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Application of a Krypton-Chlorine Excilamp To Control *Alicyclobacillus acidoterrestris* Spores in Apple Juice and Identification of Its Sporicidal Mechanism

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ABSTRACT The aim of this study was to investigate the sporicidal effect of a krypton-chlorine (KrCl) excilamp against *Alicyclobacillus 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.

IMPORTANCE *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. 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*, DNA damage, ROS generation, apple juice, inactivation mechanism, inner membrane damage, spore

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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 (1, 2). 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 (3, 4).

In 1982, however, a new type of bacterium was reported that caused spoilage in aseptically packed apple juice (5); 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-alicyclic fatty acid (6). Among various *Alicyclobacillus* species, *Alicyclobacillus acidoterrestris* is regarded as the most important spoilage microorganism because it has frequently been observed in commercially processed fruit juice products and has been associated with spoilage problems (7–10). *A. acidoterrestris* is a thermophilic, spore-forming, Gram-positive, nonpathogenic spoilage microorganism, and it is acidophilic. *A. acidoterrestris* is able to grow at a wide range of temperatures (26 to 60°C), its spores have a high thermal resistance with a d-value of 16 to 23 min at 90°C, and it can germinate in acidic products (pH 3.0 to 4.5) (11, 12). 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 (11, 13, 14).

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 to 10^5 CFU (15–17). 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 (9, 17). Therefore, it is very important to inactivate *A. acidoterrestris* before final juice products are shipped (7).

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 (3, 18). Therefore, the application of nonthermal technologies can be an alternative; among them, UV irradiation is regarded to be a great substitution for 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 (19, 20). 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 (21). However, there is always a concern that using conventional UV lamps is a potential hazard since Hg can harm humans and pollute the environment (22, 23). 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 to 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 (24–28). Excilamps emit UV radiation that is almost monochromatic from 172 to 345 nm, and several researchers have conducted studies applying various types of excilamps, such as Xe_2, KrCl, KrF, or XeBr excilamps with peak emissions of 172, 222, 248, or 282 nm, respectively, to spore control (29–33). In particular, the results of research by Pennell et al. (30) and Wang et al. (32) 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

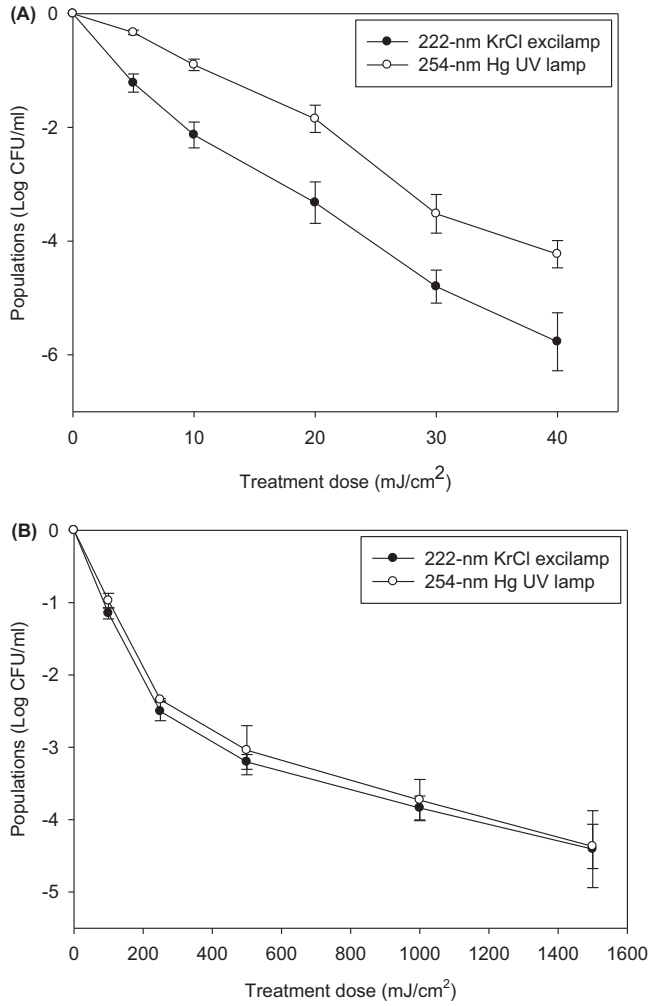

FIG 1 Populations (log CFU/ml) of the spores of *A. acidoterrestris* in PBS (A) and apple juice (B) treated with the 222-nm KrCl excilamp or 254-nm mercury (Hg) UV lamp. Each point and error bar indicate the means and standard deviations calculated from three replicates, respectively.

effect; thus, the KrCl excilamp is a highly promising candidate to effectively control *A. acidoterrestris* spores in fruit juices.

To the best of our knowledge, 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, we 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.

RESULTS AND DISCUSSION

Inactivation effect of a KrCl excilamp against *A. acidoterrestris* spores compared to an Hg UV lamp. Figure 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 a 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 the 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 (34). 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 (35, 36). Because the apple juice sample used in this study has no turbidity (0 nephelometric turbidity units [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 (37). 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}; 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 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 (38). Although apple juice, which is characterized by a greater absorption of light at 222 nm than at 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 the Wang et al. study (32) comparing the sporicidal effect of irradiation at 172, 222, and 254 nm on *Bacillus subtilis* spores under ideal conditions (deionized water) and the Pennell et al. study (30) 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, the Clauss study (31) 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.

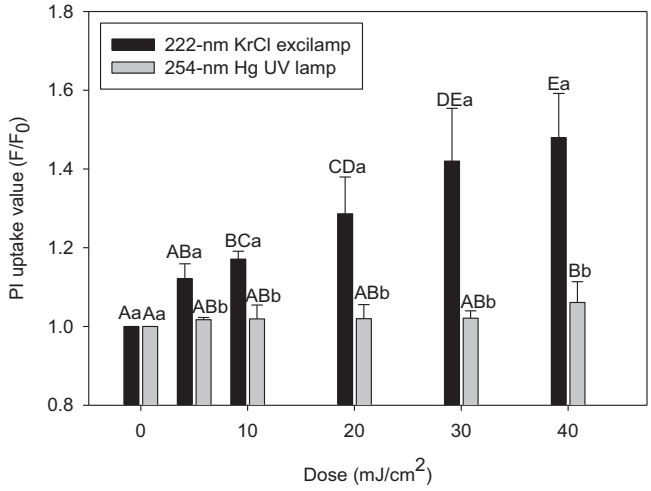

FIG 2 Levels of inner membrane permeability of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined by a PI uptake assay. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were 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 ($P < 0.05$).

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.

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 to 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 to 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 studies applying various techniques to kill spores, such as thermosonication (39), supercritical CO_2-peracetic acid treatment (40), high-pressure CO_2 treatment (41), and electron beam irradiation (42), 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 (40, 43). Therefore, the results are interpreted as the KrCl excilamp inducing inactivation of the *A. acidoterrestris* spores by damaging the spore inner membranes.

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

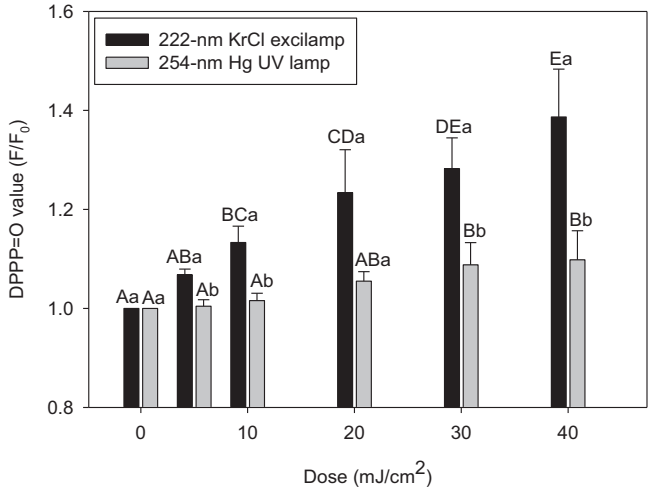

FIG 3 Levels of lipid peroxidation in the membrane of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using a DPPP probe. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data 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 ($P < 0.05$).

permeability barrier because of the high immobility and compression of lipids (44, 45), 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 (46). 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 diphenyl-1-pyrenylphosphine 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.

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) In addition, 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 (47–49). Meanwhile, reactive oxygen species (ROS) are one of the major causes of lipid peroxidation that produce physical changes in the cell membrane (50–52). Our previous study (25) comparing the inactivation dynamics of a KrCl excilamp and Hg UV lamp for vegetative bacterial cells concluded that the KrCl excilamp produced intracellular ROS, whereas 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 (53). Based on these facts, the incidence of intracellular ROS in *A. acidoterrestris* spores for each lamp treatment was measured. The results presented in Fig. 4 show that the treatments with both lamps did not significantly ($P > 0.05$) increase

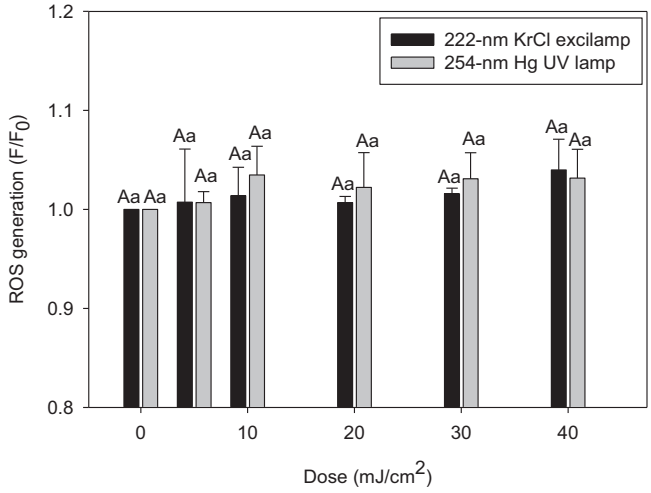

FIG 4 Levels of ROS generation within *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using a DPPP probe. The data represent averages for three independent experiments, and error bars indicate standard deviations. The data were normalized by 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 ($P < 0.05$).

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) 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 (44, 54). 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 (54, 55). 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 shown 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 (56).

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 (57–59). 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 an Hg UV lamp induced greater DNA damage than a KrCl excilamp when applied to vegetative bacterial cells (25). Our previous study (25) 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 (60). The DNA of vegetative bacterial cells absorbed the 254-nm light of the Hg

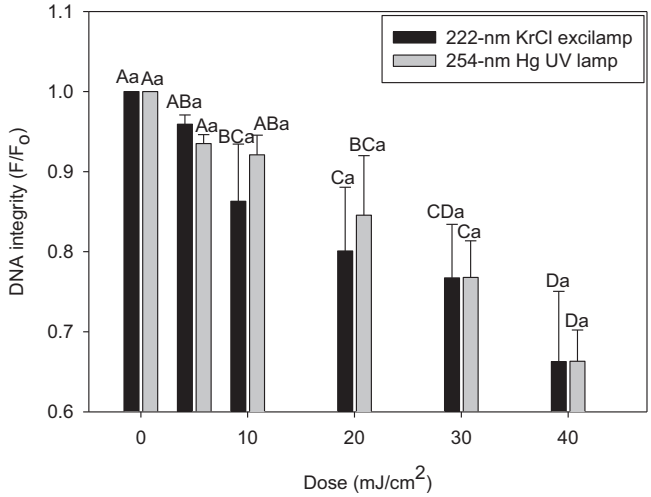

FIG 5 Levels of DNA integrity of *A. acidoterrestris* spores during treatment with the 222-nm KrCl excilamp or 254-nm Hg UV lamp determined using a DPPP probe. The data represent averages for three independent experiments, and the error bars indicate standard deviations. The data were normalized by the 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 ($P < 0.05$).

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 (61). An important feature of these proteins is that they change the structure of the DNA and increase the resistance (62). 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 (44, 63–65). 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 (24, 25, 66). 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 (53, 67). 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.

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 (2,2-diphenyl-1-picrylhydrazyl) 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 (68–72). 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 (see Table S1 in the supplemental material). This result means that the KrCl excilamp can reduce *A. acidoterrestris* spore abundance by 5-log without causing changes in the apple juice quality.

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 that 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.

MATERIALS AND METHODS

Bacterial strain. *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, South Korea) acidified to pH 3.7 using 10% tartaric acid (Junsei Chemical Co., Inc., Tokyo, Japan), maintained at 4°C, and subcultured monthly (73).

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) and incubated at 43°C for 7 days until at least 80% sporulation, as confirmed by microscopic examination. To prepare spore suspensions, 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 $\times$ *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. The concentration of spore suspensions was approximately 10^7 to 10^8 spores/ml. This inoculum was used immediately for the experiment on the day it was made.

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 the 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 or 254 nm, the absorbance values of apple juice at 222 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. At 1 to 2 h before the experiment, the samples taken from refrigerated conditions were adjusted to room temperature (22 $\pm$ 2°C) by equilibration. A petri dish (60 mm by 15 mm) with the lid removed was filled with 5 ml of each sample, and 0.1 ml of inoculum was inoculated into the sample.

Experimental setup and treatment. A DBD-driven excilamp (110 W; 29 cm by 9 cm by 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 as described in our previous study (25). 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 to 240 nm for the KrCl excilamp and from 230 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 the 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°Bx, 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 $\pm$ 2°C) while being mixed with a magnetic stirrer (1.5 cm by 0.1 cm) at 500 rpm; UV doses were calculated by multiplying the UV light intensity by the irradiation times.

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. The samples were then immediately placed 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), 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 the $\log_{10}$ CFU/ml.

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 occur only in spores, the previously stated spore inoculum was disrupted using a sonicator (Bandelin Sonoplus HD 2070; Bandelin Electronic, GmbH, Germany) at 70% of power for 2 min in a water bath containing ice and centrifuged at 10,000 $\times$ *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, the survival rates of spores with a 2-min sonication treatment for each lamp were obtained using the treatment procedure described above and compared to those of spores in PBS without sonication treatment. It was confirmed that no significant ($P > 0.05$) survival rate difference between spores with or without sonication treatment occurred (see Table S2 in the supplemental material). 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. The optical density at 600 nm (OD_{600}) of the spore suspension in PBS was measured before each treatment to normalize the analysis results.

(i) Confirmation of cell membrane damage. The fluorescent dyes PI and DPPP 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 pore formation in the cell membrane, and then can bind with nucleic acid and become a fluorescent form (74, 75). 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 (41, 76). 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) (77). 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 (78).

Following the treatments, the treated samples were reacted with PI (Sigma-Aldrich, St. Louis, MO) 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 $\times$ *g* for 10 min to collect cells. After two washes with PBS, the fluorescence intensity was measured with a spectrofluorophotometer (Spectramax M2e; Molecular Devices, Sunnyvale, CA) 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 are presented as values in which the obtained fluorescence intensity was divided by the OD_{600}.

(ii) 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 reaction with ROS (79–81). Therefore, the increased fluorescence intensity of DCF can be used to evaluate the degree of the generation of ROS within the spore (82).

After the treatments, the treated samples were reacted with CM-H_2DCFDA (Invitrogen, Carlsbad, CA) at a final concentration of 5 μM for 15 min at 37°C and centrifuged at 10,000 $\times$ *g* for 10 min to collect cells. After two washes with PBS, 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}.

(iii) 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-stranded DNA (83). 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 (84).

Following the treatments, intracellular DNA was isolated from the spores using an UltraClean microbial DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Isolated DNA was reacted with 1$\times$ SYBR green I (Molecular Probes, Eugene, OR) 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 divided by the OD_{600}.

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) value (see Table S3 in the supplemental material). After the treatment, the color, total

phenol values, and DPPH free radical scavenging activity of apple juice were measured as described in our previous study (85).

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

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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