoterrestris ATCC 49025 (same as DSM 3922) was obtained from the Korean Culture Center of Microorganisms. Cultures were grown at 43°C for 2 days on orange serum agar (OSA; MB cell, Seoul, Korea) acidified to pH 3.7 using 10 % tartaric acid (Junsei Chemical Co. Inc., Tokyo, Japan), maintained at 4°C, and sub-cultured monthly (Bae et al., 2009).

2.2. Preparation of spore inoculum

The cultures grown for 2 days at 43°C on OSA were streaked onto potato dextrose agar (PDA, pH 5.6; Difco, Becton and Dickinson company; Sparks, MD, USA) and incubated at 43°C for 7 days until at least 80 % sporulation, as confirmed by microscopic examination. To prepare spore suspensions, the 3 ml of sterile PBS was added to the surface of the PDA culture plates and smoothly rubbed with a sterile swab. The spore suspensions collected from 10 plates were centrifuged at 4,000 × g for 20 min at 4°C. After centrifugation, the supernatant was discarded, the pelleted cells were washed three times with PBS and the final pelleted cells were resuspended in 10 ml of PBS. Concentration of spore suspensions was approximately 10^7 – 10^8

spores/ml. This inoculum was used immediately for the experiment on the day it was made.

2.3. Sample preparation

In this study, pasteurized and clarified apple juice (Woongjin Foods Co., Seoul, South Korea) commercially available and PBS were used as samples to conduct experiment. These samples were kept in a refrigerator at 4°C until use. The apple juice sample acquired from a local grocery market had pH 2.93, 0 turbidity [nephelometric turbidity units (NTU)], and 12.5 sugar concentration [degree Brix (°Bx)]. To measure the absorption coefficient [α (cm^{-1})] of apple juice at 222 nm or 254 nm, the absorbance values of apple juice at 222 nm or 254 nm for several dilutions [1:10, 1:25, 1:50, 1:100, 1:250, and 1:500 (vol/vol)] were measured using a quartz cuvette with a 1 cm path length in a spectrophotometer. The slope of the absorbance value plot to the sample concentration was used to calculate the absorption coefficient. One to two hours before the experiment, the samples taken from refrigerated conditions were adjusted to room temperature (22 ± 2°C) by equilibration. A Petri dish (60 mm x 15 mm) with the lid removed was filled with five milliliters of each sample, and 0.1 ml of inoculum was inoculated into the sample.

2.4. Experimental setup and treatment

A DBD-driven excilamp (110 W; 29 cm x 9 cm x 9 cm; UNILAM, Ulsan, South Korea) filled with a KrCl gas mixture and a conventional Hg UV lamp (16 W; G10T5/4P; 357 mm; Sankyo, Japan) were used as the UV irradiation sources, and detailed specifications of these two lamps were depicted in previous study (Kang et al., 2018). The inoculated sample was placed onto a magnetic stirrer plate (TM-17R; Jeio Tech, Daejeon, South Korea), and the lamp was positioned vertically and directly toward the sample at a distance of 5 cm from the center of the sample surface. In this experimental setup, the UV intensities of the KrCl excilamp and Hg UV lamp on the sample surface were measured with a UV fiber optic spectrometer (AvaSpec-ULS2048; Avantes, Eerbeek, Netherlands) calibrated to a range of 200 to 400 nm within the UVC spectrum, and in this case, the integral irradiation intensities from 200 nm to 240 nm for the KrCl excilamp and from 230 nm to 270 nm for the Hg UV lamp were measured to include the whole intensity emitted by each UV source because it represents a sharply decreasing intensity distribution around the peak wavelength (222 nm for the KrCl excilamp and 254 nm for the Hg UV lamp); the light intensities of the KrCl excilamp and Hg UV lamp were 6.7 and 6.9 mW/cm^2, respectively. Before treatment, each lamp was operated for 15 min to stabilize, and then, the apple juice (pH 2.93,

12.5 °Brix, and 0 NTU) and the PBS were treated with the lamp at doses of 0 to 1,500 mJ/cm^2 and 0 to 40 mJ/cm^2, respectively, at room temperature (22 ± 2°C) while being mixed with a magnetic stirrer (1.5 cm x 0.1 cm) at 500 rpm; UV doses were calculated by multiplying the UV light intensity by the irradiation times.

2.5. Spore enumeration

After treatment, a water bath at 80°C for 20 min was used to inactivate surviving vegetative cells in samples so that only spores remained. Then samples immediately put in an ice-water bath. For enumeration of spores, 1-ml aliquots of each sample were 10-fold serially diluted into 9 ml of 0.2 % peptone water (PW; Bacto, Becton and Dickinson company; Sparks, MD, USA), and 0.1-ml aliquots of diluents or 1-ml aliquots of the undiluted original sample were spread onto OSA acidified to pH 3.7 using 10 % tartaric acid. Plates were incubated for 2 days at 43°C, and then, the number of spores was enumerated by counting colonies and expressed as $\log_{10}$ colony-forming units per milliliter (CFU/ml).

2.6. Identification of the inactivation mechanism of the KrCl excilamp against *A. acidoterrestris* spores

The sporicidal mechanism of the KrCl excilamp against *A. acidoterrestris* spores was investigated by analyzing the structural damage and lipid peroxidation occurrence at the spore membrane, DNA damage and generation of ROS compared to that with a conventional Hg UV lamp. To observe the phenomena that only occur in spores, the previously stated spore inoculum was disrupted using a sonicator (Bandelin Sonoplus HD 2070, Bandelin Elec., Germany) at 70% of power for 2 min in a water bath containing ice and centrifuged at 10,000 × g for 20 min at 4°C; the supernatant with debris of the disrupted vegetative cells was discarded, and the pelleted cells were washed three times with PBS to leave only spores. It was confirmed by microscopic examination that only the spores remained. Furthermore, in order to investigate whether the application of sonicator had a negative effect on the spores, survival rates of spores with 2 min sonication treatment for each lamp were obtained through the above treatment procedure and compared with those of spores in PBS without sonication treatment; it was confirmed that no significant ($P > 0.05$) survival rate difference between spores with and without sonication treatment occurred (TABLE 1). Therefore, it can be considered that the 2 min sonicator

treatment allows the removal of only vegetative cells without negatively affecting the spores, thus facilitating the analysis of the inactivation mechanisms. The previously stated spore inoculum prepared with sonicator treatment was inoculated in PBS and treated with the KrCl excilamp and Hg UV lamp at dosages from 0 to 40 mJ/cm^2. Optical density at 600 nm (OD_{600}) of the spore suspension in PBS was measured before each treatment to normalize the analysis results.

TABLE 1. Log reduction (Log_{10} CFU/ml) [a] of *Alicyclobacillus acidoterrestris* spores with or without sonication application suspended in PBS following KrCl excilamp treatment

Treatment	Sonication	Treatment dose (mJ/cm^2)				
		5	10	20	30	40
KrCl excilamp	Applied	1.22 ± 0.23	2.41 ± 0.35	3.25 ± 0.34	4.68 ± 0.16	5.32 ± 0.38
	Not applied	1.22 ± 0.16	2.14 ± 0.23	3.32 ± 0.36	4.80 ± 0.36	5.77 ± 0.63
Hg UV lamp	Applied	0.50 ± 0.12	1.02 ± 0.11	2.14 ± 0.24	3.52 ± 0.21	4.15 ± 0.09
	Not applied	0.33 ± 0.04	0.90 ± 0.10	1.85 ± 0.24	3.52 ± 0.34	4.23 ± 0.24

[a]Values are means ± standard deviations from three replications. For each lamp treatment, there were no significant ($P > 0.05$) log reduction differences between spores with and without sonication application.

2.6.1. Confirmation of cell membrane damage

The fluorescent dyes DPPP and PI were utilized to quantitatively analyze the occurrence of lipid peroxidation and structural damage of the cell membranes of spores, respectively. The PI, which does not penetrate intact cell membrane, can enter the cells when structural damage occurs such as pores formation in the cell membrane and then can bind with nucleic acid and become a fluorescent form (Breeuwer and Abee, 2000; Hewitt and Nebe-Von-Caron, 2004). Therefore, the fluorescence intensity produced from PI binding with nucleic acids within the cell can be used to evaluate the degree of structural damage in the inner membrane of spores (Mathys et al., 2007; Rao et al., 2016). The DPPP with high lipophilicity, a nonfluorescent probe, can be selectively reacted with lipid hydroperoxide within the biomembrane and then converted to fluorescent DPPP oxide (DPPP=O) (Okimoto et al., 2000). Therefore, the fluorescence intensity generated by DPPP=O can be used to evaluate the degree of the occurrence of lipid peroxidation in the spore membrane (Hashizume et al., 2013)

Following the treatments, the treated samples were reacted with PI (Sigma-Aldrich, St. Louis, MO, USA) or DPPP (Sigma-Aldrich) at a final concentration of 2.9 or 50 μM for 10 or 20 min at 37°C, respectively, and centrifuged at 10,000 × g for 10 min to collect cells. After twice washing

with PBS, the fluorescence intensity was measured with a spectrofluorophotometer (Spectramax M2e; Molecular Devices, CA, USA) at excitation/emission wavelengths of 493/630 nm for the PI uptake assay or 351/380 nm for the DPPP assay. For quantitative comparison, the results were represented as values in which the obtained fluorescence intensity was divided by the OD_{600}.

2.6.2. Measurement of intracellular ROS generation

CM-H_2DCFDA was utilized to detect ROS generation within the cells. This compound can freely enter the cell and then be hydrolyzed into the dichlorofluorescin (DCFH) carboxylate anion, which can be converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by reacting with ROS (Kalyanaraman et al., 2012; Negre-Salvayre et al., 2002; Wojtala et al., 2014). Therefore, increased fluorescence intensity of DCF can be used to evaluate the degree of the generation of ROS within the spore (Zhao, Wisniewski, et al., 2014).

Following the treatments, the treated samples were reacted with CM-H_2DCFDA (Invitrogen, Carlsbad, CA, USA) at a final concentration of 5 μM for 15 min at 37°C, and centrifuged at 10,000 × g for 10 min to collect cells. After twice washing with PBS, and the fluorescence intensity was measured

with a spectrofluorophotometer at excitation/emission wavelengths of 495/520 nm. For quantitative comparison, the results were expressed as values in which the obtained fluorescence intensity was divided by the OD_{600}.

2.6.3. DNA integrity assessment

SYBR green I (Sigma-Aldrich) was utilized to evaluate DNA integrity. SYBR green I is a probe that exhibits fluorescence when binding to double strand DNA (dsDNA) (Dragan et al., 2012). Therefore, since SYBR green I does not bind at the place of damage in the DNA strand, the degree of DNA integrity can be evaluated by using the degree of decreased fluorescence intensity (Han et al., 2016).

Following the treatments, intracellular DNA was isolated from the spores using an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated DNA was reacted with 1X SYBR green I (Molecular Probes, Eugene, OR, USA) for 15 min at 37°C. After incubation, the fluorescence intensity was measured with a spectrofluorophotometer at excitation/emission wavelengths of 485/525 nm. For quantitative comparison, the results were expressed as values in which the obtained fluorescence intensity was dividing by the OD_{600}.

2.7. Measurement of apple juice quality

Apple juice samples were treated with the KrCl excilamp at a dosage of 2,011 mJ/cm^2, the amount of energy needed to reduce the *A. acidoterrestris* spore count by 5-log (D_{5d} value). This D_{5d} value was calculated by applying the survival curve of spores in apple juice exposed to the KrCl excilamp treatment (FIG. 1B) to the prediction model, and in this case, the Weibull model equation was used as a predictive model because of its low root mean square error (< 0.22) and a high R^2 (> 0.97) values (TABLE 2).

2.8. Statistical analysis

All experiments were performed in three replicates. Obtained data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and the mean values were separated using the least significant difference (LSD) t-test. Significant differences were determined at a probability level of $P < 0.05$.

TABLE 2. Parameters of Weibull model for inactivation of *Alicyclobacillus acidoterrestris* ATCC 49025 in phosphate buffer saline (PBS) treated with a KrCl excilamp and the calculated D_{5d} value which represent dosage necessary for achieving 5-log reduction using model equation.

Parameters				D_{5d}
δ	*p*	R^2	RMSE	
31.83 ± 1.19	0.39 ± 0.02	0.97 ± 0.01	0.21 ± 0.03	2,011 ± 557

The values are means ± standard deviations for three replications. δ time to first decimal reduction; *p*, shape parameter; RMSE, root mean square error; D_{5d}, Dosage necessary to achieve a 5-log reduction.

III. RESULTS AND DISCUSSIONS

3.1. Inactivation effect of a KrCl excilamp against A. acidoterrestris spores compared with a Hg UV lamp

FIG. 1 depicts surviving populations of *A. acidoterrestris* spores in phosphate-buffered saline (PBS) (A) and apple juice (B) after treatment with a KrCl excilamp and conventional Hg UV lamp. The survival levels of *A. acidoterrestris* spores in PBS and apple juice were significantly ($P < 0.05$) decreased with increasing treatment dose from 0 to 40 mJ/cm^2 for PBS and from 0 to 1,500 mJ/cm^2 for apple juice. For *A. acidoterrestris* spores in PBS, the inactivation effect of the KrCl excilamp was significantly ($P < 0.05$) better than that of Hg UV lamp. However, unlike the results in PBS, the inactivation effect of the KrCl excilamp on *A. acidoterrestris* spores in apple juice was not significantly ($P > 0.05$) different from that of the Hg UV lamp. This result can be demonstrated by the phenomena that occur when UV light penetrates the apple juice sample. Colored compounds, organic matter, and suspended particles in fresh juice products limit UV light transmission, and this lower UV light transmission can negatively affect the performance of the UV pasteurization process (Koutchma et al., 2007). Indeed, some studies have reported that the control performance of UV light intensity was reduced as the amount of insoluble particles in the sample or the UV absorbance

increases, which is interpreted as follows: with an increasing concentration of suspended particles in the medium (increasing turbidity), the shielding effect against UV light by the particles increases, and as the medium absorbs more UV light, a lower intensity of UV light actually reaches the medium (Murakami et al., 2006; Oteiza et al., 2005). Because the apple juice sample used in this study has no turbidity (0 NTU), the inactivation effect of each lamp on *A. acidoterrestris* spores in apple juice samples can be considered to be affected by the sample absorbing the UV wavelength emitted by each lamp. According to the Bouguer-Lambert-Beer law, the intensity of light transmitted through a medium can be calculated as a value that is inversely proportional to the absorption coefficient (α) of the medium for the given wavelength of light (Willard et al., 1988). Therefore, to quantitatively compare the UV absorption characteristics of the apple juice sample of this study for each lamp, we calculated the absorption coefficient for the wavelength of each lamp. As a result, it was found that the absorption coefficient of the apple juice at 222 nm is significantly ($P < 0.05$) larger than that at 254 nm (α_{222}: 15.73 cm^{-1} and α_{254}: 6.91 cm^{-1}). That is, it can be inferred that fundamentally, the KrCl excilamp with 222 nm has a greater control capability against *A. acidoterrestris* spores than the Hg UV lamp with 254 nm does because the former showed a greater inactivation effect

than the latter against *A. acidoterrestris* spores in PBS under ideal conditions in which complete transmission at 222 nm and 254 nm was allowed; however since more absorbance occurs at 222 nm than at 254 nm in apple juice, the UV energy of the 222 nm light actually reaching the spores in apple juice is reduced more than that of the 254 nm light, and thus, both lamps showed a similar inactivation effect in apple juice. The Minamata Convention, which has gradually restricted the use of mercury in products or processes, has been signed by the United Nations Environment Program; thus, there is a growing need to develop new alternative technologies to replace conventional UV lamps containing mercury ((UNEP), 2013). Although apple juice, which is characterized by a greater absorption of light at 222 nm than 254 nm, exhibits a greater disturbance in the control effect for a KrCl excilamp than a Hg UV lamp, the KrCl excilamp can exhibit similar control effects on *A. acidoterrestris* spores in apple juice because the 222 nm wavelength shows a fundamentally greater control effect than that of the 254 nm wavelength. Therefore, this result suggests that the KrCl excilamp has the potential to replace conventional Hg UV lamps in the juice industry.

The results of Wang et al's study (Wang et al., 2010) comparing the sporicidal effect of irradiation at 172, 222, and 254 nm on *Bacillus subtilis*

spores under ideal conditions (deionized water) and Pennell et al's study (Pennell et al., 2008) comparing the inactivation effect of 222, 254, and 282 nm on *B. subtilis* spores under ideal conditions (nanopure water) have indicated that the wavelength of 222 nm exhibits the greatest inactivation effect on *B. subtilis* spores. However, Clauß's study (Clauß, 2006) comparing the inactivation effects of irradiation at 222 and 254 nm on spores of various species under ideal conditions (demineralized water) showed that the inactivation effect of 222 nm light was greater than that of 254 nm light for spores of *B. cereus* and *Thermoactinomyces vulgaris*, but for *Clostridium pasteurianum*, there was no significant difference in the inactivation effect between the two wavelengths; rather, for *Streptomyces griseus* spores, irradiation at 254 nm showed a larger inactivation effect than that at 222 nm. Therefore, for *A. acidoterrestris* spores, the KrCl excilamp with 222 nm shows a greater inactivation effect than the Hg UV lamp with 254 nm, but depending on the species of the spore, the inactivation effect between the two lamps can vary.

Understanding the inactivation principle of the KrCl excilamp on *A. acidoterrestris* spores can be an important basis for practical application of this excilamp to industries that want to control *A. acidoterrestris* spores or

perform further research on it, and it is crucial to identify the inactivation mechanism of the KrCl excilamp against *A. acidoterrestris* spores.

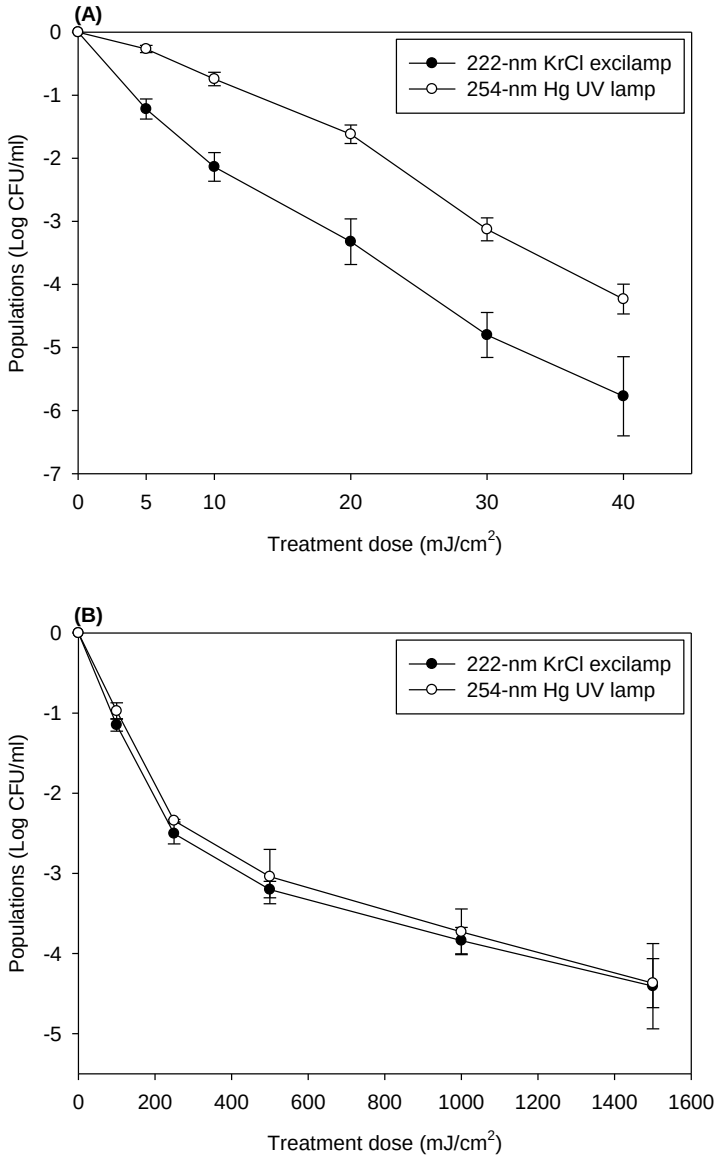

Fig. 1. Populations (Log CFU/ml) of the spores of *A. acidoterrestris* in PBS (A) and apple juice (B) treated with the 222-nm KrCl excilamp or 254-nm mercury (Hg) UV lamp. Each point and error bar indicate the means and standard deviations calculated from three replicates, respectively.

3.2. Identification of the inactivation mechanism of the KrCl excilamp against A. acidoterrestris spores

3.2.1. Inner membrane damage of A. acidoterrestris spores

To identify the inactivation mechanism of the KrCl excilamp against *A. acidoterrestris* spores, the inner membrane damage of spores was first examined to assess where the damage occurred. As shown in FIG. 2, the propidium iodide (PI) uptake value of *A. acidoterrestris* spores significantly ($P < 0.05$) increased after the KrCl excilamp irradiation compared with that of the untreated control, and the values significantly ($P < 0.05$) increased as the treatment dose increased. On the other hand, treatment with the Hg UV lamp did not make the PI uptake value significantly ($P > 0.05$) different from that of the untreated control until the 30 mJ/cm^2 treatment, and there was a significant ($P < 0.05$) slight increase in the PI uptake value compared with that of the untreated control after the 40 mJ/cm^2 treatment. These results indicate that compared to the Hg UV lamp, the KrCl excilamp effectively damages the inner membrane of *A. acidoterrestris* spores. Several researches applying various techniques to kill spores such as thermosonication (Fan et al., 2019), supercritical CO_2-peracetic acid treatment (Setlow et al., 2016), high-pressure CO_2 treatment (Rao et al., 2016), and electron beam irradiation (Fiester et al., 2012) have also found that the increased permeability leads to

the inner membrane damage of spores after treatment; thus, it can be considered that this form of membrane damage has a major role in decreasing the viability of spores. Such observation suggests that spores with damage to the inner membrane are no longer able to germinate or that even if spores can germinate, they do not exhibit proper metabolism because the plasma membrane of the germinated spore is derived from the damaged inner membrane, eventually leading to death (Cortezzo et al., 2004; Setlow et al., 2016). Therefore, the results are interpreted as the KrCl excilamp inducing inactivation of the *A. acidoterrestris* spores by damaging the spore inner membranes.

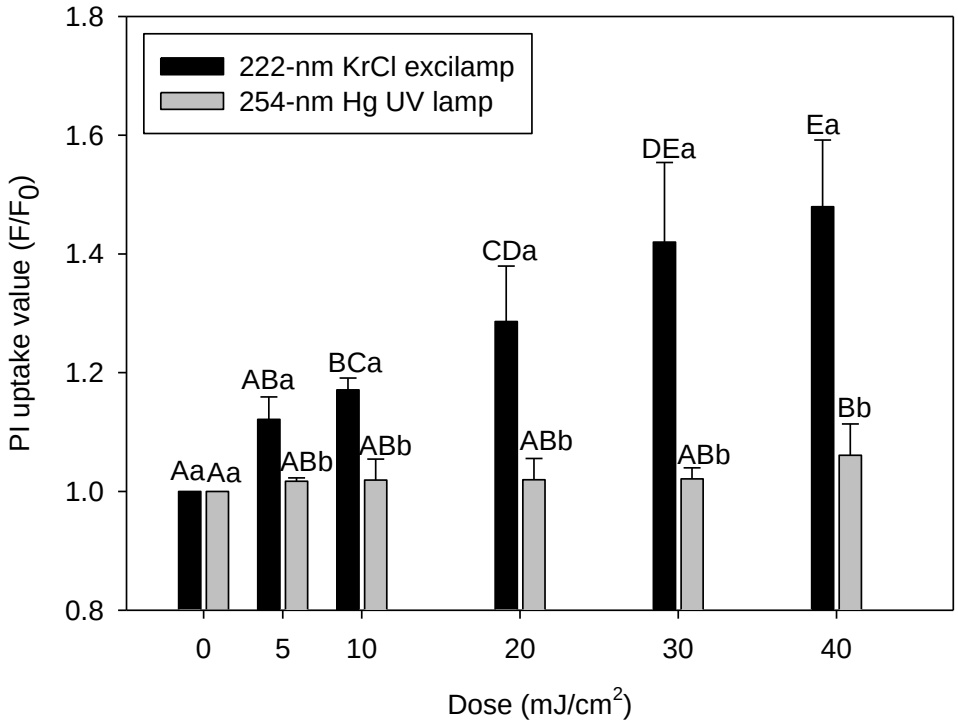

Fig. 2. Levels of inner membrane permeability of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined by a PI uptake assay. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were normalized by using the following formula: (fluorescence value after treatment/OD_{600}, F) / (fluorescence value for untreated control/OD_{600}, F_0). Different capital letters for each lamp treatment indicate significant differences ($P < 0.05$). Different lowercase letters for the same treatment dose indicate significant differences ($P < 0.05$)

3.2.2. Generation of lipid peroxidation of the inner membrane

To understand in more detail how the KrCl excilamp causes an increase in permeability inducing inner membrane damage to *A. acidoterrestris* spores, it is necessary to confirm which material of the inner membrane undergoes what form of change leading to this increased permeability. It is generally known that the spores' inner membrane layer acts as a strong permeability barrier because of the high immobility and compression of lipids (Planchon et al., 2011; Setlow, 2006), and the incidence of lipid peroxidation in the cell membrane induces a decrease in cell membrane potential and fluidity, which in turn leads to increased permeability (Gutteridge, 1995). Based on these facts, we considered that the increased permeability of the inner membrane may be related to lipid peroxidation and subsequently confirmed the occurrence of lipid peroxidation in the inner membrane of *A. acidoterrestris* spores. Accordingly, FIG. 3 indicates that the KrCl excilamp treatment significantly ($P < 0.05$) increased the DPPP=O value compared to that of the untreated control, and this value increased significantly ($P < 0.05$) as the treatment dose increased. On the other hand, the Hg UV lamp did not significantly ($P > 0.05$) increase the DPPP=O value compared to that of the untreated control until the 20 mJ/cm^2 treatment, and this value increased significantly ($P < 0.05$) from the 30 mJ/cm^2 treatment.

Although DPPP does not specifically act on lipid hydroperoxides in the spore inner membrane but also on that in the outer membrane, since this result shows a similar tendency as the results of the inner membrane destruction in FIG. 1, it is reasonable to infer the following: the KrCl excilamp treatment causes lipid peroxidation in the inner membrane of *A. acidoterrestris* spores, which induces inner membrane damage by increasing membrane permeability, eventually leading to death.

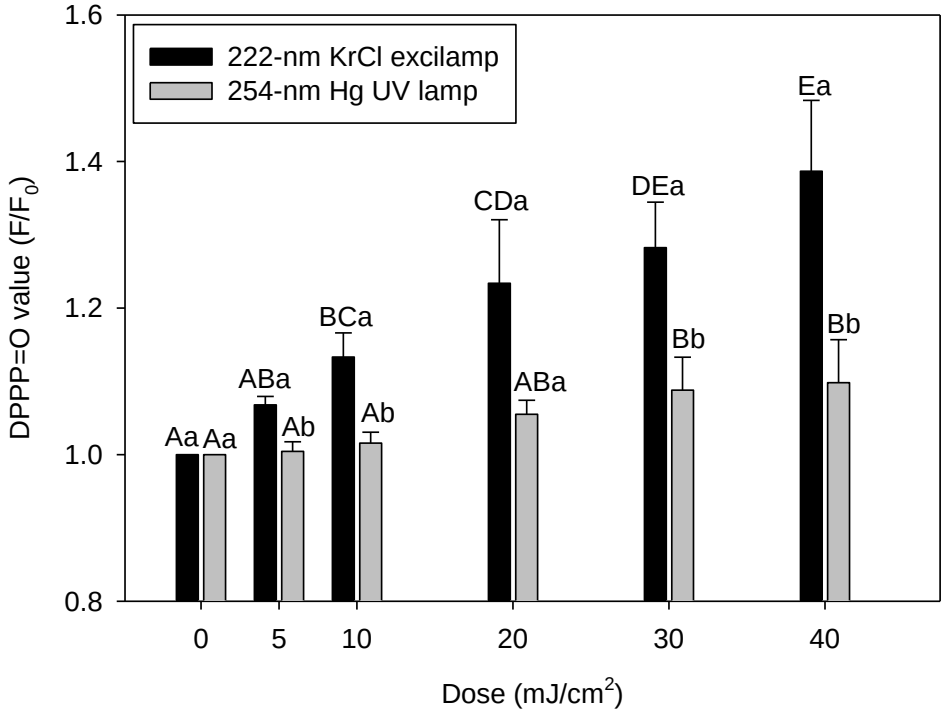

Fig. 3. Levels of lipid peroxidation in the membrane of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using a DPPP probe. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were normalized by using the following formula: (fluorescence value after treatment/OD_{600}, F) / (fluorescence value for untreated control/OD_{600}, F_0). Different capital letters for each lamp treatment indicate significant differences ($P < 0.05$). Different lowercase letters for the same treatment dose indicate significant differences ($P < 0.05$).

3.2.3. Intracellular ROS generation

UV irradiation can cause cellular damage in two ways: (i) UV radiation can be absorbed directly by cellular components, inducing photoinduced reactions that can cause cellular damage. (ii) Additionally, when UV radiation is absorbed by cellular materials acting as photosensitizers, photoionization can cause the formation of excited states or radicals that can cause cellular damage (Costanzo et al., 1995; Gao et al., 2008; Pattison and Davies, 2006). Meanwhile, reactive oxygen species (ROS) are one of the major causes of lipid peroxidation that produce physical changes in the cell membrane (Gaunt et al., 2006; Joshi et al., 2011; Premanathan et al., 2011). Our previous study (Kang et al., 2018) comparing the inactivation dynamics of a KrCl excilamp and Hg UV lamp for vegetative bacterial cells concluded that the KrCl excilamp produced intracellular ROS, while the Hg UV lamp did not because chromophoric amino acids, the major cellular components that act as photosensitizers, absorb more of the 222 nm radiation than 254 nm radiation (Bensasson et al., 2013). Based on these facts, the incidence of intracellular ROS in *A. acidoterrestris* spores for each lamp treatment was measured. The results of FIG. 4 show that the treatments with both lamps did not significantly ($P > 0.05$) increase the intracellular ROS values compared to those of the untreated control. Contrary to our expectations, these results

indicate that the KrCl excilamp and the Hg UV lamp do not generate intracellular ROS in *A. acidoterrestris* spores. These spores contain a large amount of pyridine 2,6-dicarboxylic acid [dipicolinic acid (DPA)] complexed with calcium ions in the spore core, which lowers the water content and thus makes the core of the spore experience dehydration conditions; it is known that the reduced water content of the core prevents UV radiation from producing ROS (Moeller et al., 2009; Setlow, 2006). This effect can be demonstrated by the fact that since the photochemical reaction of water or oxygen molecules by UV treatment leads to ROS generation, a decreased water content limits this reaction, so it is difficult to generate UV-induced ROS within spores containing a low water content (Attri et al., 2015; Moeller et al., 2009). Therefore, the 222 nm KrCl excilamp is inferred to induce cell damage only by the direct absorption of UV radiation without causing indirect cell damage induced by ROS due to the spore characteristics, that is, a low water content that makes ROS generation difficult. The results in FIG. 4 show that the KrCl excilamp induces higher lipid peroxidation incidence than the Hg UV lamp does, which can be explained by the fact that phospholipids absorb more 222 nm light than 254 nm light; thus, more photoinduced reactions of phospholipids occur with irradiation at 222 nm, leading to more lipid peroxidation (Spector et al., 1996).

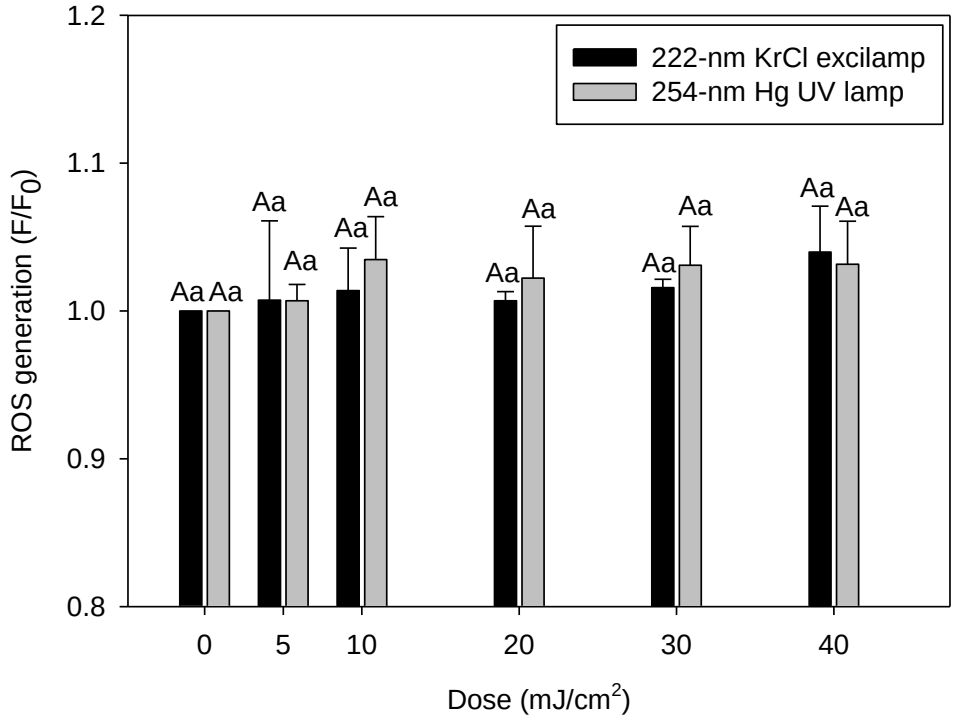

Fig. 4. Levels of ROS generation of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using ROS detection assay by CM-H_2DCFDA. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were normalized by using the following formula: (fluorescence value after treatment/OD_{600}, F) / (fluorescence value for untreated control/OD_{600}, F_0). Different capital letters for each lamp treatment indicate significant differences ($P < 0.05$). Different lowercase letters for the same treatment dose indicate significant differences ($P < 0.05$).

3.2.4. DNA damage

UV radiation can also induce spore inactivation by causing damage to the spore DNA because this damage interferes with cellular processes essential for vital functions such as DNA transcription or replication, leading to cell death (Britt, 2004; Lindahl, 1993; Rastogi et al., 2010). Therefore, to confirm the effect of the KrCl excilamp on DNA, the degree of DNA damage of spores was measured for each lamp treatment. The results of FIG. 5 indicate that the DNA integrity was significantly ($P < 0.05$) affected by the KrCl excilamp and Hg UV lamp and that the DNA integrity decreased significantly ($P < 0.05$) with increasing treatment dose of each lamp. However, it can be seen that the effect on the DNA integrity between the two lamps was not significantly different. Interestingly, the tendency of this result is different from our previous finding that a Hg UV lamp induced greater DNA damage than a KrCl excilamp when applied to vegetative bacterial cells (Kang et al., 2018). Our previous study (Kang et al., 2018) interpreted this result in that the degree of UV absorption of DNA increases gradually from 220 nm and shows its peak value at 260 nm (Taylor, 1994). The DNA of vegetative bacterial cells absorbed the 254 nm light of the Hg UV lamp more than the 222 nm light of the KrCl excilamp, eventually leading to more DNA damage by the Hg UV lamp. Even though the KrCl

excilamp, which produces intracellular ROS in vegetative bacterial cells, does not produce intracellular ROS in *A. acidoterrestris* spores, the spore DNA damage induced by the KrCl excilamp is not significantly different ($P > 0.05$) from that of the Hg UV lamp, which is a distinct feature that differs from DNA damage mechanisms in vegetative bacterial cells.

Spore DNA is packed with a group of nonspecific DNA-binding proteins termed small, acid-soluble proteins (SASPs), which cause the spore DNA to tightly pack into a toroidal morphology with an A-like conformation (Yang et al., 2017). An important feature of these proteins is that they change the structure of the DNA and increase the resistance (Sella et al., 2014). Indeed, many studies have reported that SASPs protect spores from a variety of treatments such as UV irradiation, because the damage to DNA saturated with SASPs is reduced (Setlow and Setlow, 1987; Setlow, 1988, 2006, 2007). Moreover, several studies, including ours, have found that the KrCl excilamp inactivates enzymes within cells, while the Hg UV lamp does not inactivate enzymes or has a lower inactivation capacity than the KrCl excilamp (Ha et al., 2017; Kang and Kang, 2019; Kang et al., 2018). This phenomenon of the KrCl excilamp showing greater damage to enzymes than the Hg UV lamp is observed because the major amino acids and peptide backbones of the proteins absorb more of the 222 nm radiation than the 254 nm radiation

(Bensasson et al., 2013; Kerwin and Remmele Jr, 2007). Based on these facts, it can be inferred that the KrCl excilamp induced functional denaturation of SASPs in *A. acidoterrestris* spores, interfering with the DNA saturation of the proteins, reducing the resistance of the DNA, and eventually making the DNA more sensitive to the 222 nm wavelength; thus, the KrCl excilamp and Hg UV lamp showed similar levels of DNA damage in the *A. acidoterrestris* spores despite the inherent characteristic of DNA absorbing more of the 254 nm wavelength than the 222 nm wavelength. To analyze these effects in more detail, further studies are needed to provide more specific evidence by comparing DNA damage after exposure of the KrCl excilamp and Hg UV lamp using *A. acidoterrestris* spores with the genes encoding SASPs deleted.

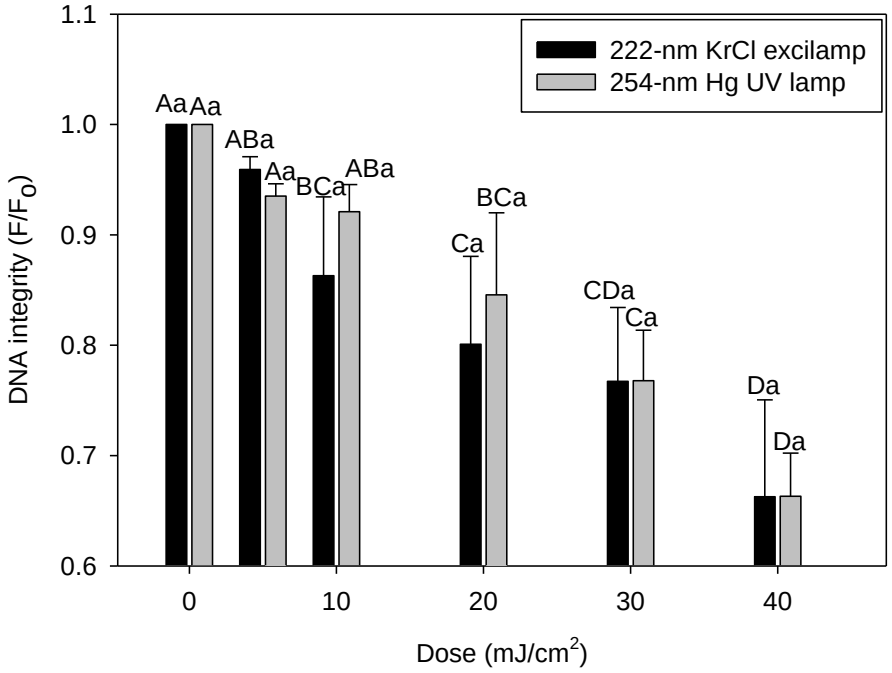

Fig. 5. Levels of DNA integrity of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using DNA quantification assay by SYBR green I. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were normalized by using the following formula: (fluorescence value after treatment/OD_{600}, F) / (fluorescence value for untreated control/OD_{600}, F_0). Different capital letters for each lamp treatment indicate significant differences ($P < 0.05$). Different lowercase letters for the same treatment dose indicate significant differences ($P < 0.05$).

3.3. Apple juice quality after KrCl excilamp treatment

To investigate the effect of the KrCl excilamp on apple juice quality, color (L*, a*, and b*, indicating color lightness, redness, and yellowness, respectively), total phenols, and DPPH free radical scavenging activity were chosen as quality attributes and measured because these parameters have usually been investigated to assess the quality change of apple juice in several studies conducting apple juice disinfection (Abid et al., 2013; Islam et al., 2016; Liao et al., 2018; Song et al., 2015; Zhao, Wang, et al., 2014). After the KrCl excilamp treatment at 2,011 mJ/cm^2, the color, DPPH free radical scavenging activity, and total phenols of apple juice were not significantly ($P > 0.05$) changed compared to those of the untreated control (TABLE 3). This result means that the KrCl excilamp can reduce *A. acidoterrestris* spore abundance by 5-log without causing changes in the apple juice quality.

Table 3. Changes of quality parameters (color, DPPH radical scavenging activity, and total phenols) in apple juice after KrCl excilamp treatment of 2,011 mJ/cm^2

	Quality parameters[a]				
	Color			DPPH radical scavenging activity (%)	Total phenols (mg GAE/liter)
	L*	a*	b*		
Untreated control	26.33 ± 0.17	0.06 ± 0.02	1.83 ± 0.03	84.70 ± 0.33	408.44 ± 11.00
Treated sample	26.33 ± 0.03	0.05 ± 0.01	1.81 ± 0.01	85.31 ± 0.31	404.56 ± 3.42

[a]Values are means ± standard deviations from three replications. All values within same column represented no significant ($P > 0.05$) difference.

IV. CONCLUSION

Since the KrCl excilamp has a fundamentally much higher sporicidal effect against *A. acidoterrestris* spores than the Hg UV lamp does, it was able to exhibit an inactivation effect comparable to that of the Hg UV lamp for inactivating *A. acidoterrestris* spores in apple juice, even though the 222 nm wavelength of the KrCl excilamp is absorbed more by the apple juice than the 254 nm wavelength of the Hg UV lamp is. The obtained experimental results suggest that the KrCl excilamp has a high potential to be utilized for controlling *A. acidoterrestris* spores in the apple juice industry by replacing traditional UV lamps containing harmful Hg. In addition, a greater inactivation effect of the KrCl excilamp than that of the Hg UV lamp against *A. acidoterrestris* spores is interpreted as the former induced similar spore DNA damage compared to that of the latter, but the KrCl excilamp caused more lipid peroxidation in the inner membrane, leading to greater membrane damage. The results of these inactivation mechanisms are expected to be used as important data for establishing an effective industrial application

strategy to control *A. acidoterrestris* spores using a KrCl excilamp or for conducting relevant studies.

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VI. 국문초록

열처리에 대한 내성이 높고 낮은 산성환경에서 발아할 수 있는 *Alicyclobacillus acidoterrestris* spore 는 주스산업에서 매우 중요하게 관리되어야 하는 균 중의 하나이다. 적절히 제거되지 않았을 경우, 균은 증식하여 불쾌한 화학 냄새를 발생시키는 구아이아콜을 형성하여 제품의 상업적 가치를 현저하게 떨어뜨리기 때문이다. 본 연구의 목적은 *A. acidoterrestris* spore 를 제어하는 기술로써 크립톤-염소 (KrCl) 엑시램프와 수은(Hg)을 포함하는 자외선 (Ultraviolet, UV) 램프의 효과를 비교하고 저감화 메커니즘에 대하여 알아보고자 한다. 먼저 사과주스에서 *A. acidoterrestris* spore 에 대한 222-nm KrCl 엑시램프와 254-nm 수은 자외선램프의 효과를 비교해보았다. 사과주스에서 KrCl 엑시램프의 222-nm 파장이 수은 자외선 램프의 254-nm 파장보다 더 잘 흡수됨에도 불구하고, *A. acidoterrestris* spore 를 제어하는 효과는 두 처리 간에 유의미한 ($P > 0.05$) 차이는 없었다. 즉, 사과주스에서 높은 흡광계수 (absorption coefficient)를 가짐에도 불구하고 KrCl 엑시램프의

저감화 효과가 뛰어남을 알 수 있었다. 이는 이상적인 조건인 인산 완충 식염수 (phosphate buffered saline)에서도 확인되었다.

222-nm KrCl 엑시램프의 저감화 기작은 세포막과 DNA 손상을 분석함으로써 조사되었다. KrCl 엑시램프는 세포막의 지질과산화를 발생시켜 세포막 손상을 유도한 것으로 나타났다. 그러나 세포막의 지질과산화는 222-nm 의 활성산소 (reactive oxygen species, ROS)의 생성이 없음으로 보아 직접적인 빛 흡수에 의해 유도되었음을 알 수 있었다. 222-nm KrCl 엑시 램프도 254-nm 수은 자외선 램프와 유의미하게 ($P > 0.05$) 다르지 않은 DNA 데미지를 발생시켰다. 게다가 KrCl 엑시 램프로 사과주스의 *A. acidoterrestris* spore 를 5 로그 만큼 저감화하는 동안 사과주스의 유의적인 ($P > 0.05$) 품질 변화는 나타나지 않았다.

따라서 본 연구는 222-nm KrCl 엑시 램프가 *A. acidoterrestris* spore 를 제어하기 위한 수단으로 적용될 수 있음을 제시하였다. 또한 제어 원리의 분석 결과는 식품 산업 및 관련된 추후 연구를 수행하기에 중요한 기초 자료로 활용될 수 있을 것으로 예상된다.

주요어: 222-nm KrCl 엑시 램프, *Alicyclobacillus acidoterrestris,* spore, 저감화 기작, 사과주스

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