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Development of non-thermal microbial inactivation techniques: application of ohmic heating and irradiation of UVC by light-emitting diode

（非熱的殺菌技術の開発：

通電加熱と発光ダイオードによる UVC 照射）

Hokkaido University
Graduate School of Agriculture

Division of Biosystems Sustainability
Doctor Course

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Chapter 1

General Introduction

1.1. Microbial inactivation techniques in food processing

The most important aspect in microbial inactivation is to secure food safety by reducing the number of spoilage bacteria and also eliminating pathogenic bacteria in foods. However, microbial inactivation has to be managed to balance securing food safety and minimizing degradation of food quality. The most common technique for microbial inactivation is presently thermal processing. Food products are normally processed at high temperature for ensuring microbiological food safety. However, excessive higher thermal treatment with high pressure causes thermal deterioration of the food materials such as change in flavor, texture, taste, and nutrient composition. In addition, conventional thermal processing that usually conducts by external heating could cause thermal damage on foods by overheating. Because the time for sufficient heat transfer into the center of food is normally required for long time in a real processing scale, thermal damage is inevitable (Pereira, 2009). Thus, minimally thermal processing technique and/or non-thermal processing technique will play a key role in preventing thermal deterioration or processed foods.

Recently, electromagnetic technologies in food processing have emerged to replace the traditional conventional thermal processesing (Vicente and Castro, 2007). Electromagnetic technologies in food processing could shorten heating time and/or reduce heating temperature. Internal heating produres including ohmic heating and microwave heating are promising

alternatives to conventional external heating. These technologies do not need external heating resource and thermal energy, because heat is generated directly inside of the food. This heating generation could overcome excessive cooking duration due to efficient internal heating of food. In addition to internal heating methods, non-thermal technologies such as ultrasounds, high pressure processing, pulsed electric fields and ultra violet irradiation for inactivation microorganism have emerged to improve quality of processed food products. These technologies would avoid thermal degradation of food quality and contribute to producing high quality processed food that retains fresh taste, flavor, and nutritious. In order to produce such the high quality processed food, utilizing those emerging thermal and non-thermal technologies will play an important role. In this chapter, we introduced various emerging microbial inactivation techniques which would be alternative to conventional external heating.

1.2. Non-thermal effect in novel thermal processing technologies

Internal heating method, which is generating heat directly in food itself, such as ohmic heating, microwave heating, and ultra sound would be alternatives to conventional external heating. Due to internal heating of food, rapid and uniform heating can be realized. Although microbial inactivation effect of these methods is commonly relied on the thermal effect, there would be additional microbial inactivation effect by electricity and/or microwave (Cho et al. 1999; Kuhnert, 2002; Pereira, et al., 2007; Somavat et al., 2012; Somavat et al., 2013). Therefore, these heating methods would have potential to contribute to shorten heating time and reduce heating temperature.

1.2.1. Ohmic heating

Ohmic heating is the process of passing alternating electrical current (AC) through food products such as liquid and liquid-particulate food system. Heat is generated and distributed internally in the foods when an electrical current is passed through a food due to the electrical resistance of the food. The defining characteristics of ohmic heating are the frequency and waveforms of the electric field, and the presence of electrodes that contact the material. The most important factor is the electrical conductivity of the product and its temperature dependence (Sarang, 2008). A major advantage claimed for ohmic heating is its ability to heat materials rapidly and uniformly, including products containing particulates, resulting in less thermal damage to the product in comparison to conventional heating (Pereira, 2007). In ohmic heating, the principal mechanisms of microbial inactivation are supposed to thermal in nature. However, Ohmic heating is expected to inactivate bacteria not only by thermal effects, but also by non-thermal effects via electricity (Cho et al., 1999; Pereira et al., 2007; Baysal and Ichier, 2010; Somavat et. al., 2013; Park et al., 2013). Park et al. (2013) compared the inactivation effect of ohmic heating on *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in peptone water and apple juice with those of conventional external heating. They found that the electric effect of ohmic heating was a very important factor for reducing process times and temperatures by enhancing levels of inactivation of these bacteria. Pereira et al. (2007) compered the effect of both techniques on *E. coli* in goat milk and *Bacillus licheniformis* in cloudberry jam. They found that ohmic heating could shorten *D*-values of *E. coli* and *B. licheniformis* and *Z*-value of *E. coli*. On the other hand, *Z*-value of *B. licheniformis* was not

significantly different. Cho et al. (1999) concluded that spore inactivation during ohmic heating was primarily due to the thermal effect but there was an additional killing effect caused by the electric current.

1.2.2. Microwave heating

Microwaves are part of the electromagnetic spectrum and are considered to be that radiation ranging in frequency from 300MHz to 300GHz. This nonionizing electromagnetic radiation is absorbed at molecular level and manifests as changes in vibrational energy of the molecules or heat (Baniki et al., 2003). Microwave heating is more efficient in terms of the energy usage. Microwave heating produces a higher temperature homogeneity in objective food and realizes considerably more rapid heating than those of conventional heating sources (Kuhnert, 2002). Moreover, bacterial cells could be killed by not only thermal effect but also by non-thermal effect during microwave heating. Microwaves cause different microbial inactivation effects depending on field strength, frequencies, wave forms, modulation and duration of exposure (Rai et al., 1994). Kuhnert (2002) found the death rates of *Escherichia coli* exposed to microwave irradiation were higher than those obtained in conventional heating at 45, 47 and 50 °C. Although the author could not specify the exact mechanism of the non-thermal effect by microwaves, the author proposed that the microwaves caused ions to accelerate and collide with other molecules or caused dipoles to rotate and line up rapidly with alternating electric field resulting in a change in secondary and tertiary structure of proteins of microorganisms. Banik et al., (2003) reported that microbiological studies involving

microwave irradiation have resulted in the following two conflicting conclusions; cell death was solely the result of heat produced by microwave irradiation; death was due to not only heat but also microwave electric field.

1.2.3. Ultrasounds

Ultrasonic waves (0.1–20 MHz) have emerged in recent years in food processing due to their ability to influence the physical or microbiological properties of food. Ultrasonic waves propagating in a liquid medium causes cavitation, which has been attributed to the main mechanism for cell disruption (Sala et al., 1995). This lethal effect would be due to the extreme pressure variations caused by implosions or bubble collapse. Implosion also releases localized high temperatures or hot spots. Therefore, ultrasonic is considered both a thermal and a non-thermal treatment by effect of its mechanism of action (Feng and Yang, 2010). Although microorganisms can withstand high pressures, they are incapable of withstanding quick alternating pressures produced during cavitation. While ultrasound could inactivate vegetative bacterial cells, insignificant reduction of bacterial spores has been reported even with an extended ultra sound treatment (Guan and Hoover, 2005). While Ultrasound can be used in the product sanitization process to improve the efficacy of the washing process (Guan and Hoover, 2005). Ultrasonic waves are effective in inactivating vegetative microbial cells, especially when used in conjunction with other treatments including heat, pH modification, and chlorination. Seymour (2002) demonstrated that ultrasound combined with chlorinated water reduced the population of *Salmonella* Typhimurium on iceberg lettuce by 1.7 log cycles

compared to 0.7 log cycle reduction by ultrasonic alone. Several researchers have observed an enhancement in the nutritional value of ultrasonicated foods while others have shown a reduction after sonication and the loss was exacerbated during subsequent storage (Banerjee et al., 1996; Portenlänger and Heusinger,1992; Silver, 2007).

1.3. Non-thermal effect in emerging non-thermal food processing

Non-thermal technologies for inactivating microorganisms have been developed in the world because the demand of fresh and natural food products was increased. There is a great need for a non-thermal method for inactivating microorganisms that is economical, compact, energy efficient, safe, and socially and environmentally acceptable and does not adversely affect nutrition, texture, and flavor of the treated foods. The following sections detail non-thermal food processing techniques that have been recently commercialized or are close to being commercialized.

1.3.1. High hydrostatic pressure processing (HHP)

HHP is an emerging non-thermal food processing technology whereby foods are subjected to high hydrostatic pressure, generally in the range of 100-1000 MPa, at or around room temperature. The application of HHP on foodstuffs is currently a subject of major interest for both food preservation and food preparation once it inactivates vegetative microorganisms by using pressure rather than heat to achieve pasteurization (Rendueles et al., 2011). Microorganisms related to food such as vegetative bacteria, human infectious viruses, fungi,

protozoa and parasites could be significantly reduced when subjected to high pressure (Patterson, 2005; Vanlint et al., 2012; Whitney et al., 2007). HHP contributes to extending the shelf-life of processed foods and reduction of numbers of pathogenic bacteria while retaining the food's inherent color, flavor, nutrients and texture (Farkas and Hoover, 2000; San Martin et al., 2002). The application of HHP has been explored by numerous food research institutions as well as the food industries with the goal of enhancing the safety, quality, nutritional, and functional properties of a wide variety of foods with minimal deleterious effects on their nutritional and organoleptic characteristics (Jung et al., 2011; Tewari, 2007). The mechanism of microbial control and inactivation is supposed to be a combination of processes such as the breakdown of non-covalent bonds in large macromolecules, biochemical effects, effects on the genetic mechanisms of cells, morphological changes and the disruption and permeabilization of the cell membrane (Patterson et al., 2005; Considine et al., 2008).

1.3.2. Pulsed electric fields (PEF)

PEF processing involves the application of high-voltage pulses to foods located between a series of electrode pairs. The electrical fields (generally at 20–80 kV/cm) are achieved through capacitors that store electrical energy from direct current power supplies (Guan and Hoover, 2005). When a short electric pulse (1–100 μsec) was applied to food, there was a pronounced lethal effect on microorganisms (Ohlsson and Bengtsson, 2002). The precise mechanisms by which the microorganisms are destroyed by electric fields are not fully understood although it is considered thought that cell inactivation occurs by several

mechanisms, including the formation of pores in cell membranes (Toepfl et al., 2007), formation of electrolytic products or highly reactive free radicals, oxidation and reduction reactions within the cell structure that disrupt metabolic processes, disruption of internal organelles and structural changes (Barbosa-Canovas et al., 1999), and production of heat produced by transformation of induced electrical energy. The antimicrobial efficacy of the PEF process varies as a function of numerous processing parameters, including the electric field strength, number of pulses, pulse duration, pulse shape, processing temperature, and physiological state of the bacteria. Other factors that also influence the degree of inactivation include the temperature of the food, pH, ionic strength, and electrical conductivity (Vega-Mercado et al., 1999). Several reports have presented the promising PEF- induced inactivation of microorganisms on food matrices (Cserhalm et al, 2011; Ou et al., 2017; Sotelo et al., 2018). PEF research has mostly focused on the inactivation of microorganisms suspended in various pumpable non-particulate foods with free flowing characteristics including fruit juices, liquid eggs, milk, and pea soup (Vega-Mercado et al., 1999).

1.3.3. Ultra violet (UV) irradiation

UV is one of the range of electromagnetic wave. The UV spectrum is customarily divided into three regions: UV-A with a wavelength of 320–400 nm, UV-B with a wavelength of 280–320 nm, and UV-C with a wavelength of 200–280 nm (Clydesdale et al., 2001; Sharma, 2010). UV-C possesses germicidal properties, for example, UV-C irradiation at a dose rate of 1000 J/m^2 or more, exhibitas as much as 4-log reductions of bacteria, yeasts, and viruses (Neetoo

and Chen, 2014). The mechanism of inactivation and cell death is considered absorption of UV by DNA and RNA (Sharma, 2010). The effect of UV on microorganisms depends on a multitude of factors (Sharma, 2010). For example, the range of wavelengths used to irradiate the cells, treatment time, treatment intensity, and target species will affect the lethality of the process (Sharma, 2010). UV radiation may denature proteins, enzymes, and aromatic amino acids, leading to changes in the composition of the food (Neetoo and Chen, 2014). The application of UV light has been used in three areas: air disinfection, liquid sterilization and inhibition of microorganisms in surface (Bintsis et al., 2000). One of the main limitations of UV is its low penetration (Falguera et al., 2011). The presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effect (Falguera et al., 2011). In addition, the main source of UVC has been UV lamp which contains mercury and fragile lamp. Furthermore, UV irradiation can change the flavor profile in certain products (Neetoo and Chen, 2014). UV light not only causes several undesirable chemical reactions, but can bring about deterioration in product quality. The use of UV in food industry has been limited because of these reasons. We need to overcome these negative aspects of UV irradiation in the practical use although UV irradiation can non-thermally and efficiently inactivate bacteria.

1.4. Objectives of this study

The novel thermal and non-thermal technologies mentioned above would be able to contribute to produce high quality processed foods through inactivating microorganism by non-thermal effect. In the present study, we focused on ohmic heating as an alternative thermal treatment, and focused on UV-C irradiation as a promising non-thermal treatment. In particular, we focused on UV-C light emitting diode (UVC-LED) as a new UV source and investigated the usage of UVC-LED for non-thermal treatment.

Accordingly, objective of this study was evaluation of microbial inactivation effect by non-thermal energy for minimizing deterioration of the food by heat. In chapter 2, we investigated the non-thermal effects of Ohmic heating on the inactivation of bacterial spores and evaluated the effects of the magnitude of the electric fields and electric frequency. We evaluated the effect of electricity on bacterial spores by comparing inactivation effect of ohmic heating with that of conventional external heating at the same heating temperature. From chapter 3 to chapter 5, we evaluated the performance of UVC-LED by investigating the effect of distance between UVC-LED and irradiated material and the effect of temperature on UV intensity of UVC-LED. In addition, we investigated the non-thermal effects of UVC-LED for practical usage such as water, ice and vegetable. In particular, we aimed to develop a new value such as inactivation of microorganism in ice and disinfection of raw food just before eating by non-thermal energy.

Chapter 2

Effects of Ohmic Heating on Thermal Inactivation of Bacterial Spores

2.1. Introduction

The essential goal of microbial inactivation is to secure food safety by reducing spoilage and eliminating pathogenic bacteria. Microbial inactivation is simultaneously required to secure food safety and maintain food quality. Thermal processing is the most common technology for microbial inactivation. However, high-temperature treatments induce the deterioration of food, resulting in changes in flavor, taste, and texture or the loss of nutrients. To overcome the limitations of thermal processing, various non-thermal microbial inactivation techniques, such as high-pressure processing and high electric field pluses, ultrasonic sonic, have been developed. However, without heating foods excessively, most non-thermal processing methods do not completely inactivate bacterial spores, and accordingly do not sufficiently meet the needs of the food industry (Cserhalmi et al., 2002; Raso et al., 2006; Evelyn and Silva, 2016).

Ohmic heating (OH) is a heating procedure in which an alternating electric current is applied to foods. OH rapidly and uniformly heats materials (Jun and Sastry, 2005) and achieves close to 100% energy transfer efficiency because heat is generated and distributed internally in foods (Sastry and Salengke, 1998). OH has the potential to reduce thermal damage and retain the quality of processed foods (Leizerson and Shimoni, 2005; Yildiz-Turp et al., 2013), unlike conventional external heating (CH), owing to the highly efficient temperature increase.

In addition, OH is expected to inactivate bacterial spores not only by thermal effects, but also by non-thermal effects via electricity (Cho et al., 1999; Pereira et al., 2007, Baysal and Icier, 2010; Somavat et al., 2012; Somavat et al., 2013). Cho et al. (1999) compared the effects of OH and CH on Bacillus subtilis spores at various temperatures and concluded that *Bacillus subtilis* spores heated in water with 0.1% NaCl at 92.3°C showed a significant reduction in the D-value under OH treatment. However, there was no significant difference in D-values between OH and CH at 88.0°C. Moreover, Cho et al. (1999) concluded that the z-value is not significantly different between the two methods. Pereira et al., (2007) compared the effects of OH with those of CH on *B. licheniformis* spores at various temperatures and concluded that *B. licheniformis* spores heated in cloudberry jam at 70°C, 75°C, and 80°C showed a significantly reduced D-values under OH treatment. However, there was no significant difference in D-values between OH and CH at 90°C and the z-values for the two methods were not significantly different. In contrast, the effect of OH was not significantly ($P > 0.05$) different from that of CH on *B. licheniformis* spores inactivation (Tola and Ramaswamy, 2014).

Although OH is expected to have non-thermal effects on bacterial spores and contribute to reductions in the food heating temperature and time, the results of previous studies have been variable. Conflicting results may be explained by unstable or fluctuating electric field stress during OH treatment. It is technically difficult to maintain a constant temperature and apply a constant electric field simultaneously because the temperature of the heating medium increases as a constant electric current is applied. In previous studies, the electric field has been increased or decreased to maintain a constant the temperature (Cho et al., 1999; Pereira et al.,

2007, Baysal and Icier, 2010; Somavat et al., 2012; Somavat et al., 2013). In the present study, an electric field was applied constantly and continuously by maintaining the heating medium at the boiling point. Electric field stress was constantly applied to bacterial spores using the procedure examined in the present study. The objective of this study was to clarify the non-thermal effects of OH on the inactivation of *B. subtilis* spores when a constant electric field is applied. In addition, the effects of the magnitude of the electric fields and electric frequency on *B. subtilis* spore inactivation were evaluated.

2.2. Materials and methods

2.2.1. Bacterial strains

Bacillus subtilis (NBRC13719) was used as a representative bacterial spore because NBRC13719 has the highest thermal resistance strain among *B. subtilis* strains in our laboratory. A stock culture maintained on nutrient agar (Difco, Becton-Dickinson and Company, Sparks, MD, USA) slants at 3°C was inoculated into glucose broth (GB; 2 g of beef extract, 3g of yeast extract, 10 g of peptone, 5 g of dextrose, 5 g of sodium chloride, and 1 liter of deionized water, pH 7.0) and incubated at 37°C for 48 h for the activation of the bacterial cells.

2.2.2. Preparation of spore suspension

To induce the sporulation of *B. subtilis* cells, an aliquot of activated bacterial cells was inoculated on a nutrient agar plate and then incubated at 37°C for 24 h. The colonies that formed on the nutrient agar were inoculated into 25 ml of Difco Sporulation Medium (8 g of

Bacto nutrient broth (Difco), 10 ml of KCl, 10 ml of $MgSO_4 \cdot 7H_2O$, 1 M NaOH (pH to 7.6), 1 ml of $Ca(NO_3)_2$, 1 ml of $MnCl_2$, 1 ml of $FeSO_4$, and 1 liter of deionized water) and incubated at 37°C for 48 h. Greater than 90% of sporulation was obtained, as verified by observations of refractile spores under phase-contrast microscopy. The culture was centrifuged at 3000 × g for 20 min. Recovered spores were washed with distilled water 4 times with repeated centrifugation for 10 min at 3000 × g. Between the second and third centrifugation steps, the suspension was pasteurized at 80°C for 20 min to eliminate vegetative cells. The suspensions were stored at 4°C until use.

2.2.3. Ohmic heating (OH) treatment

A schematic diagram depicting the OH treatments is shown in Fig. 2-1. The heating unit consisted of titanium square electrodes (length: 110 mm, width: 110 mm, thickness: 2 mm) in contact with the sample, a Teflon vessel (thickness: 10 mm, height: 110 mm, width: 123 mm, depth: 55 mm), and a thermocouple (K-type) inserted at the center of the Teflon vessel with a capacity of 300 ml. The distance between the two titanium square electrodes was 30 mm.

For the OH treatment, a 250-ml aliquot sodium chloride aqueous solution (0.05, 0.2 and 0.6% w/v) was added to the Teflon vessel and heated to the boiling point (101°C) by applying a constant electric field. Once the desired temperature was reached in the salt water, 1 ml of spore suspension (~10^7 CFU/ml) was added to 250 ml of the heating medium. The electric field intensity (5, 10, and 20 V/cm) and the frequency (20, 40, and 60 kHz) were controlled by the power supply. Every 2 min, a 1-ml aliquot was collected from the heating medium and kept in

a plastic tube in an ice bath until the microbiological analysis (< 20 min).

In many studies of the inactivation of bacterial spores using OH, sporular liquid in the medium is heated to a targeted temperature and the temperature is maintained by OH and CH. In contrast, in this study, sporular liquid was added to the heated medium at the targeted temperature to ensure identical temperature histories for the OH and CH treatments.

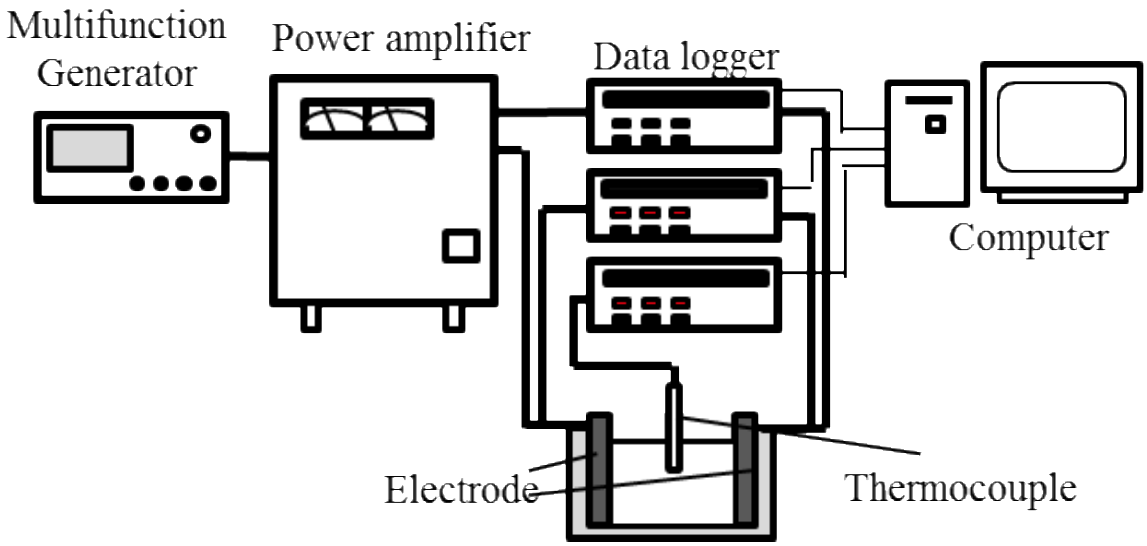


Fig. 2-1. Schematic diagram of the experimental ohmic heating apparatus.

2.2.4. Conventional external heating (CH) treatment

For the CH treatment, a 250-ml aliquot of sodium chloride aqueous solution (0.05, 0.2, and 0.6% w/v) was heated in a glass beaker using a heating-magnetic stirrer to the boiling point (101°C). After the temperature reached 101°C, 1 ml of spore suspension (~107 CFU/ml) was added to 250 ml of the heated water.

2.2.5. Microbiological analysis

To determine the number of surviving spores at each sampling point (every 2 min) during the heat treatment, appropriate serial dilutions (1:10) were performed with sterile 0.1% peptone water and were plated on duplicate tryptic soy agar plates. The plates were then incubated at 37°C for 24 h.

2.2.6. Survival kinetics analysis

Survival kinetics were analyzed by using the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT; Ver. 1.6), a freely available add-in for Microsoft Excel (Geeraerd et al., 2005). The Weibull model, the log-linear regression model, and the log-linear regression plus shoulder model were used.

The Weibull model is represented by the following formula:

$$\text{Log } S(t) = \log \frac{N(t)}{N_0} = -\left(\frac{t}{\delta}\right)^p \qquad (1)$$

where S (t) is the momentary survival ratio and N(t) and N0 are the momentary and initial counts at 101°C, respectively. The initial counts (N_0) were viable cell counts at the time zero. The parameters δ and p represent the survival rate and kinetics curvature, respectively. The parameter δ is the first time at which a ten-fold reduction in the viable cells is observed, and p is the shape parameter.

The log-linear regression model is described by the following formula:

$$\text{Log } S(t) = \log \frac{N(t)}{N_0} = -\frac{k_{max} \cdot t}{\ln(10)} \qquad (2)$$

where S(t) is the momentary survival ratio and N(t) and N_0 are the momentary and initial

counts at 101°C, respectively. The parameter *kmax* is a first-order inactivation constant. Therefore, the traditional decimal reduction time (*D*-value) can be calculated from the *kmax* parameter according to the equation: $D = 2.303/\text{kmax}$.

The log-linear regression with shoulder model is described by the following formula:

$$\text{Log}\, S(t) = \log \frac{N(t)}{N_0} = -\frac{k_{max} \cdot t}{\ln(10)} + \frac{k_{max} \cdot Sl}{\ln(10)} - Log\left(1 + e^{(k_{max} \cdot Sl)} - 1\right) \cdot e^{(k_{max} \cdot t)} \qquad (3)$$

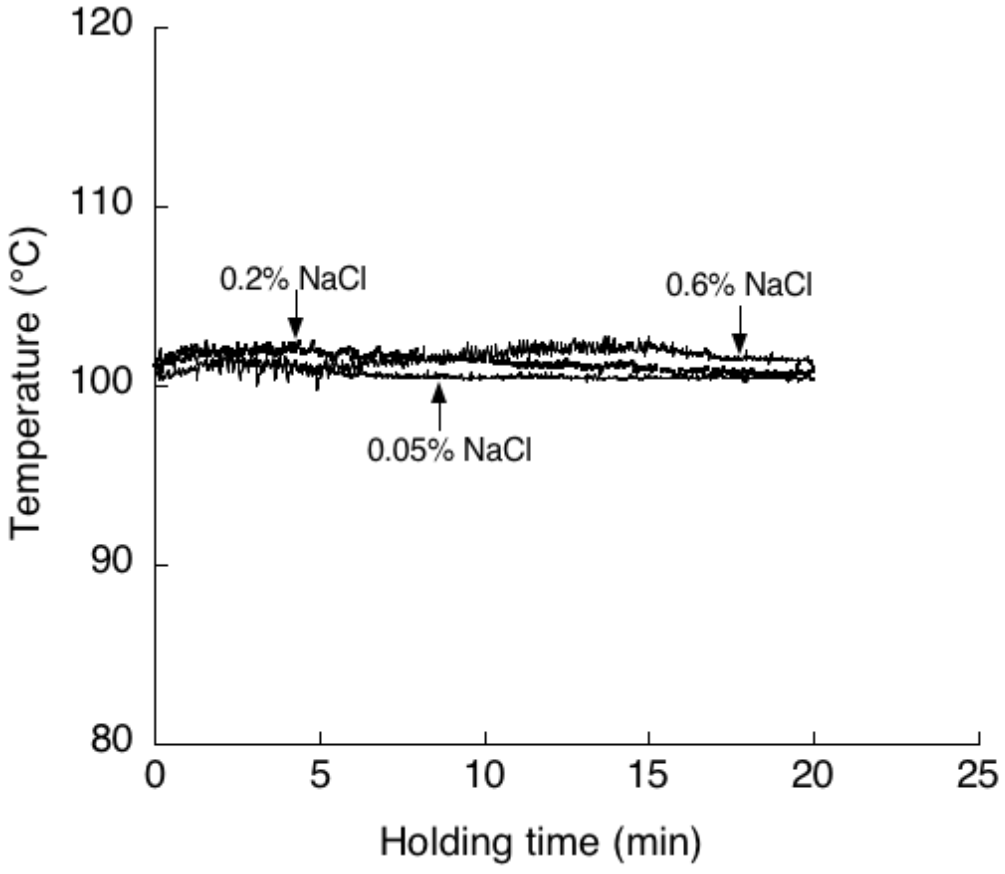
where *kmax* is the first-order inactivation constant and *Sl* is the shoulder length. This model describes the survival curves based on two parameters, i.e., the shoulder length (*Sl*), defined as the time until exponential inactivation begins, and the inactivation rate (*kmax*), defined as the slope of the exponential portion of the survival curve. Therefore, the traditional decimal reduction time (*D*-value) can be calculated from the *kmax* parameter, as mentioned above.

Statistical analysis. All experiments were repeated three times and each data point was determined based on duplicate plates counts. Data from three samples subjected to each treatment in each of three independent replicate experiments were analyzed. For the statistical evaluation, significant differences ($P < 0.05$) in the survival ratio between the OH and CH treatments at each heating time and the parameters for the model fits for OH and CH were analyzed using the Tukey–Kramer method. The statistical analysis was conducted using the R statistical environment (Ver.3.2.4 for Mac OS URL; http://www.R-project.org)

2.3. Results

The temperature were 101°C and the electric fields were constantly applied during OH (Fig. 2-1). The electric fields were constantly applied during OH (Fig. 2-2). Electric field conditions for OH were controlled by adjusting the sodium chloride concentration. As the sodium chloride concentration increases, an electric current flows more easily and the intensity of the electric field declines. The sodium chloride concentration did not affect the inactivation of *B. subtilis* spores during heating at the boiling point (data not shown). Therefore, we concluded that sodium chloride concentrations of 0.05–0.6% do not significantly influence the survival kinetics of *B. subtilis* spores. In the following, we treated the average of the data from sodium chloride concentrations of 0.05–0.6% as the date of external CH.

(a)



(b)

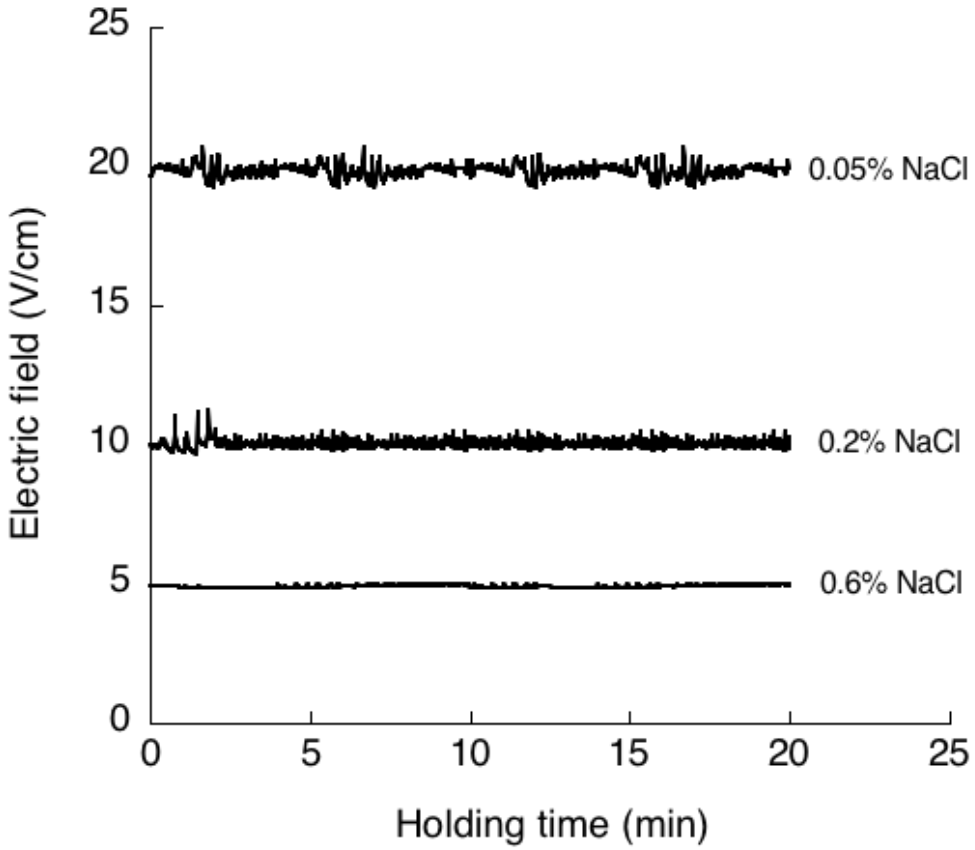


Fig. 2-2. Temperature history (a) and applied electric field history (b) for solutions with various concentrations of sodium chloride solution during ohmic heating.

Greater inactivation effects on *B. subtilis* spores were observed for all of the electric field conditions (5, 10, and 20 V/cm) for OH than for external CH (Fig. 2-3). As the intensity of the electric field increased, a greater, more rapid log-reduction in viable cells was observed. In particular, the OH treatment with 20 V/cm showed significantly ($P < 0.05$) greater log-reductions at 8, 10, 12, 14, and 16 min than those obtained for the CH treatments. However, there were no significant differences in log-reduction between CH and OH for 20 V/cm at 18 and 20 min.

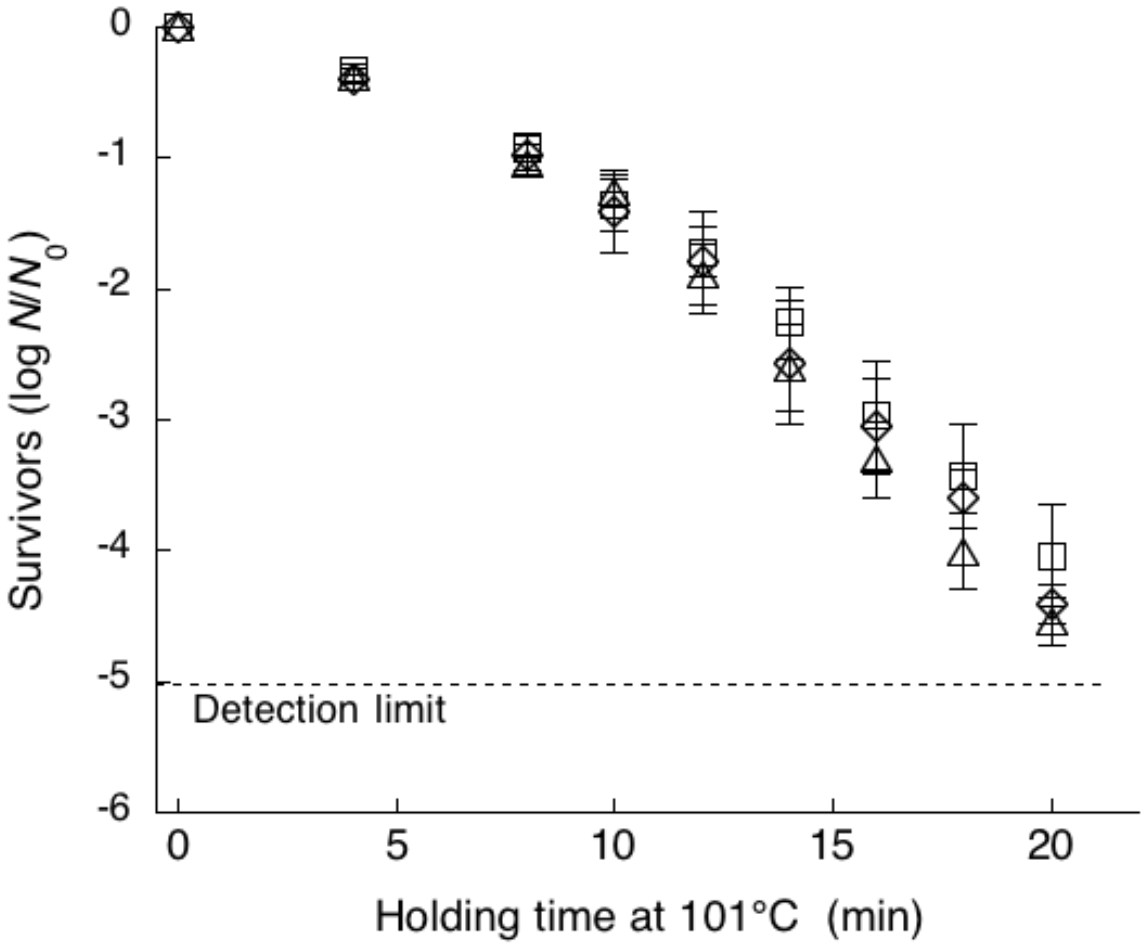


Fig. 2-3. Survival kinetics of *Bacillus subtilis* in a sodium chloride solution (0.05 (○), 0.2 (◇), 0.6 (□) %). The results are presented as means from triplicate experiments, and the error bars indicate standard errors. Means with different letters are significantly different ($P < 0.05$).

In the log-linear model, the log-linear-plus shoulder model, and the Weibull model, kmax of OH tended to be higher and δ of OH tended to be lower than those of CH (Table. 2-1). In the Weibull model and the log-linear model, there were significant differences ($P < 0.05$) in δ and *kmax* between OH at 20 V/cm and CH. These results showed that B. subtilis spores were

inactivated more efficiently as the electric field intensity increased.

As the frequency increased during OH treatment at 20 V/cm, OH showed a trend toward a faster and greater log-reduction in viable *B. subtilis* spores (Fig. 2-4). In particular, the OH with 60 kHz completely inactivated *B. subtilis* spores at 14–16 min, while viable spores remained after treatment with OH at 20 kHz and 40 kHz for the same treatment duration. However, there were no statistically significant differences ($P > 0.05$) in surviving *B. subtilis* spores (Fig. 2-4) and the fitted model parameters between frequencies (Table. 1). Although there were no significant differences among the frequencies, the time until complete inactivation of *B. subtilis* spores was apparently frequency-dependent.

Table.2-1 Survival kinetics parameters for *B. subtilis* spore inactivation at 101°C in sodium chloride aqueous solution with or without ohmic heating at different electric fields and frequencies.

					Ohmic heating		
		Conventional	5 V/cm	10 V/cm	20 V/cm	20 V/cm	20 V/cm
Fitted model		heating	20 kHz	20 kHz	20 kHz	40 kHz	60 kHz
Weibull	R_2	0.97±0.02	0.96±0.01	0.98±0.01	0.93±0.03	0.89±0.04	0.80±0.23
	δ	8.49±1.93 a	6.05±0.34 ab	8.13±0.98 ab	5.15±0.81 b	4.58±0.78 b	5.40±0.87 b
	p	1.77±0.35 a	1.37±0.08 a	2.04±0.49 a	1.36±0.03 a	1.29±0.27 a	1.63±0.42 a
Log-linear	R_2	0.92±0.02	0.95±0.01	0.89±0.04	0.92±0.02	0.89±0.03	0.79±0.17
	k_{max}	0.53±0.10 a	0.60±0.03 ab	0.61±0.06 ab	0.73±0.12 b	0.70±0.13 ab	0.79±0.07 b
Log-linear +shoulder	R_2	0.97±0.01	0.97±0.01	0.97±0.01	0.95±0.02	0.91±0.04	0.80±0.24
	SL	6.11±1.46 a	4.24±0.50 a	6.18±1.01 a	4.19±0.37 a	3.44±1.96 a	4.10±1.93 a
	k_{max}	0.72±0.12 a	0.72±0.05 ab	0.90±0.19 ab	0.89±0.15 ab	0.89±0.25 ab	1.07±0.32 b

Values are the mean ±SE from three replicate trials. Within the same row, means with different letters are significantly different ($P < 0.05$)

δ: rate parameter of the Weibull model

p: shape parameter of the Weibull model

k_{max}: first-order inactivation rate

SL: shoulder period

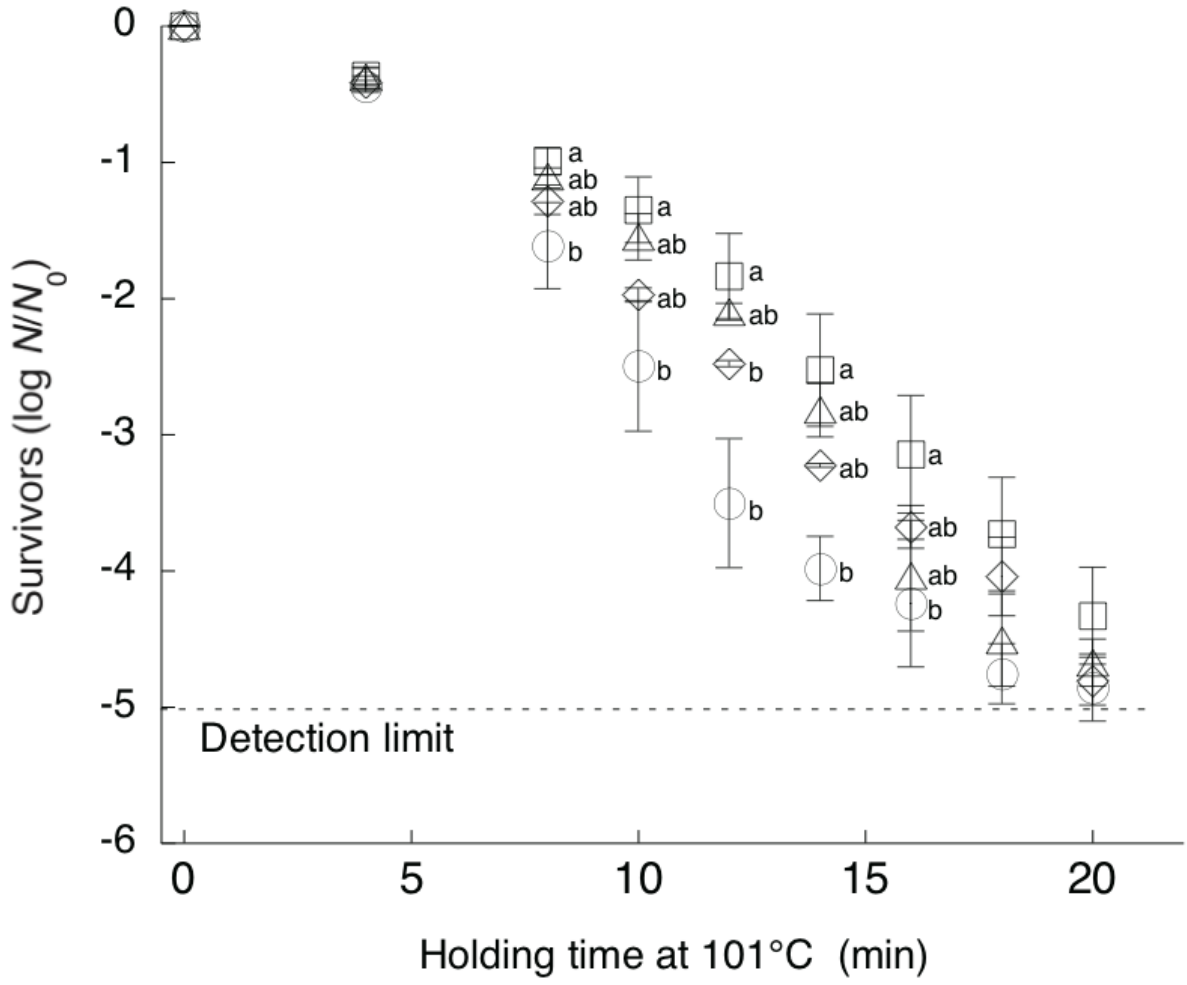


Fig. 2-4. Survival kinetics of *Bacillus subtilis* in a sodium chloride solution treated by conventional external heating (□) at 101°C and ohmic heating at 5 V/cm (◇), 10 V/cm (△), and 20 V/cm (○) at 101°C and at 20 kHz. The results are presented as means from triplicate experiments, and the error bars indicate standard errors. Means with different letters are significantly different ($P < 0.05$).

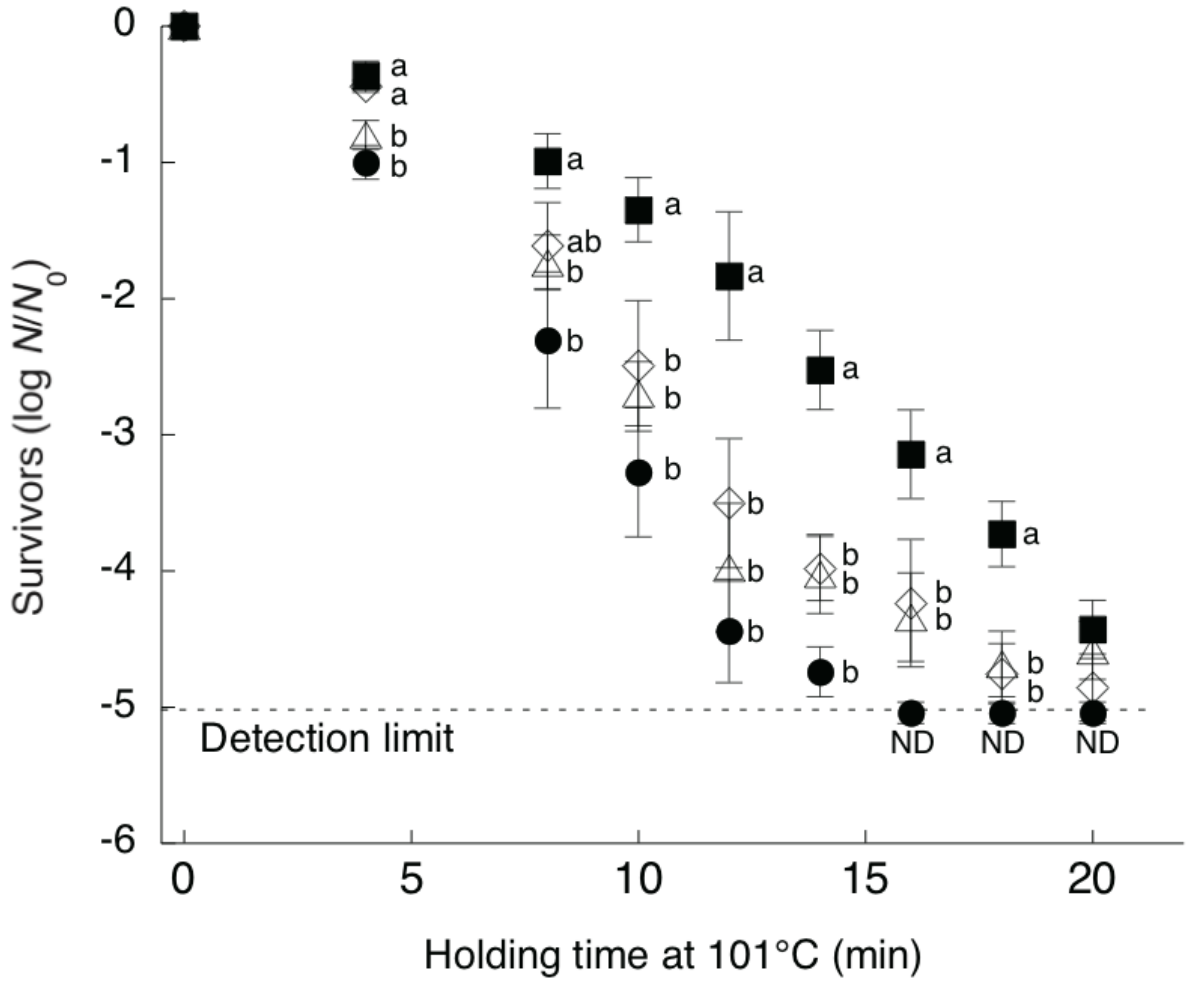


Fig. 2-5. Survival kinetics for *B. subtilis* in a sodium chloride solution treated by conventional external heating (■) at 101°C and ohmic heating at 20 V/cm with 20 kHz (◇), 40 kHz (△), and 60 kHz (●) at 101°C. The results are presented as means from triplicate experiments, and error bars indicate standard errors. Means with different letters are significantly different ($P < 0.05$).

2.4. Discussion

A few studies have compared the efficiency of bacterial spores inactivation between OH and CH. Cho et al. (1999) studied the inactivation kinetics of inactivation of B. subtilis spores using OH and CH and reported that OH has a greater inactivation effect than CH. Moreover, Cho et al. (1999) indicated that bacterial spore inactivation during ohmic heating is primarily attributed to the thermal effect, but an additional killing effect occurs via the electric current. In the present study, the electric field and frequency had additional effects because *B. subtilis* was inactivated not only by OH but also by CH. *B. subtilis* would be damaged by heat as well as increases in the electric field and frequency.

The additional effect of the electric field on bacteria may be primarily explained by a related phenomenon, electroporation. High electric fields, such as pulsed electric fields (> 10 kV/cm), caused electroporation and low frequencies (usually 50–60 Hz) allow cell walls to build up charges and form pores (USA-FDA United States of America, 2015). Park and Kang (2013) studied the effects of electric field-induced ohmic heating on the inactivation of *Escherichia coli* O157:H7, *Salmonella* enterica serovar Typhimurium, and *Listeria monocytogenes* in buffered peptone water and apple juice at 30 V/cm and 60 V/cm. The authors found that bacterial reduction differed significantly between OH and CH treatments. Moreover, propidium iodide values (an index of cell membrane damage) were significantly different between OH and CH treatments. Yoon et al. (2002) investigated the effects of OH on the structure and permeability of the cell membrane of Saccharomyces cerevisiae. They found that the amount of exuded protein increased significantly as the electric field increased from 10 to

20 V/cm, while the amount of exuded protein increased when higher frequencies (600 Hz, 6 kHz, and 60 kHz) were applied. Thus, vegetative bacterial cells and yeast cells would be damaged by higher electric fields and frequencies similar to electroporation.

However, the responses of bacterial spores to various electric fields and frequencies are unclear. Baysal and Icier (2010) studied the effect of electric field (30, 40, and 50 V/cm) on *Alicyclobacillus acidoterrestris* spores heated by OH. Baysal and Icier (2010) reported that while there were significant differences among the electric fields at 70°C, there were no significant differences among the electric fields at 80°C and 90°C. Somavat et al. (2012) indicated that 10 kHz treatment results in comparatively more effective inactivation than 60 Hz for *Geobacillus stearothermophilus* spores. In contrast, Somavat et al. (2013) observed that 60 Hz treatments for *B. coagulans* spores tend to more efficiently inactivate spores than 10 kHz treatments.

Because a constant electric field was not applied to maintain the temperature in order to avoid over-heating in these previous studies, the effects of electric fields are expected to be variable. In contrast, in the present study, a constant electric field was applied at the boiling point during OH treatment and a higher electric field and frequency increased the non-thermal effect of OH on *B. subtilis*. These results demonstrated that the continual application of a certain electric field would increases non-thermal effects on bacterial spores. Moreover, the effect of frequency on the inactivation of spores is pronounced under a constant certain electric field.

Although the mechanism by which electric field and frequency influence bacterial spores

is unclear, it is common for ion molecules, such as hydrogen ions, to be drawn by electric fields and polar substances, such as water molecules, vibrate in response to electric fields. These attractive forces and vibrations may cause the changes in cell structure or the leakage of intercellular substances in bacterial spores. The heat resistance of bacterial spores may decline above a certain frequency, which may explain why higher frequencies increase the non-thermal effects of OH on *B. subtilis*. In the future, it is necessary to investigate the effects of electric field and frequency on other kind of bacterial spore types by applying a contrast electric field.

In conclusion, the results of the present study indicated that non-thermal *B. subtilis* spore inactivation results from OH when an electric field is constantly applied. The OH conditions with higher electric field intensities and higher frequencies improve the efficiency of *B. subtilis* spore inactivation and reduce the heating time to inactivate *B. subtilis* spores. Consequently, although we will need to examine the effect of OH on food quality and/or sensory evaluation, the OH treatment would have a potential for reducing thermal treatment time for ensuring food safety.

Chapter 3

Inactivation of bacteria in water and ice using an ultra violet C light-emitting diode

3.1. Introduction

Ice is widely used to cool foods, such as drinking water, seafood, and fresh produce. Ice is mainly made by home freezers or by ice-making machines in restaurants, cafes, and ice-making companies. Importantly, ice must be microbiologically and chemically safe because consumers eat ice directly or eat foods that come in direct contact with ice. However, ice can be microbially contaminated (Moore et al., 1953; Tsuno et al., 1984; Falcão, 2002; Falcão et al., 2004; Lateef et al., 2006; Chavasit et al, 2011; Gerokomou et al., 2011; Economou et al., 2016) and thus can cause food poisoning (Khan et al., 1994; Chavasit et al., 2011). Gerokomou et al. (2011) investigated bacterial contamination in 100 ice samples were collected at 10 different retail points in the region of Epirus in Greece. They found the presence of Escherichia coli and Salmonella in 15% and 4% of ice samples, respectively, ranging from 10^2 to 10^3 CFU/ml. Lateef et al. (2006) evaluated the microbiological safety of commercial ice using ice collected from four ice manufacturing factories in Ogmonoso, Nigeria. They found that all the samples were microbially contaminated and that the microbial load ranged from 1.88 to 3.20 $\times 10^4$ CFU/ml.

Microorganisms can be contaminated in ice owing to the poor quality of source water used, and/or a lack of hygiene during production or handling (Moore et al., 1953). Moreover, Burnett et al. (Burnett et al., 1994) indicated that ice-making machines can be contaminated

due to seeding from the mains supply, faulty plumbing allowing backflow from the drains, and irregular cleaning of ice machines. Once ice-making machines are contaminated, contaminated ice will be produced until ice-making machines are cleaned.

In order to prevent food poisoning by ice, sufficiently hygienic water should be used, and ice-making machines should be cleaned regularly. However, both of these processes require additional work and utilize chemicals that can be problematic. Additionally, it is particularly difficult to evaluate the safety of water and cleaning of ice-making machines, which can be individually owned or automatic vending machines. Therefore, development of an inactivation technique for microorganisms in ice may be the most effective approach for preventing ice contamination. However, because such a technique must be non-thermal and nondestructive technique, no studies have yet reported the direct microbial inactivation of ice.

Ultra violet (UV) irradiation is a common non-thermal microbial inactivation technique. UV light covers a wavelength spectrum from 100–380 nm and is subdivided into three regions by wavelength: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm) (Song et al., 2016). UV radiation is believed to inactivate microorganisms by acting directly on the DNA in the cell, blocking cellular reproduction. In general, UVC radiation is believed to have high inactivation effects because DNA mainly absorbs UV radiation from 200–300 nm with an absorbance peak around 260 nm. UV treatment can inactivate all types of microorganisms because UV acts directly on DNA. However, the main UV sources are mercury lamps, which are fragile and contain toxic mercury. In contrast, UV light-emitting diodes (UV-LEDs) have recently emerged as new source for UV irradiation. UV-LED has several advantages such as

compactness, robustness, faster start-up time, less energy consumption, longer lifetime, ability to turn on and off with high frequency, and lack of mercury (Würtele et al., 2011). However, few studies have evaluated the microbial inactivation effect by UV-LED. Song et al. (2016) reviewed the effects UV dose by UV-LED on various microorganisms from published literature and concluded there are many cases where the results are inconsistent because there has been a lack of uniformity in research materials and methods. Different UV-LEDs have various radiation patterns, such as emission spectra, viewing angles, and radiation distributions. Similarly, there have been few studies comparing the effects of UV-LEDs with those of conventional UV lamps. Bowker et al. (2011) compared the inactivation effect of a 254-nm UV lamp with 275-nm and 255-nm of UVC-LEDs in which the UV intensities were 0.34, 0.094–0.11, and 0.049–0.060 mW/cm^2, respectively. The log reduction for the same UV dose increased with the increase in UV intensity. Bowker (2011) assumed that lower UV intensity may cause the lower inactivation rates, despite using the same UV dose. Thus, fundamental knowledge of the effects of UVC-LEDs on microbes in ice is necessary for evaluation of its applicability.

Accordingly, in the present study, we evaluated the effects of a UVC-LED for bacterial inactivation in ice. The objectives of this study were to evaluate the performance of the UVC-LED and to investigate the effects of the UVC-LED on bacterial inactivation in the ice. Our results provide insights into the performance and practical applicability of UVC-LED irradiation in the inactivation of microorganisms in ice.

3.2. Materials and methods

3.2.1. Bacterial strains

E. coli (ATCC 25922), four strains of *E. coli* O157:H7 (RIMD 0509939, RIMD 05091896, RIMD 05091897, and HIPH 12361), five strains of *S.* Typhimurium (RIMD 1985007, RIMD 1985009, ATCC29057, ATCC29629, and ATCC29630), and six strains of *Listeria monocytogenes* (ATCC13932, ATCC15313, ATCC19111, ATCC19117, ATCC19118, and ATCC35152) were used in this study. These strains were maintained at −80°C in tryptic soy broth (TSB) containing 10% glycerol. A platinum loop was used to transfer the frozen bacterial cultures by scratching the surface of the frozen culture into tryptic soy agar (TSA) plates. The inoculated plates were incubated at 37°C for 24 h, and an isolated colony of each bacterium was then transferred to fresh 5 ml TSB in a sterile plastic tube. The cultures were transferred using loop inocula at two successive 24-h intervals to obtain a more homogeneous and stable cell population. Grown cells were collected by centrifugation (3000 × g, 10 min), and the resulting pellet was washed by sterile 0.1% peptone water three times and subsequently resuspended in 10 ml sterile 0.1% peptone water, corresponding to approximately $10^8 - 10^9$ CFU/ml. Strains of three pathogenic species, except *E. coli* ATCC 25922, were combined to prepare culture cocktails for use in experiments. The suspensions were stored at 5°C until use.

3.2.2. Sample preparation

For distilled water-inactivation experiments, an aliquot of 1 ml of *E. coli* ATCC 25922 suspension ($10^8 - 10^9$ CFU/ml) was added to 50 ml distilled water in a glass beaker such that

the initial concentration of the inoculum was approximately $10^7 - 10^8$ CFU/ml. For an ice-inactivation experiments, an aliquot of 1 ml of *E. coli* ATCC 25922 suspension and the cocktail suspensions of each pathogen were added to 25 ml distilled water in a glass beaker. The inoculated distilled water was placed into an ice cube tray (length: 30 mm, width: 30 mm, thickness: 30 mm) and then frozen at −80°C for 5 to 6 h. The initial concentration of E. coli and other pathogens in the ice was approximately $10^6 - 10^7$ CFU/ml, because viable bacterial numbers were reduced by one log during the freezing process.

3.2.3. Experimental apparatus

One UVC-LED module (UVC-EC910ZA; Panasonic Photo lighting, Co. Ltd., Osaka, Japan; diameter: 5.5 mm, height: 0.2 mm) with a wavelength of 270–280 nm was connected onto a direct-current power supply (GW Instek, Taipei, Taiwan). The electric current and voltage were 120 mA, 9－10 V, respectively. The UVC-LED and UV lamp were placed in the vertical direction relative to the sample. The intensity of UVC was adjusted by varying the distance between the samples and the LED. The intensity of the UV was determined with a spectrometer (UV-37SD; Custom, Tokyo, Japan). The UV intensity was maintained constant for 30 min. A low-pressure UV lamp (Handy UV lamp SUV-16; As one, Osaka, Japan; length: 336 mm, width: 82.3 mm, height: 65 mm) with a peak wavelength of 254 nm was used to compare the effects of the UVC-LED. The UV lamp was housed in a UV collimated beam apparatus (Bolton et al., 2003).

3.2.4. Distilled water-inactivation experiment by UVC

Water samples containing *E. coli* ATCC 25922 in a glass beaker were irradiated by UVC-LED irradiation at various UV intensities (0.084, 0.025, 0.013, 0.007, and 0.005 mW/cm^2) or by UV lamp at a UV intensity of 0.025 mW/cm^2 at room temperature (20－25°C). A 1-ml aliquot was collected by pipetting at each sampling time during UVC irradiation treatment.

3.2.5. Ice-inactivation experiment by UVC

In this experiment, all ice samples (width: 30 mm, depth: 30 mm, height: 28 mm) on the petri dishes were irradiated at －30°C in a deep freezer. Ice samples containing *E. coli* ATCC 25922 were irradiated by a UVC-LED of various UV intensities (0.084, 0.025, 0.013, 0.007, and 0.005 mW/cm^2) or by a UV lamp at a UV intensity of 0.025 mW/cm^2 at room temperature (20－25°C). Ice samples containing pathogens, such as *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, were irradiated by a UVC-LED at 0.084 mW/cm^2. Irradiated ice samples were completely melted at room temperature. A 1-ml aliquot of sample was collected from melted ice. Samples were irradiated with UVC for a maximum of 30 min.

3.2.6. Microbiological analysis

After UV irradiation treatment, to determine the number of surviving bacteria at each UV dosages, appropriate serial dilutions (1:10) were performed with sterile 0.1% peptone water and were plated on duplicate TSA plates. The plates were then incubated at 37°C for 24－48 h.

Survival kinetics analysis. The sensitivity of microorganisms to UV radiation can be evaluated by following formula (Hijnen et al., 2006):

$$\log \frac{N(t)}{N_0} = -k \times \text{UV dose} \qquad (1)$$

where *N(t)* and N_0 are the momentary and initial counts, respectively. The initial counts (N_0) were viable cell counts at the time zero. The parameter *k* (cm^2/mJ) is the inactivation rate from the linear portion of the relationship between log inactivation and the applied UV dose.

3.2.7. Statistical analysis

All experiments were repeated three times, and each data point was determined based on duplicate plates counts. Data from three samples subjected to each treatment in each of three independent replicate experiments were analyzed. To determine statistical significance ($P < 0.05$) between the UV treatment conditions and type of pathogenic bacteria, we used Students' paired t-tests in the case of comparisons with two groups, such as the UVC-LED versus the UV lamp, and we used Tukey-Kramer's method for multiple comparison tests of comparisons of more than three groups, such as UV intensities and type of pathogenic bacteria. All statistical analyses were conducted using the R statistical environment (Ver.3.2.4 for Mac OS URL; http://www.R-project.org).

3.3. Results

3.3.1. Performance of the UVC-LED

Changes in the irradiation intensity of the UVC-LED depending on the irradiation distance were examined. As shown in Fig. 3-1, the UV intensity decreased as the irradiation distance became longer. While the UV intensity was 0.084 mW/cm^2 at a distance of 2 cm, the UV intensity was decreased by 0.005 mW/cm^2 at a distance of 10 cm. We also evaluated the durability of the UVC-LED as another aspect of the performance of the UVC-LED. Changes in the UV intensity of the UVC-LED for 30 min at room temperature and －30°C are shown in Fig. 3-2. The UV intensity was almost constant during the 30-min irradiation, and temperature did not affect the intensity of the UVC-LED.

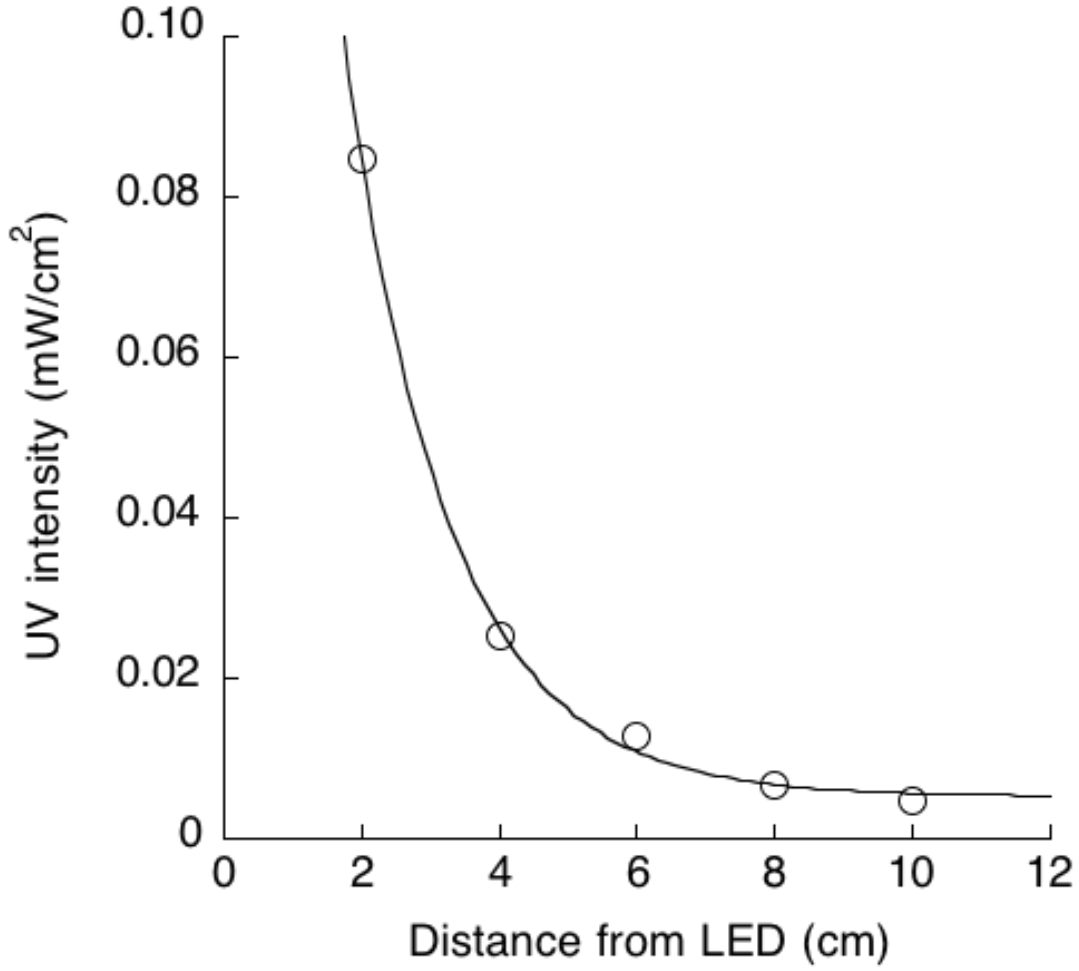


Fig. 3-1. Changes in the intensity of UVC-LED depending on the distance between UVC-LED and the objective.

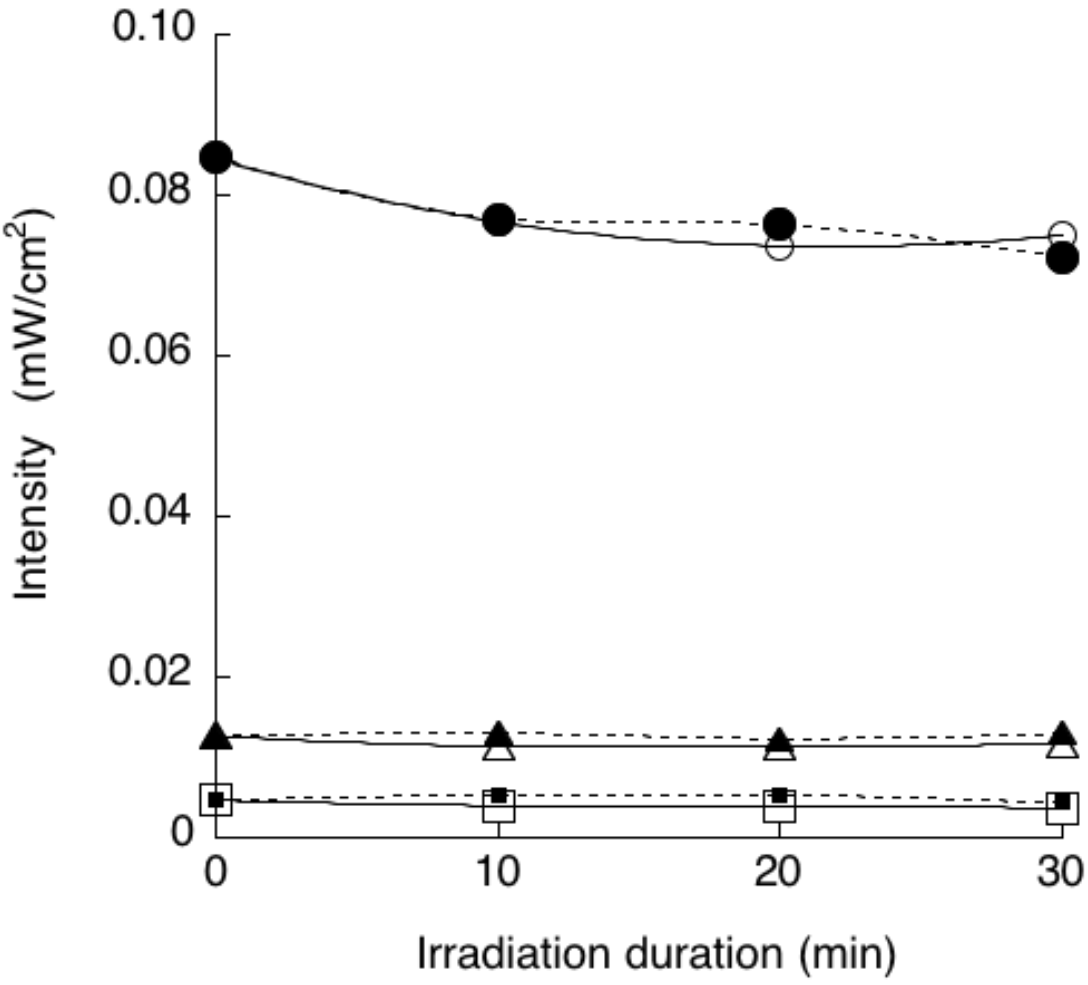


Fig. 3-2. The intensity of UVC-LED when the distance and temperature changes. 2 cm (○), 6 cm (△), and 10cm (□) at room temperature and 2 cm (●), 6 cm (▲), and 10cm (■) at –30°C.

3.3.2. Inactivation of *E. coli* and pathogens in distilled water by UVC irradiation

E. coli ATCC 25922 in distilled water decreased as the UV dose of the UVC-LED increased (Fig. 3-3). The reduction of *E. coli* depended on the UV dose at all UV intensities except for 0.084 mW/cm^2. *E. coli* ATCC 25922 was inactivated completely at 4 mJ/cm^2 when the UV intensity was 0.084 mW/cm^2. In contrast, *E. coli* was completely inactivated at 1–2 mJ/cm^2 when other UV intensities were applied. The kinetic parameter inactivation rate *k* value

of the UVC-LED at a 0.084 UV intensity of mW/cm^2 was significantly lower ($P < 0.05$) than that at other UV intensities (Table. 3-1).

Furthermore, we compared the performance of the UVC-LED with that of a conventional UV lamp at a UV intensity of 0.025 mW/cm^2 (Fig. 3-4). The UVC-LED could inactivate *E. coli* ATCC 25922 in distilled water more efficiently than the UVC lamp at the same UV dose (Fig. 3). The *k* values of UVC-LED and that of UV lamp at 0.025 mW/cm^2 were significantly different (4.27 ± 0.49 versus 0.50 ± 0.02, respectively; $P < 0.05$; Table. 3-1).

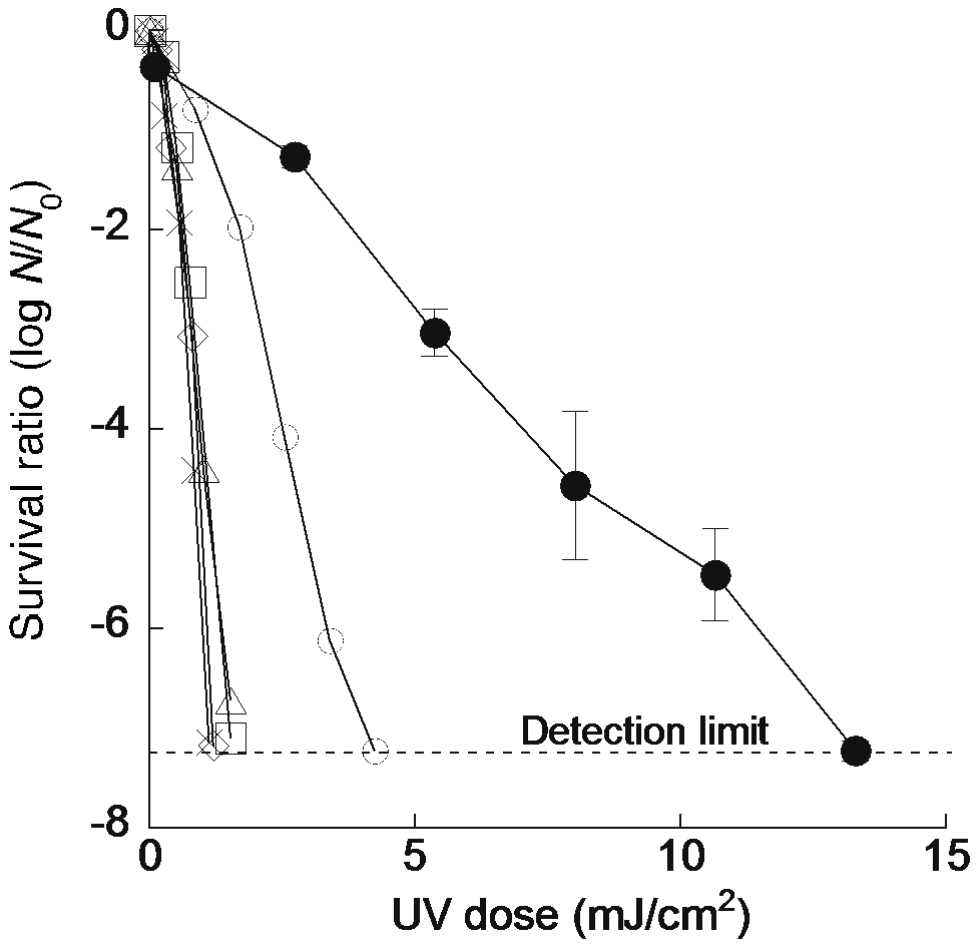


Fig. 3-3. Changes in the survival ratio of *E. coli* ATCC 25922 in distilled water irradiated by UVC-LED at each UV intensities of 0.085 mW/cm^2 (○), 0.025 mW/cm^2 (△), 0.013 mW/cm^2 (□), 0.007 mW/cm^2 (◇), and 0.005 mW/cm^2 (×), and irradiated by a UV lamp (●) at a UV intensity of 0.025 mW/cm^2 at room temperature.

Table.3-1 UV sensitivity of *E. coli* ATCC 25922 in distilled water for UVC-LED and low-pressure UV lamp.

	UVC-LED					UV lamp
	UV intensity (mW/cm^2)					
	0.084	0.025	0.013	0.007	0.005	0.025
k (cm^2/mJ)	1.69±0.14 $_{ad}$	4.27±0.49 $_{bc}$	3.66±0.42 $_{b}$	5.02±0.20 $_{bc}$	5.45±0.11 $_{c}$	0.50±0.02 $_{d}$
R^2	0.94±0.02	0.95±0.01	0.96±0.01	0.89±0.03	0.91±0.01	0.97±0.01

Values are the mean ±SE from three replicate. Within the same column, means with different letters are significantly different ($P < 0.05$)

k: Inactivation rate from the linear portion of relationship between log inactivation and the applied UV dose

3.3.3. Inactivation of E. coli and other pathogens in ice by UVC irradiation

We next investigated the inactivation effects of the UVC-LED on bacterial pathogens in ice cube. As shown in Fig. 3-4, the reduction of *E. coli* ATCC 25922 in ice depended on the UV dose at all UV intensity conditions, unlike the case in distilled water (Fig. 3-4). *E. coli* ATCC 25922 required a higher UV dose to be inactivated in ice (160 mJ/cm^2) than that in distilled water (1–4 mJ/cm^2). However, the UVC-LED could inactivate *E. coli* ATCC 25922 in ice more efficiently than the UVC lamp (Fig. 3-5), similar to the results observed in distilled water. There were significantly differences ($P < 0.05$) between the survival ratios of pathogens with the UVC-LED and the UV lamp at UV dose of 1.52, 6.08, and 45.8 mJ/cm^2, but there were no significant differences at 15.2 mJ/cm^2.

Furthermore, a contamination level of 10^6–10^7 CFU/ml of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in the ice was efficiently inactivated by UVC-LED

irradiation (Fig. 3-6). Although the reduction trends of pathogens in ice by the UVC-LED appeared to differ slightly, there were no significantly differences among pathogens at each UV dose.

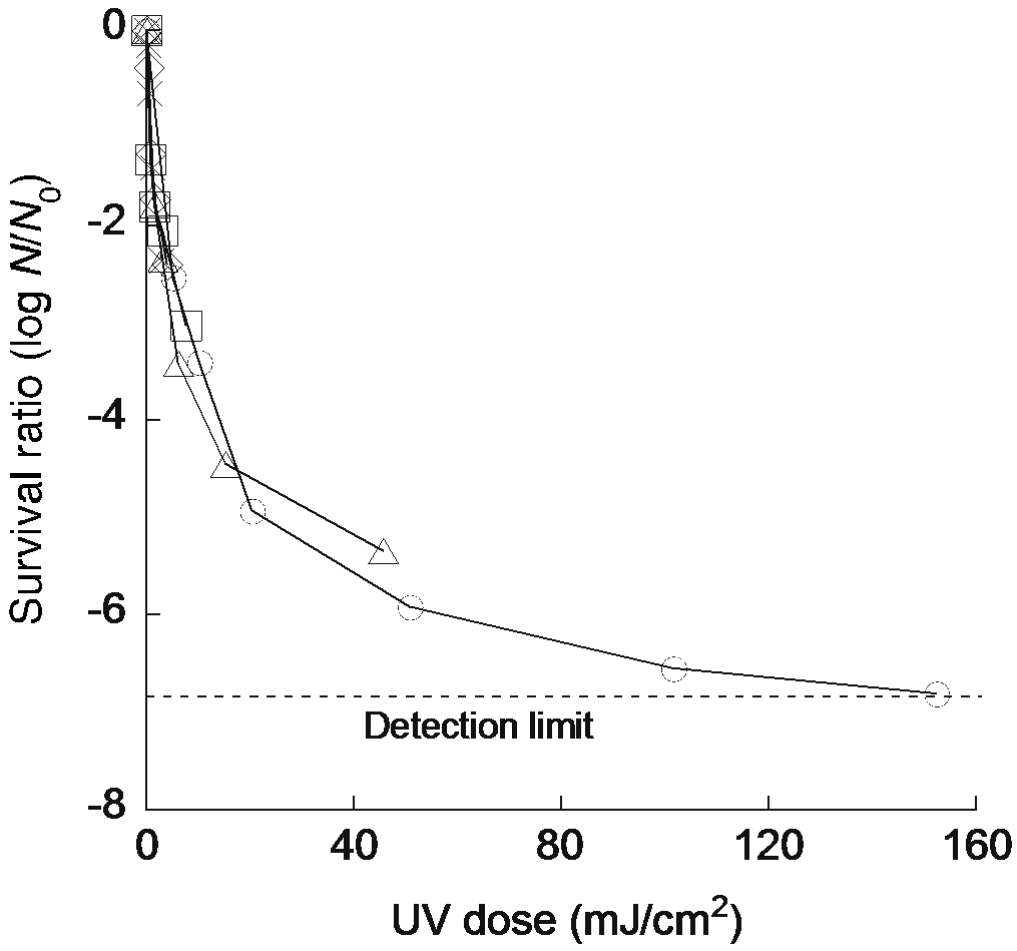


Fig. 3-4. The reduction of *E. coli* ATCC 25922 on the ice at different UV intensities of 0.085 mW/cm^2 (○), 0.025 mW/cm^2 (△), 0.013 mW/cm^2 (□), 0.007 mW/cm^2 (◇), and 0.005 mW/cm^2 (×) at –30°C.

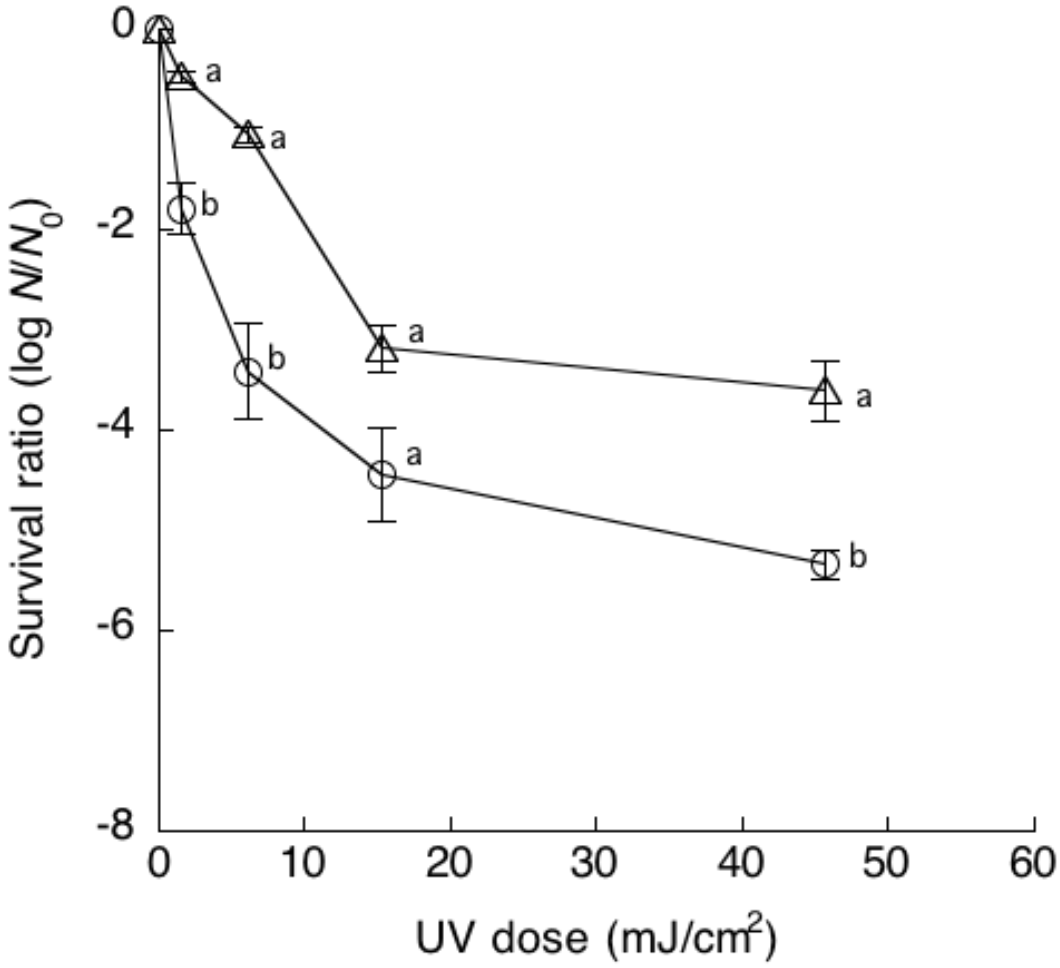


Fig. 3-5. Comparison of the inactivation effects of *E. coli* ATCC 25922 in ice between UVC-LED (○) and a conventional UV lamp at a UV intensity 0.025 mW/cm^2 (△) at –30°C.

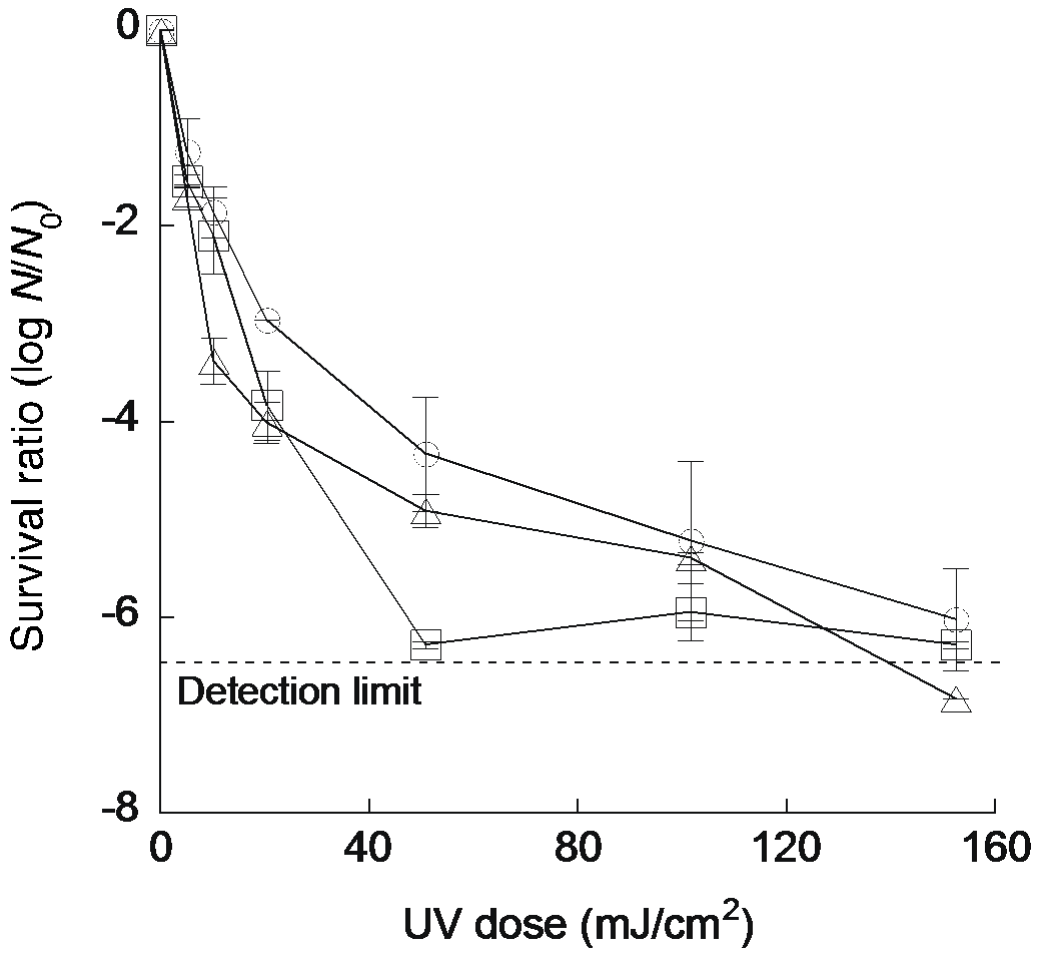


Fig. 3-6. Comparison of the inactivation effects of *E. coli* O157:H7 (○), *S.* Typhimurium (△), and *L. monocytogenes* (□) in ice by UV irradiation using UVC-LED at a UV intensity of 0.085 mW/cm^2 at –30°C.

3.4. Discussion

In this study, we evaluated the ability of a UVC-LED to inactivate pathogens in distilled water and ice. The results indicated that the microbial inactivation effect of the UVC-LED depended on the UV dose at all UV intensity conditions in distilled water and ice, and the UVC-LED could more efficiently inactivate *E. coli* ATCC 25922 in distilled water and ice than the

UV lamp.

Notably, *E. coli* ATCC 25922 in distilled water was not inactivated more efficiently at higher intensities such as 0.084 mW/cm^2, compared with that at other UV intensities. Notably, microbial inactivation by UV irradiation appeared to be dependent on UV dose. However, Sommer (1998) reported that a higher UV intensity and shorter exposure time were more effective for *E. coli* inactivation than a lower UV intensity and longer exposure time when the UV dose is the same, inconsistent with our current results. This discrepancy may be related to the inability of the sample to absorb the UV light at an intensity of 0.084 mW/cm^2 due to the linearity of UVC-LED. Kim et al. (2015) reported that light from UV-LEDs converges at one point vertically, whereas UV lamps scatter light over a large area. Song. et al. (2016) reported that UV-LED is a point source and that uniform irradiance is not expected on the water sample surface. Distilled water could not be uniformly irradiated by the UVC-LED when the distance between distilled water and the UVC-LED was short because the UVC-LED light exhibited straightness. Thus, the UVC-LED may not be able to inactivate microorganisms in a liquid efficiently when the distance between the UVC-LED and irradiated material is small. Notable, distance did not affect the inactivation of *E. coli* ATCC 25922 in the ice, potentially owing to scattering of light. Indeed, UV light could be dispersed throughout ice by bumping into molecular crystals in ice, whereas UV light was passed straight into water. Thus, our current findings supported that the efficiency of the UVC-LED was different depending on the material being irradiated when the UVC-LED and sample were close.

In the present study, the UVC-LED could more efficiently inactivate *E. coli* ATCC 25922

in distilled water and ice than the UVC lamp, consistent with the finding of Chatterley et al. (2010) and Kim et al. (2015). Chatterley et al. (2010) compared the log reduction of E. coli K12 by irradiation from low-pressure lamps (254 nm) and LEDs (265 nm) and concluded that the UV-LED could slightly improve inactivation compared with the UV lamp, although there was no significant difference at a 95% confidence level. Kim et al. (2015) studied the reduction levels of *E. coli* O157:H7, *S.* Typhimurium, and L. monocytogenes spread on selective media after treating at a 254-nm UV lamp or the 266-nm UV-LED. They concluded that UV-LED treatment at a UV dose of 0.7 mJ/cm^2 inactivated nearly all inoculated pathogens, whereas the UV lamp resulted in 3.06-, 1.42-, and 0.34-log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, which were significantly less ($P < 0.05$) than the UV-LED inactivation levels at the same dose. However, Song et al. (19) compared the average of k values for E. coli inactivation using a UV lamp at 254 nm with the k values of UV-LEDs at different wavelengths (255, 265, 272, 275, and 280 nm) from previous studies and concluded that the average k value of the UV lamp was 0.506 cm^2/ml, which was higher than those of UV-LEDs (0.170–0.422 cm^2/ml). Thus, the results of comparisons of inactivation by UV-LEDs and UV lamps have been inconsistent, potentially because of a lack of uniformity in research materials and methods, including individual differences in UV-LEDs. However, our results showed the potential of UVC-LEDs to inactivate bacterial cells efficiency compared with conventional UV lamps.

In this study, the reduction of pathogens in ice by UVC-LED depended on the type of bacteria. Although L. monocytogenes was completely inactivated at 40 mJ/cm^2, 10^2 CFU/ml

of *E. coli* O157:H7 and S. Typhimurium survived, even at 100 mJ/cm^2. In general, gram-positive bacteria, such as L. monocytogenes are more resistant to UV light than gram-negative bacteria, such as *E. coli* O157:H7 and *S.* Typhimurium, providing an explanation for these finding. However, Koutchma (2009) assumed that the UV sensitivities of *L. monocytogenes*, and *Salmonella* in liquid foods were similar to UV sensitivity of E. coli. Kim et al. (2015) studied the viable-count reduction levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* spread on selective media after treatment with a 254-nm UV lamp or 266-nm UV-LED. They found that the inactivation level was lowest for *L. monocytogenes*, similar to our study.

One unit of the UVC-LED used in this study inactivated 10^6–10^7 CFU/ml of the pathogens, although the effect was depended on the type of bacterial strain. These strains are highly pathogenic and can cause food poisoning when present at only 10^1–10^2 CFU/ml. Notably, a UV dose of more than 160 mJ/cm^2 is needed to prevent the food poisoning if the initial pathogen concentrations of more than 10^7 CFU/ml would rarely be encountered in commercial ice (Nichols et al., 2000; Gerokomou et al., 2011).

Finally, we found that bacterial pathogens in ice were not always inactivated completely at a UV dose of 160 mJ/cm^2. We assumed that the cloudiness of the ice, produced by air bubbles, could explain to this result. In general, the penetration of light is lower when the liquid has the greater color or turbidity. The presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effects (Fan and Geveke, 2007). Moreover, the cloudiness of ice could prevent the penetration of UV light into the ice. The ice used in the present study

was not clear because we intended to obtain fundamental data for evaluating the microbial inactivation effect of UVC-LED in commercial ice.

the efficiency of the UVC-LED. In the future, we will need to investigate the effects of cloudiness of ice, the number of UVC-LED modules used for treatment, and changing the angle of UVC-LED on the results of bacterial inactivation.

In summary, our results demonstrated that the use of UVC-LEDs rather than UV lamps may contribute to efficient microbial control in ice and to ensure the safety of ice in the food industry.

Chapter 4

Maximize the characteristics of UVC-LED light-emitting diode for hygienic ice production

4.1. Introduction

UV irradiation is a common non-thermal and non-destructive microbial inactivation technology. UV light treatment is commonly used for liquid sterilization, air disinfection, and inhibition of microorganisms in surface (Falguera et al., 2011). UV treatment has several advantages such as relatively inexpensive equipment, easy to use, and wide spectrum of lethal effect of microorganism (Bintsis et al., 2000). On the other hand, UV has been limited for applying to food industry because of the limitation of the shape of mercury lamp and its low penetration influencing by the characteristics of the irradiated material (Koutchma et al., 2007; Koutchma, 2009; Falguera et al., 2011). The presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effects (Fan et al., 2007) and solid food such as vegetable, fruit, meat is disinfected only its surface (Guerrero-Beltran and Barbosa-Canovas, 2004; Allende et al., 2006; Manzocco et al., 2011; Lim and Harrison, 2016). In addition, bacteria are not inactivated efficiently when the distance between UVC apparatus and irradiated material is large because UV intensity depends on distance (Song et al., 2016). Thus, developing UV-irradiation system which can shed UV light on food efficiently is important for efficient disinfection.

Although there are weak points of UVC irradiation as described above, the strong and wide spectrum effect on various microorganisms under appropriate usage condition has long

been reported by numerous literature. The issues of UV irradiation are summarized in the influence of unevenness and shade of objectives. However, if these issues could be turned over technically, the technique of UVC irradiation would be more worth applying to food processors for ensuring microbiological food safety. To maximize the effect of UVC irradiation, conventional UV lamp technologies would not enable to respond to the industrial demands. Thus, we focused on an emerging technology of UVC irradiation by light-emitting-diode (LED).

UVC-LEDs have recently emerged as a new source for UVC irradiation. UVC-LEDs would allow novel and flexible design of UV inactivation devices because UV-LED has several advantages such as compactness (5 − 9 mm diameter) (Chatterley and Linden, 2010), robustness, faster start-up time, less energy consumption, longer lifetime, ability to turn on and off with high frequency, and lack of mercury (Würtele et al., 2011). UVC-LEDs could be located on various places because of its compactness and have the potential to irradiate from multiple sides. Irradiation from multiple sides could inactivate microorganism efficiently because that way would help to evenly irradiate on food and would reduce the influence of shade area where microorganism could hide. Thus, we hypothesized that the bactericidal effect would be maximized using multiple UVC-LEDs from different angles.

In the previous study, we focused on inactivation of pathogenic bacteria such as Escherichia coli O157:H7, Salmonella Typhimurium, and Listeria monocytogenes in ice by using UVC-LED (Murashita et al., 2017). As a result, a UVC-LED irradiation enable to demonstrate more efficient bactericidal effect in ice cube than that of UV lamp irradiation.

While basic bactericidal effects by UVC-LED for ice have been investigated, the optimal procedure for bacterial inactivation will be needed for a practical usage.

In the present study, we aimed at examining the inactivation effect on pathogenic bacteria in ice by using multiple UVC-LED modules and multiple irradiation directions of UVC-LED. Furthermore, a bench-scale UVC irradiation apparatus that is installed eight UVC-LED modules was developed for maximizing bactericidal effect in ice. We determined the bactericidal performance of multiple irradiation directions and/or multiple intensities of UVC-LED to be practically used in the future.

4.2. Materials and methods

4.2.1. Bacterial strains

Four strains of *E. coli* O157:H7 (RIMD 0509939, RIMD 05091896, RIMD 05091897, and HIPH 12361), five strains of *S.* Typhimurium (RIMD 1985007, RIMD 1985009, ATCC29057, ATCC29629, and ATCC29630), and six strains of *L. monocytogenes* (ATCC13932, ATCC15313, ATCC19111, ATCC19117, ATCC19118, and ATCC35152) were used in this study. These strains were maintained at −80°C in tryptic soy broth (TSB) containing 10% glycerol. A platinum loop was used to transfer the frozen bacterial cultures by scratching the surface of the frozen culture into tryptic soy agar (TSA) plates. The inoculated plates were incubated at 37°C for 24 h, and an isolated colony of each bacterium was then transferred to fresh 5 ml TSB in a sterile plastic tube. The cultures were transferred using loop inocula at two successive 24-h intervals to obtain a more homogeneous and stable cell

population. Grown cells were collected by centrifugation (3000 × g, 10 min), and the resulting pellet was washed by sterile 0.1% peptone water three times and subsequently resuspended in 10 ml sterile 0.1% peptone water, corresponding to approximately $10^8 - 10^9$ CFU/ml. Strains of three pathogenic species were combined to prepare culture cocktails for use in experiments. The suspensions were stored at 5°C until use.

4.2.2. Sample preparation

An aliquot of 1 ml of the cocktail suspensions of each pathogen were added to 25 ml distilled water in a glass beaker. The inoculated distilled water was placed into an ice cube tray (length: 30 mm, width: 30 mm, thickness: 30 mm) and then frozen at －80°C for 5 to 6 h. The initial concentration of pathogens in the ice sample (width: 30 mm, depth: 30 mm, height: 28 mm) was approximately $10^6 - 10^7$ CFU/ml, because viable bacterial numbers were reduced by one log during the freezing process.

4.2.3 Experimental apparatus

UVC-LED modules (UVC-EC910ZA; Panasonic Photo lighting, Co. Ltd., Osaka, Japan; diameter: 5.5 mm, height: 0.2 mm) with a wavelength of 270–280 nm was connected on a direct-current power supply (GW Instek, Taipei, Taiwan). The electric current and voltage were 120 mA, 9－10 V per one UVC-LED, respectively. As the same direction condition, 1–3 UVC-LEDs was placed in the vertical direction relative to the sample. As different direction conditions (Fig.4-1), 1–3 UVC-LEDs placed in the vertical and horizontal direction relative to

the sample. The distance between ice and UVC-LED was 6 cm in all experiment. The intensity of the UV was determined with a spectrometer (UV-37SD; Custom, Tokyo, Japan). The UV intensity was maintained constant for 30 min.

A bench-scale UVC-LED test apparatus was rectangular device and contained eight UVC-LEDs modules (Fig. 2 + picture).

An ice, which contained pathogens, on the plastic table (diameter: 5.4 mm, height: 1.0 mm) was placed in the center of the UVC-LED apparatus.

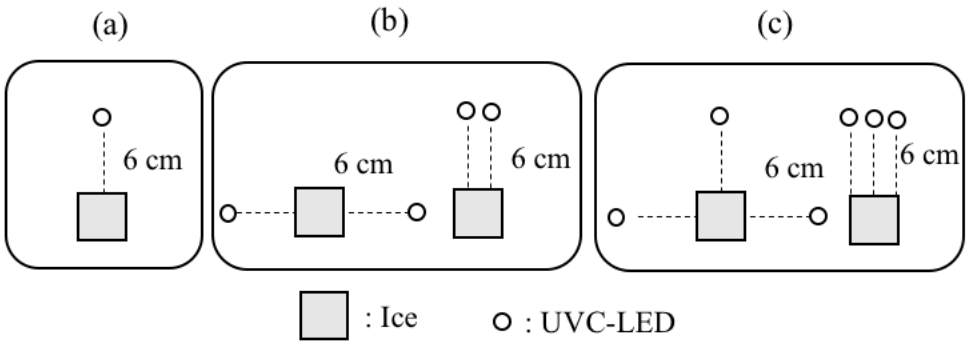


Fig. 4-1. A schematic diagram of installation of multiple UVC-LEDs. (a): using one UVC-LED, (b): using two UVC-LEDs from same or different directions, (c): using three UVC-LEDs from same and different directions.

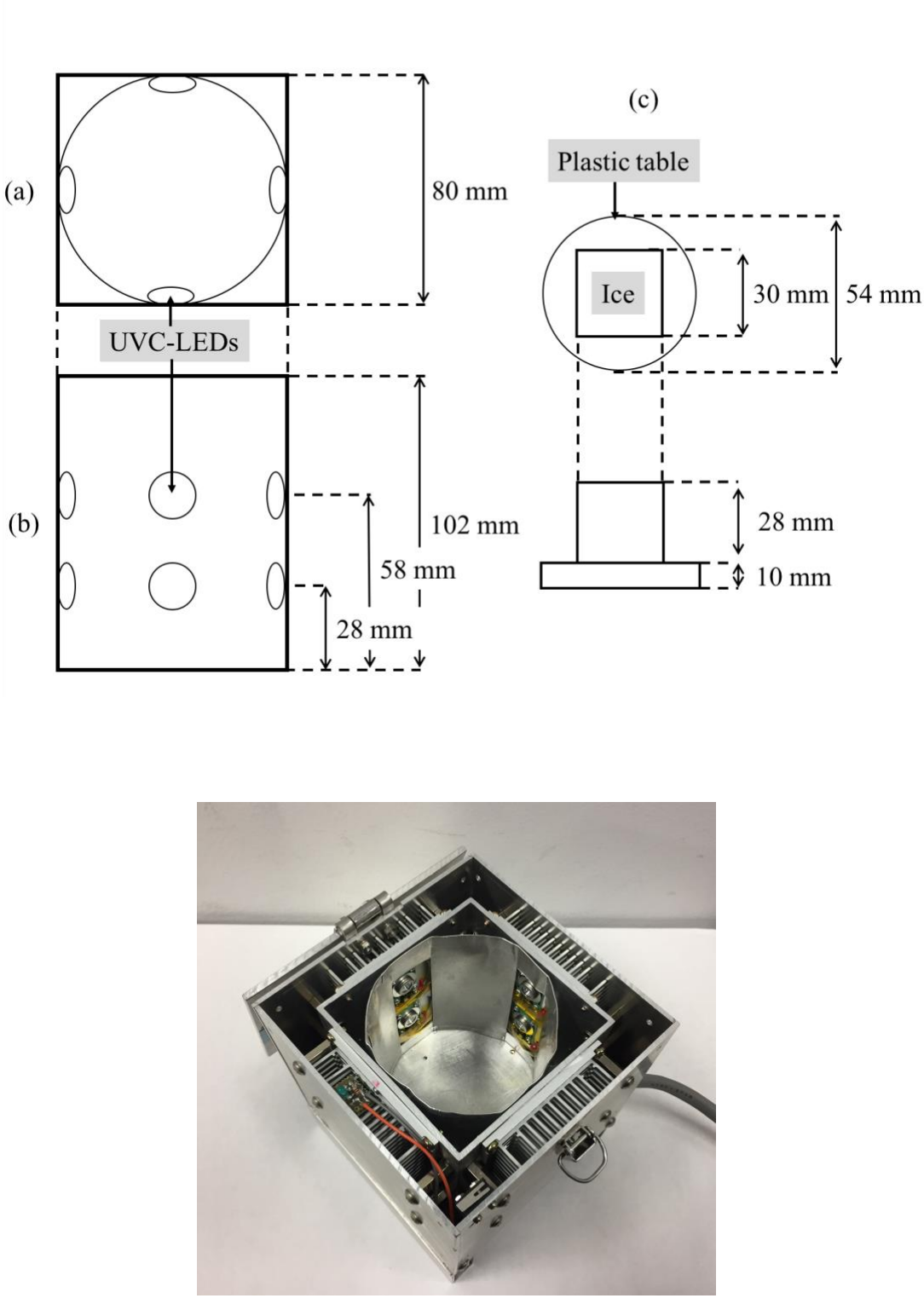


Fig. 4-2. A schematic diagram of the bench-scale test UVC apparatus. (a) upper side, (b) and sides, (c) inside of the apparatus.

4.2.4. Effect of multiple UVC-LEDs and multiple irradiation directions on bacterial inactivation in ice

In this experiment, all ice samples placed on the petri dishes were irradiated at －30°C in a deep freezer. Ice samples containing pathogens, such as *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, were irradiated by one, two, or three UVC-LEDs. Each UVC-LEDs has the UV intensity of 0.013–0.015 mW/cm^2. UVC irradiation was conducted for 30 min at the maximum. After UVC irradiation, irradiated ice samples were taken from the deep freezer and completely melted at room temperature. An aliquot of 1-ml aliquot of sample was collected from melted ice.

4.2.5. UVC irradiation by bench-scale test UVC-LED apparatus

Ice sample inoculating *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, on the plastic table was placed in the center of the UVC-LED apparatus which installed eight UVC-LEDs and irradiated at －30°C in a deep freezer. Irradiated samples were taken from the UVC-LED apparatus at 1, 4, 10, and 20 min and completely melted at room temperature. An aliquot of 1-ml sample was collected from melted ice.

4.2.6 Microbiological analysis

After UV irradiation treatment, to determine the number of surviving bacteria at each UV dosage, appropriate serial dilutions (1:10) were performed with sterile 0.1% peptone water and plated in duplicate on TSA plates. The plates were then incubated at 37°C for 24－48 h.

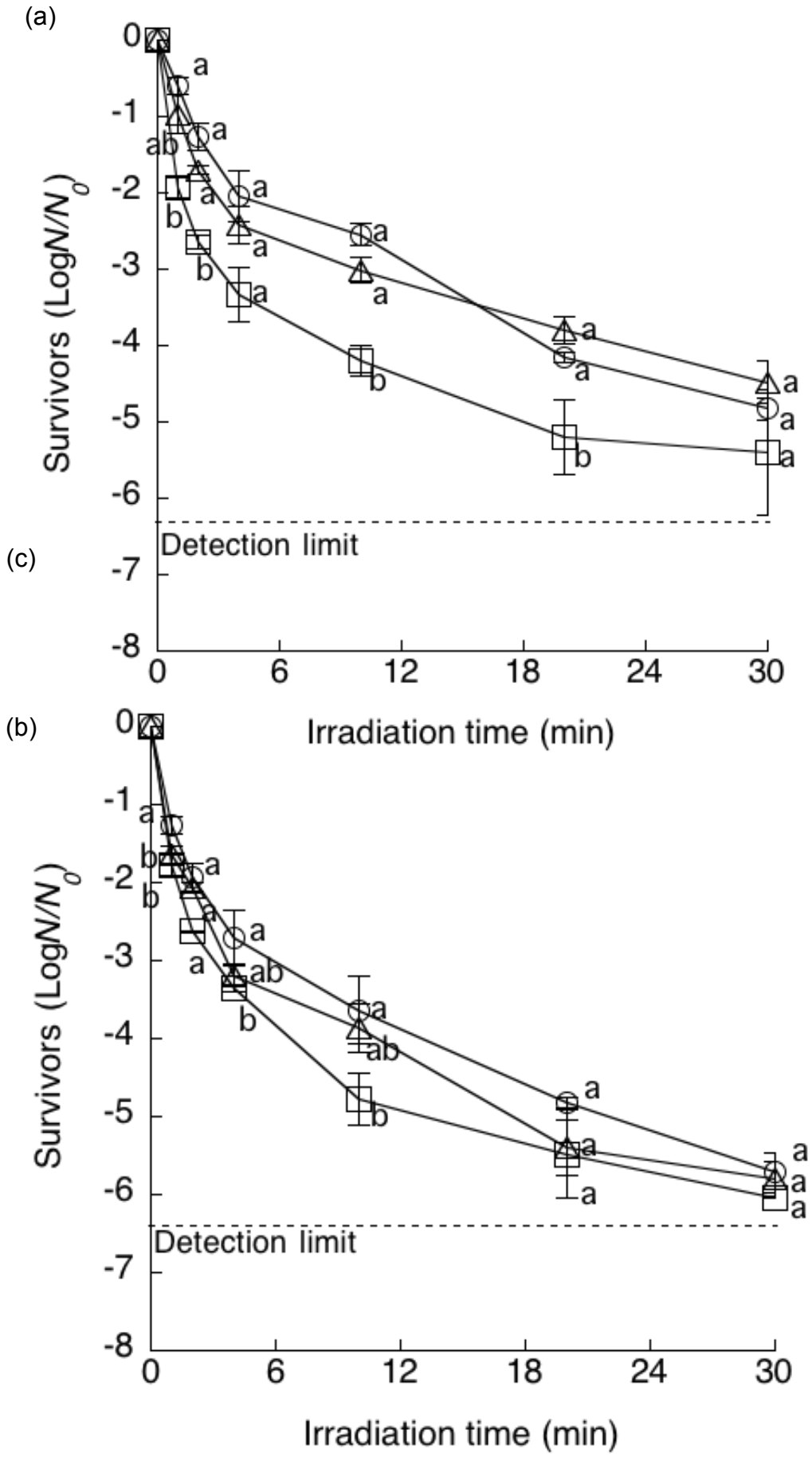
4.2.7. Statistical analysis

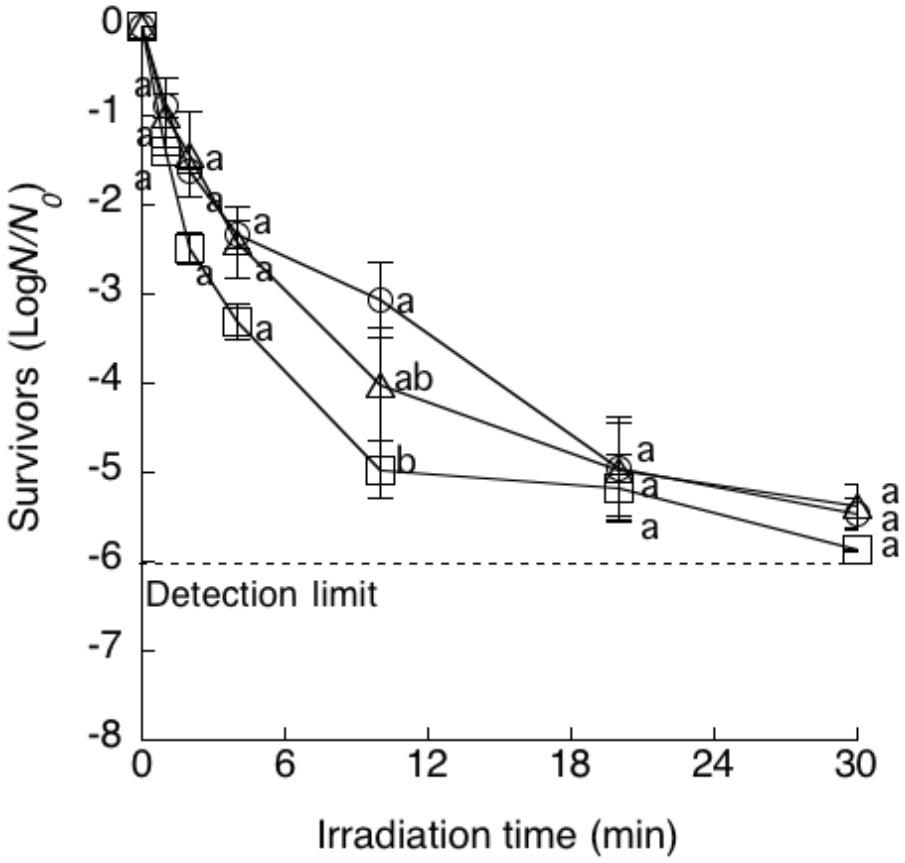
All experiments were repeated three times, and each data point was determined based on duplicate plates counts. Data from three samples subjected to each treatment in each of three independent replicate experiments were analyzed. To determine statistical significant ($P < 0.05$) between the UV treatment conditions and type of pathogenic bacteria, we used Students' paired t-tests in the case of comparisons with two groups, such as the effect of the same direction of UVC-LED versus different direction of UVC-LED, and we used Tukey-Kramer's method for multiple comparison tests of comparisons of more than three groups, such as inactivation of pathogenic bacteria by UVC apparatus. All statistical analyses were conducted using the R statistical environment (Ver.3.2.4 for Mac OS URL; http://www.R-project.org).

4.3. Result

4.3.1. Effect of UV intensity on bacterial inactivation in ice by using different numbers of UVC-LEDs from same direction

E. coli O157:H7, *S.* Typhimurium, and *L. monocytogenes* in ice were inactivated efficiently by the UVC irradiation from the same direction as the number of UVC-LED increased (Figs. 4-3). There seemed to be a slight difference in the sensitivity to UVC irradiation within 10 min treatment between kind of pathogenic bacteria. However, as the irradiation duration was longer, there was no significant difference in sensitivity to UVC between the kind of bacteria. Furthermore, longer irradiation duration that means higher UV dose did not show significant difference between the used number of UVC-LED.


(a)
(c)
(b)
Survivors (LogN/N_0)
Irradiation time (min)
Detection limit
0
-1
-2
-3
-4
-5
-6
-7
-8
0
6
12
18
24
30



Figs. 4-3. The changes in the viable bacterial cell counts during UVC irradiation by one LED (○), two LEDs (△), and three LEDs (□) placed above ice (6cm) from the same direction. Each UVC-LED has UV intensity of 0.013–0.015 mW/cm^2. (a) *E. coli* O157:H7, (b) *S.* Typhimurium, (c) *L. monocytogenes*.

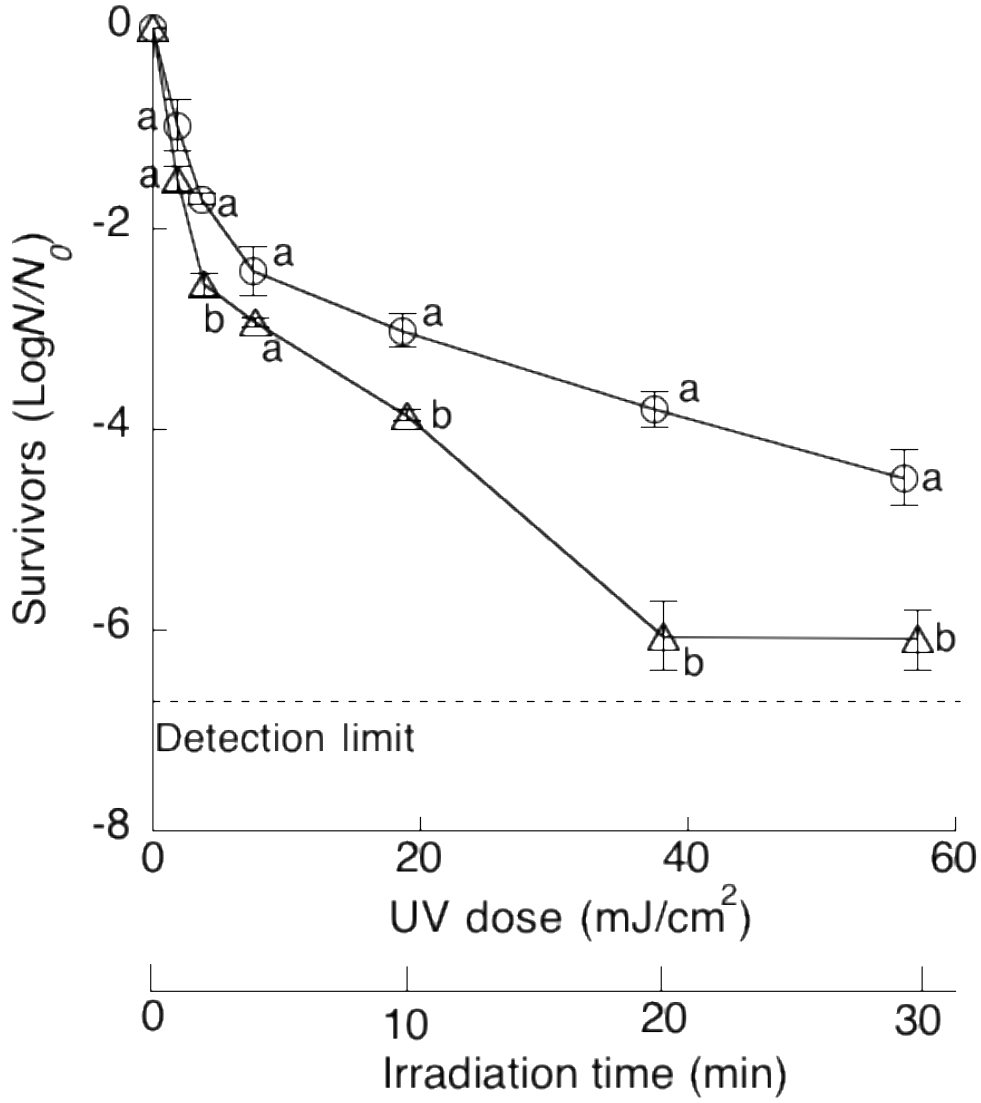
4.3.2. Effect of UVC irradiation from different directions on bacterial inactivation in ice

E. coli O157:H7 and *S.* Typhimurium were more efficiently inactivated by two UVC-LEDs irradiations from different directions than those of the same direction of those (Fig. 4-4a, 4-4b). In particular, two UVC-LEDs irradiation from the different directions showed significantly ($P < 0.05$) greater log-reductions of *E. coli* O157:H7 at 3.75, 18.8, 56.3 mJ/cm^2

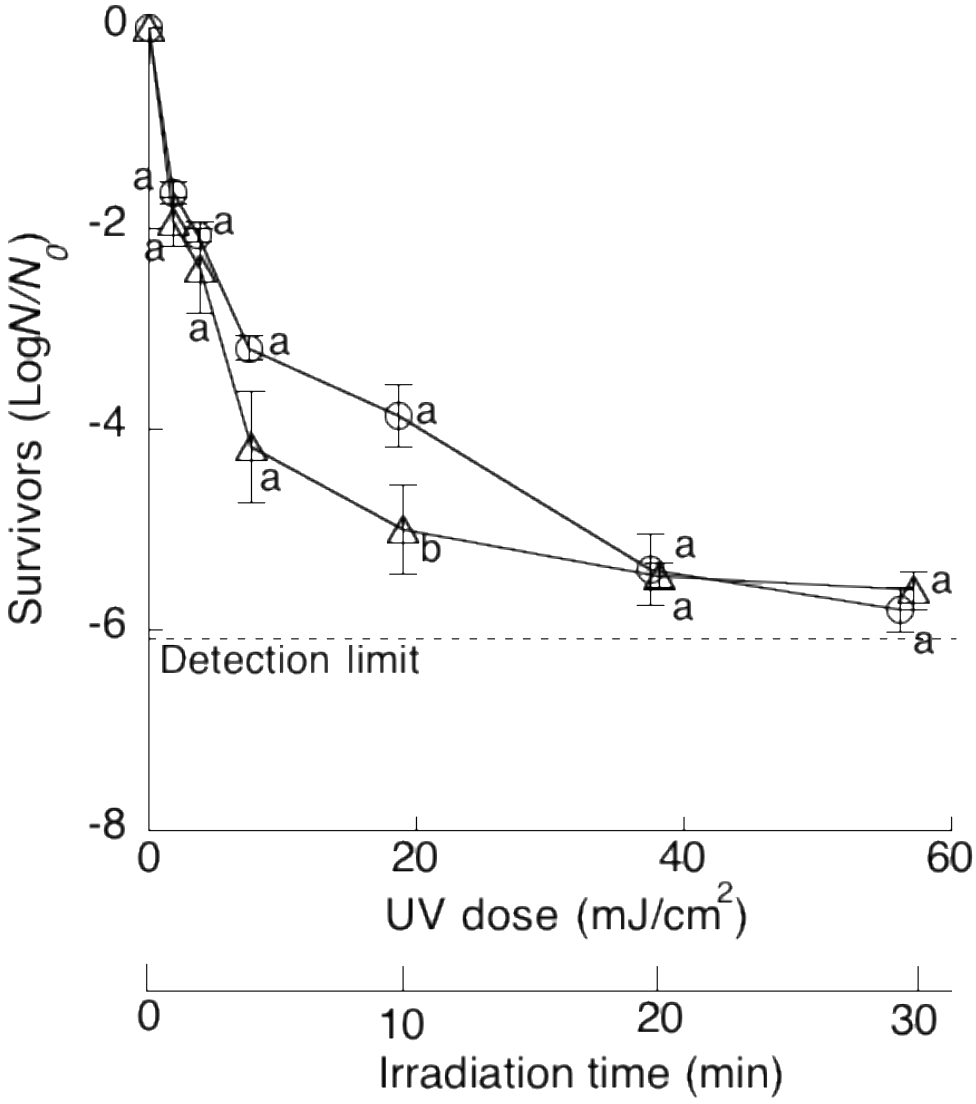
and *S.* Typhimurium at 18.8 mJ/cm^2 than those of irradiation from the same direction. However, there were no significant difference ($P < 0.05$) in survivors of *L. monocytogenes* regardless of the irradiation directions at each UV dose (Fig. 4-4c).

The similar reduction trends were found in the case of three of UVC-LEDs irradiation, although there were no significant differences ($P > 0.05$) in survivors of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* at each UV dose regardless of the irradiation directions of three UVC-LEDs except for *S.* Typhimurium at 10.6 mJ/cm^2 (Figs. 4-5). *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* was completely inactivated by UVC irradiation at 80, 60, 60 mJ/cm^2, respectively, from the different directions. In contrast, these bacteria were alive by three UVC-LED irradiations at the same UV dose by the same direction.

(a)


Survivors (LogN/N_0)
0
-2
-4
-6
-8
a
a
a
a
a
a
b
a
b
b
b
Detection limit
0
20
40
60
UV dose (mJ/cm^2)
0
10
20
30
Irradiation time (min)

(b)


0
-2
-4
-6
-8
Survivors (LogN/N_0)
a
a
a
a
a
a
a
a
a
b
a
a
a
Detection limit
0
20
40
60
UV dose (mJ/cm^2)
0
10
20
30
Irradiation time (min)

(c)

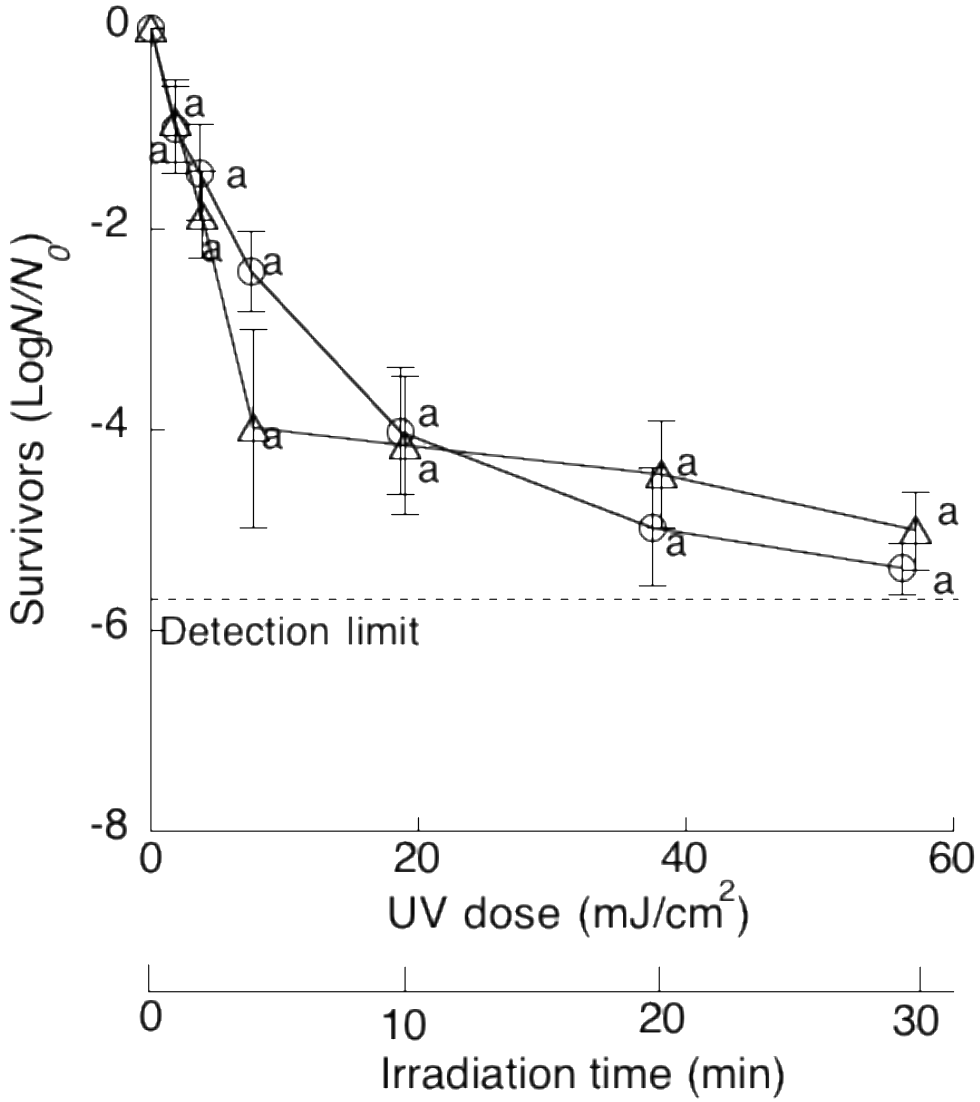
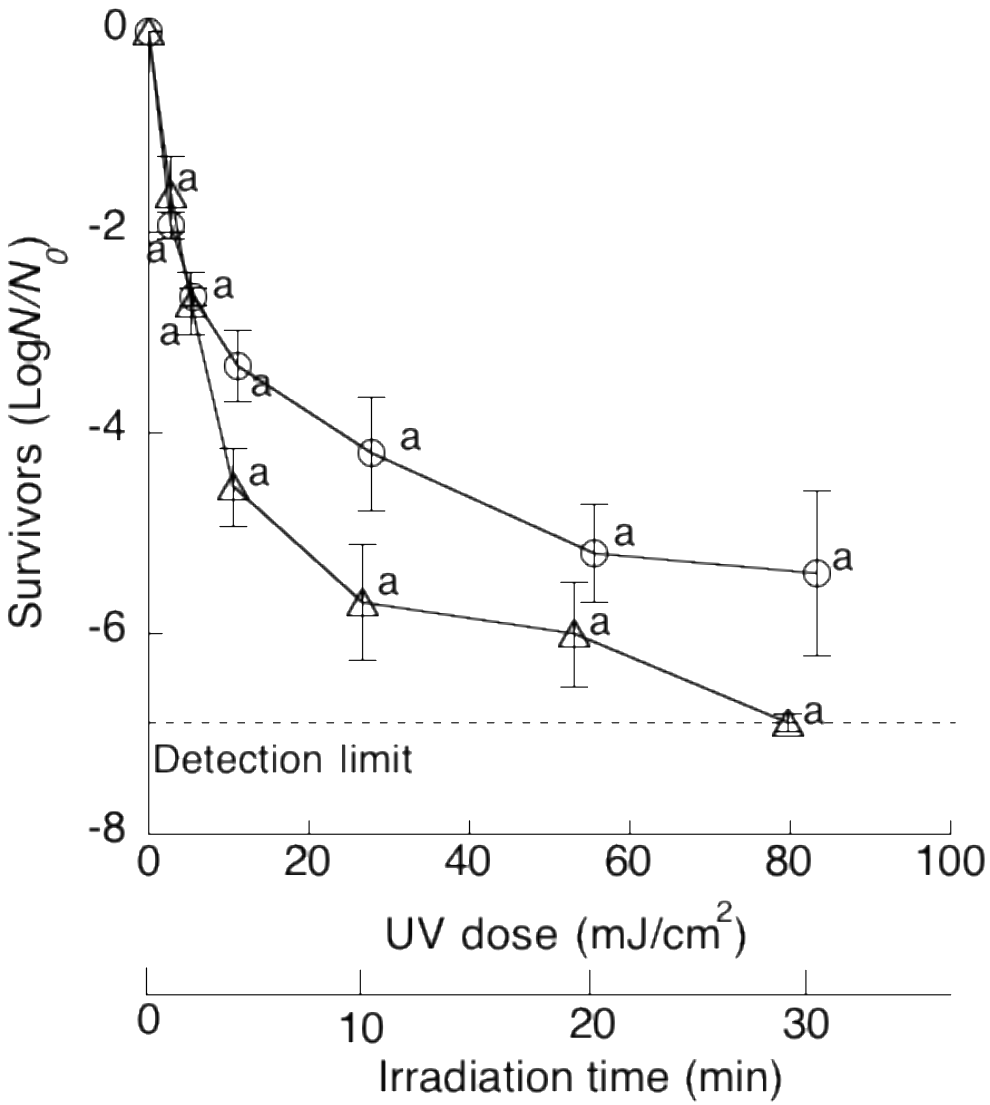
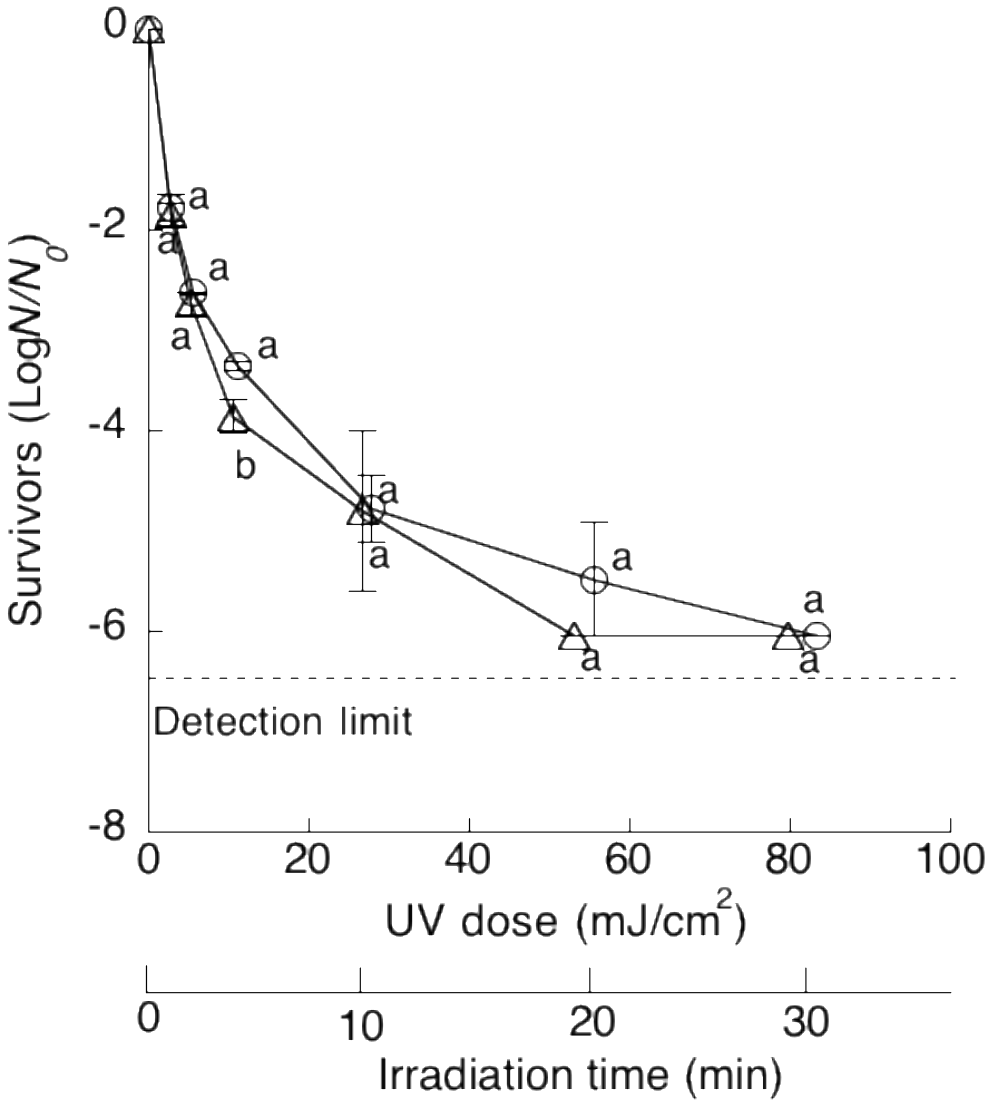


Fig. 4-4. The changes in the viable cell counts in ice during UVC irradiation at -30˚C by using two UVC-LEDs from same (○) or different directions (△). Each UVC-LED has UV intensity of 0.013–0.015 mW/cm^2. The error bars represent mean values with standard deviation (n = 3). Values with different letter means statistical significant difference. (a) *E. coli* O157:H7, (b) *S.* Typhimurium, (c) *L. monocytogenes*.

(a)


0
-2
-4
-6
-8
Survivors (LogN/N_0)
a
Detection limit
0
20
40
60
80
100
UV dose (mJ/cm^2)
0
10
20
30
Irradiation time (min)

(b)


Survivors (LogN/N_0)
0
-2
-4
-6
-8
a
a
a
a
a
a
b
a
a
a
a
a
Detection limit
0
20
40
60
80
100
UV dose (mJ/cm^2)
0
10
20
30
Irradiation time (min)

(c)

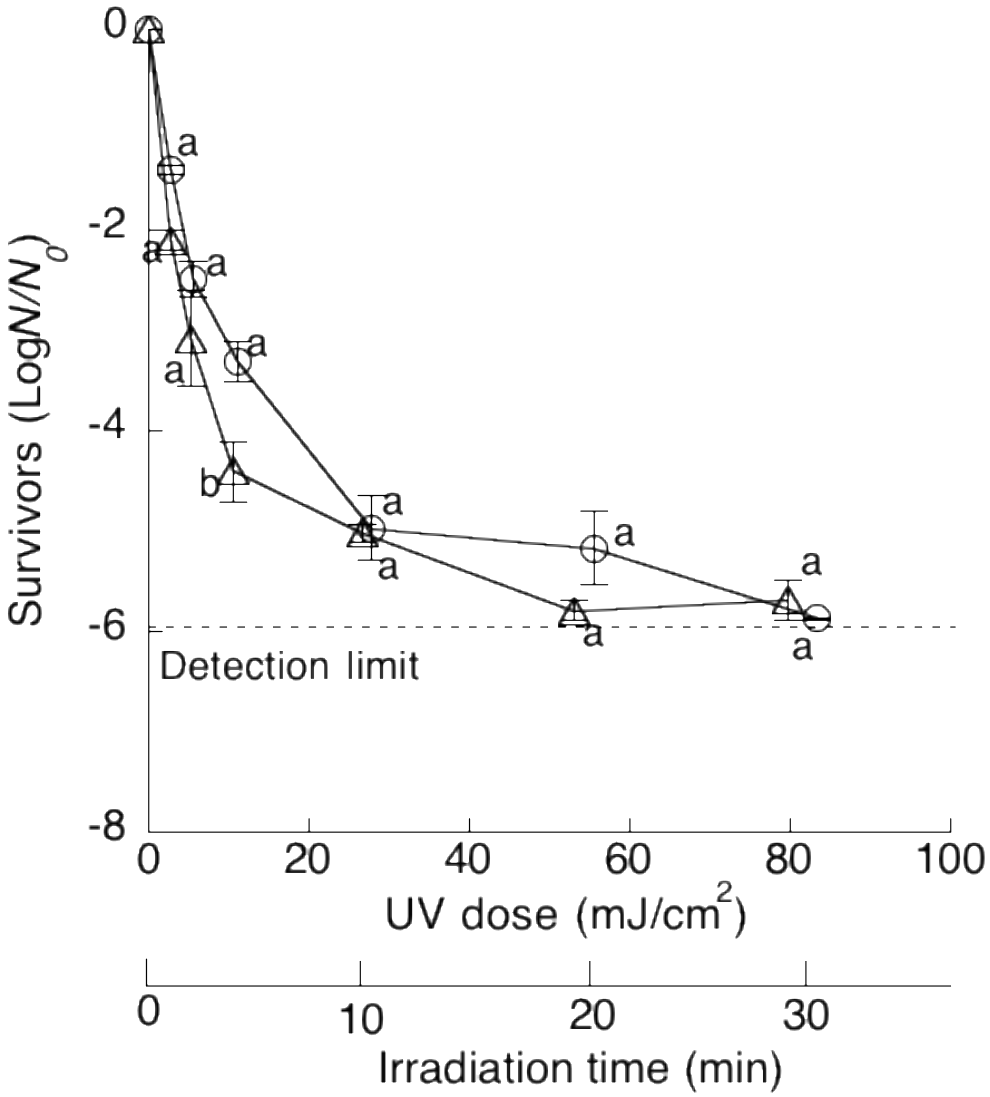


Fig. 4-5. The changes in the viable bacterial cell counts in ice during UVC irradiation at -30˚C by using three UVC-LED from the same (○) and different (△) directions. Each UVC-LED has UV intensity of 0.013–0.015 mW/cm^2.The error bars represent mean values with standard deviation (n = 3). Values with different letter means statistical significant difference. (a) *E. coli* O157:H7, (b) *S.* Typhimurium, (c) *L. monocytogenes*.

4.3.3. Performance of bench-scale UVC-LEDs test apparatus for ice

All the pathogenic bacteria in ice were more efficiently inactivated by UVC-LEDs apparatus than 1-3 of UVC-LEDs (Figs. 3, Fig.6). All the pathogenic bacteria were completely inactivated (> 6.2 log) by 20 min UVC-LED irradiations in the test apparatus. There were no significant differences in survival kinetics ($P > 0.05$) among three kinds of bacteria.

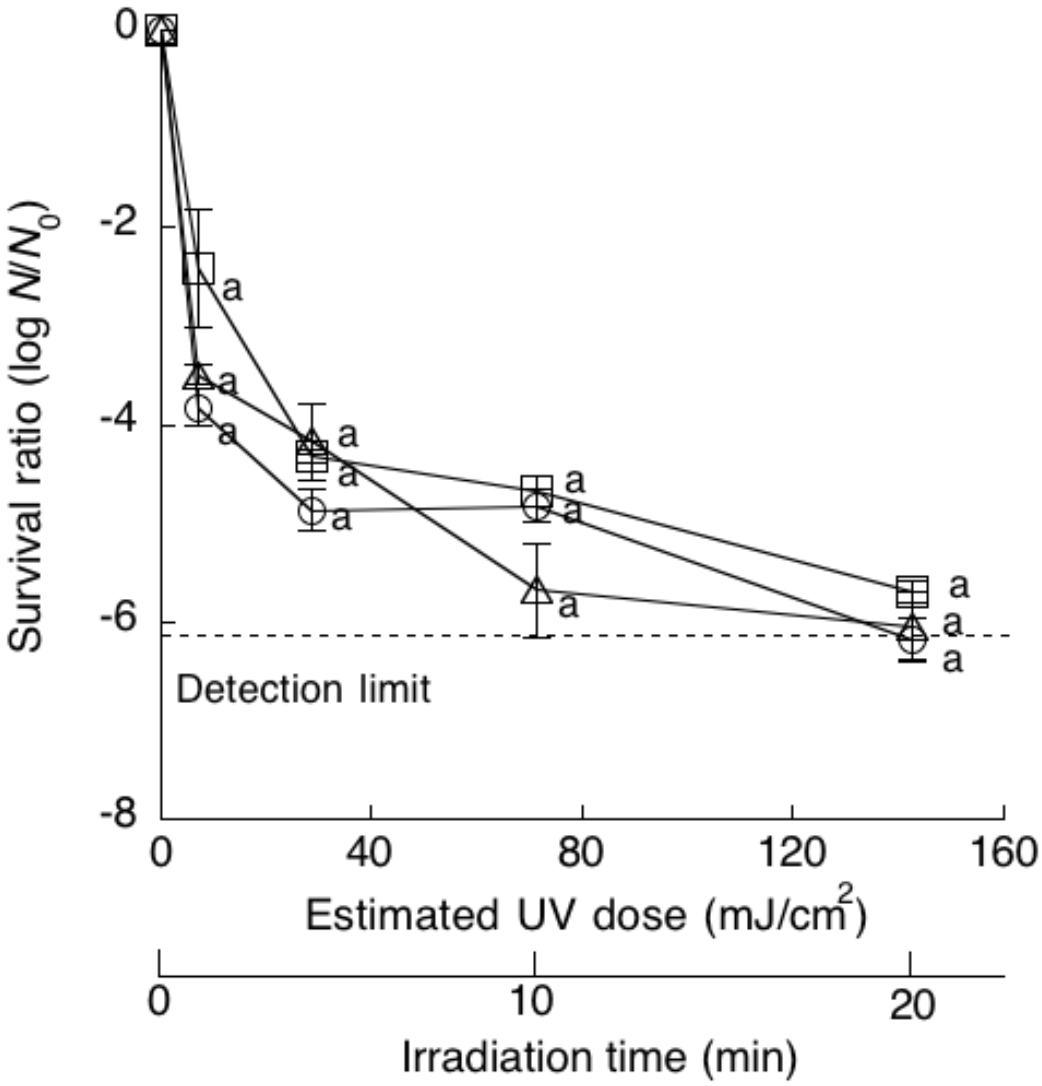


Fig. 4-6. The changes in the viable cell counts of *E. coli* O157:H7 (○), *S.* Typhimurium

(△), *L. monocytogenes* (□) in ice during UVC irradiation at -30°C by using a bench-scale test UVC-LED apparatus that has eight UVC-LEDs placed on different directions. Estimated UV intensity was 0.119 mW/cm^2. The error bars represent mean values with standard deviation (n = 3). Values with different letter means statistical significant difference.

4.4 Discussion

In this study, we evaluated the bacterial inactivation effect of multiple UVC-LEDs and effect of different irradiation direction of UVC-LEDs in ice. The results indicated that the reductions of survival *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in ice were more efficient as the number of UVC-LED increased when the irradiations of UVC-LEDs were from the same direction. Commonly, microbial inactivation by UVC irradiation are dependent on UV dose, which is defined as the product of UV intensity and exposure time (Sommer et al., 1998). In our previous study, we evaluated the effect of UV intensity (0.084, 0.025, 0.013, 0.007, and 0.005 mW/cm^2) of a UVC-LED on pathogenic bacteria in ice and we found that inactivation effect was depended on UV dose at all UV intensity conditions (Murashita et al., 2017). Thus, increasing the number of UVC-LEDs during UVC irradiation from the same direction would have similar effect to increasing the intensity of a UVC-LED.

The UVC irradiation using two UVC-LEDs from different directions demonstrated more efficient bacterial inactivation effect than those from the same direction. This result indicated that the UVC light could be easily transmitted internal ice by different direction of UVC-LEDs. However, in the case of using three UVC-LEDs, there was no significant difference ($P > 0.05$)

regardless of the irradiation direction. The result suggested that the difference of the irradiation direction would not influence on bacterial inactivation when UV intensity was sufficient. Because using three UVC-LEDs showed higher UV intensity than that of two of UVC-LEDs, the penetration of the UVC light by using three UVC-LEDs would not be different regardless of the irradiation direction. Thus, increase of the number of UVC-LED and using multiple irradiation directions had potential to inactivate microorganism more efficiently than the same direction of UVC-LED, while the effect of irradiation from different directions was small under sufficient UV intensity. UVC-LED will be suitable for locating more places and irradiating from many directions than UV lamp because of its compactness. The results obtained in the present study suggest that using multiple UVC-LEDs and irradiation from multiple direction will enable to eliminate irradiation shade and irradiate evenly and efficiently to the objectives. As a result, highly efficient bacterial inactivation would be realized by using multiple UVC-LEDs.

Based on the results obtained in the present study, we developed a bench-scale test UVC-LED irradiation apparatus that was installed eight UVC-LED in different directions to enable to be irradiated from various aspects. UVC irradiation by the test UVC-LED apparatus decreased by $10^4 - 10^5$ CFU/ml of pathogenic bacteria in ice as is within 5 min under -30˚C, while it took 20 min to kill all the bacteria completely in ice. Using multiple (eight) UVC-LEDs and the irradiation from multiple directions would enhance the bacterial inactivation by the UVC irradiation. The bacterial cells located near cloudiness of ice produced by air bubbles would not be efficiently inactivated. Because the penetration of light is lower when the liquid

has greater color or turbidity, the presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effects (Fan and Geveke, 2007). Thus, the cloudiness of ice could prevent the penetration of UV light into the ice.

In conclusion, UVC-LEDs will have a potential to enhance hygienic ice production based on the results presented in this study. Although UV irradiation has disadvantage that lightproof food is disinfect only its surface (Falguera et al., 2011) and it was less effective on rough surface cuts of meat because bacteria were partly shielded from radiation (Stermer et al., 1987), the UVC irradiation using multiple UVC-LEDs from multiple directions enables to overcome the weakness of conventional UV irradiation. Thus, the findings presented here for ice would be applied to other various foods as well.

Chapter 5 Disinfection of vegetables by using UVC-LED

5.1. Introduction

Distribution process of agricultural commodities, meat and meat products, fish and fishery products to consumers are recently very complex. There would be many chances of contamination of pathogens for most foods during distribution and processing. The number of accident by food poisoning per year in Japan had been more than 1000 since 2000 to 2016 (Fig. 5-1). In particular, seafoods, composite cooked foods, meats and vegetables are main source of food poisoning in 2016 (Fig. 5-2). Inactivation, removing, and/or eliminating of pathogenic microorganisms just before eating will play a key role in ensuring microbiological food safety. Raw meats and fishes are commonly heated (cooked) just before eating at restaurant and/or home. However, there are no enough method to inactivate microorganisms which contaminate raw food such as vegetable. The simple way to disinfect raw vegetable is washing in portable water. However, the efficacy of this treatment is few. 10-fold to 100-fold reductions can sometimes be achieved by that treatment (Beuchat et al., 1998). Chlorine has been used as the most effective sanitizers to assure the safety of vegetables. However, there is a trend in eliminating chlorine from food process because of concern about the environmental and health risks associated with the formation of carcinogenic halogenated disinfection by-products (Allenda eta al., 2008, Gii et al., 2009). Most of the current studies have been focused on the search for alternative sanitizers based on assuring the quality and safety of the fresh-cut produce (Gii et al., 2009). UVC offers several advantages to food processors as it does not leave a residue, does not have legal restrictions and does not require extensive safety equipment

to utilize (Yaun et al, 2004). There are several studies about effectiveness of UV lamp radiation to reduce the surface population of microorganisms on vegetables (Yaun et al., 2004, Allenda et al., 2006, Gil et al., 2009, Manzocco et al., 2011, Daniela and Gustavo, 2013,). However, UVC irradiation has some disadvantages that it could inactivate microorganism in only surface of vegetable because of low penetration (Fan and Geveke, 2007) and excessive irradiation could cause color change (Kim et al., 2013). On the other hand, the effect of UVC-LED on vegetable has not been cleared. It has been known that the effect of UVC-LED is different from that of UV lamp (Song., et al 2016, Murashita et al., 2017). Accordingly, in this study, we investigated the effect of UVC-LED on bacteria in vegetable and on the quality of raw food, simultaneously. UVC-LED would contribute to ensure the safety of raw food by inactivating bacteria just before eating because UVC-LED could non-thermally inactivate bacteria.

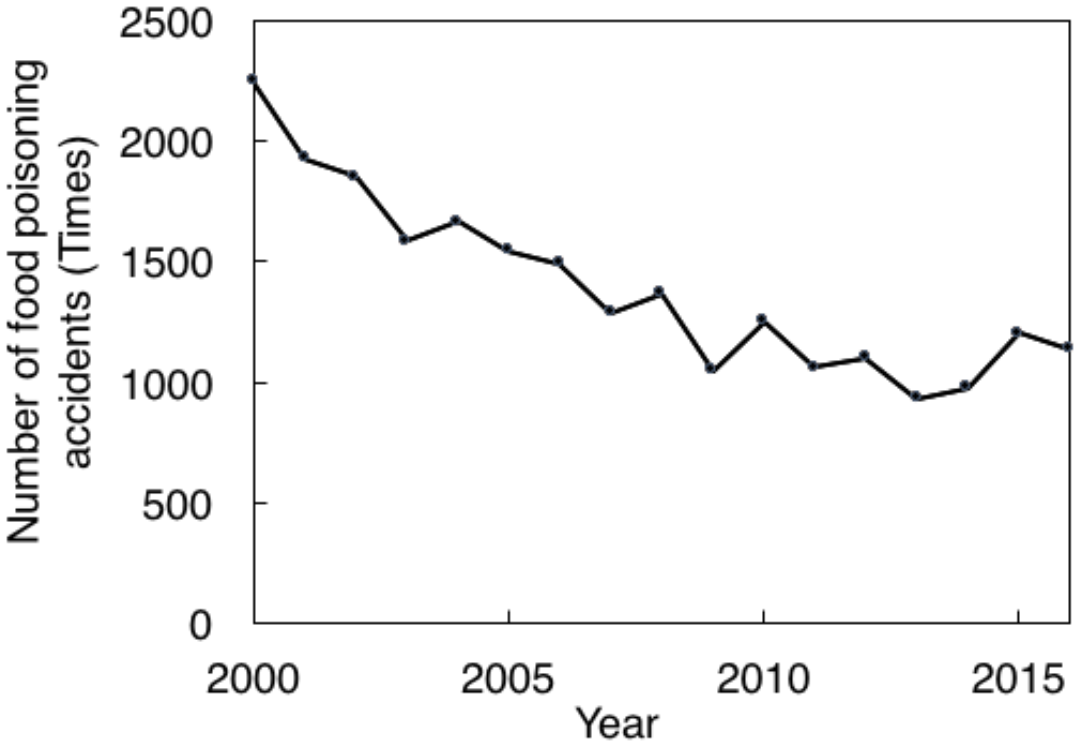


Fig. 5-1. Number of food poisoning accidents in Japan.

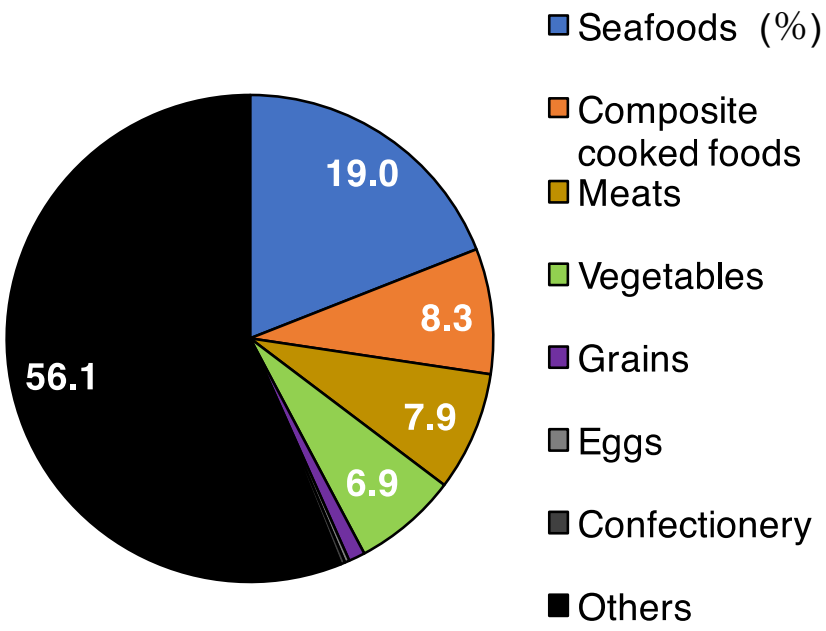


Fig. 5-2. The source of food poisoning in Japan (2016).

5.2. Materials and method

5.2.1. Bacterial strains

E. coli ATCC259222 was used as a hygienic indicator of bacterial contamination in a food sanitation. Bacterial suspension was prepared as same as the previous chapter.

5.2.2. Sample preparation

Cherry tomato and lettuce were purchased from a local super market in Sapporo, Hokkaido. These vegetables were preserved in a fridge before used on experiment. The calyx of the cherry tomato was removed and the lettuce leaves were cut into approximately 3 × 3 cm with sterile knife. Cherry tomato and lettuce were rinsed with water to remove dirt and dust. Water was wiped by sterile paper towel. Then, an aliquot of 30 μL of bacterial suspension

(approximately $10^8 - 10^9$ CFU/ml) were spotted on the surface of the vegetables. After inoculating, vegetables were air-dried at ambient temperature for 2 h in a biological safety cabinet.

5.2.3. UVC irradiation treatment

Inoculated area of the sample vegetable was irradiated for maximum 10 min by one UVC-LED module that was placed in the vertical direction. The distance between the UVC-LED and the sample was 6 cm and the UV intensity was 0.013 mW/cm^2. After irradiation, the UVC-treated tomatoes and lettuces were individually placed in a sterile stomacher bag with 0.1% peptone water (20 mL). These vegetables were manually shaken and hand-rubbed at the inoculation spot for 1 min according to UV lamp inactivation of vegetable (Lim et al, 2016). The suspension was serially diluted by 0.1% peptone water and then plated on TSA plates in duplicate as well as the previous chapter.

5.2.4. Firmness and color measurement

Firmness and color (L*, a*, and b*) of cherry tomatoes and lettuces after irradiation by UVC-LED for 0, 4, and 10 min were determined in order to identify quality change by UVC. Firmness was determined by a rheometer (Rheotech, Tokyo, Japan). The samples were punched by a cylindrical probe with 3 mm diameter. Crosshead speed was set at 3 cm/min. Force–distance curves were obtained from the penetration tests and firmness was taken as the force (N) required to puncture the sample.

Color of samples were measured by a colorimeter (CR-400; Konica minolta, Tokyo, Japan). The instrument was standardized against a white tile before measurement. Color was expressed in L*, a*, and b* parameters.

5.3. Results and discussion

The UV-C irradiation by UVC-LED with 10 mJ/cm^2 reduced the number of *E. coli* inoculated on tomatoes and lettuce by 2 log-cycles (Fig. 5-3). This reduction was considerably less than those in distilled water and in ice. Because bacterial suspension would be absorbed into vegetables and just only bacteria in surface would be inactivated. Commonly, solid foods such as vegetables, fish, meats, are disinfected only its surface because UV light does not penetrate into their insides.

Yaun et al., (2004) found that Salmonella inoculated on tomatoes were reduced by 2.19 log CFU/tomato when a maximum UV-C dose of 24 mJ/cm^2 by using a UV lamp. Song et al. (2011) reported that UV-C at 500 mJ/cm^2 decreased S. Typhimurium on light red cherry tomatoes by 2.58 logs using UV lamp. Disinfection of vegetable by non-thermal effect of UV irradiation might be around 2-3 log reduction. Smoothness of vegetable surface would affect on the results. UV radiation does not penetrate most opaque materials, it was less effective on rough surface such as round steak because bacteria were partly shielded from the radiation (Stermer et al, 1987). Bermúdez-Aguirre et al. (2013) also mentioned the effect of smoothness of vegetable on microorganism inactivation. Bermúdez-Aguirre et al. (2013) compared the effect of UV lamp on *E. coli* in carrots, lettuce, and tomatoes and they found *E. coli* in tomatoes

were more efficiently inactivated than that in carrots and lettuce. They mentioned because tomatoes have a smooth surface and the protective sites are limited, microorganisms were exposed to the radiation and inactivation was achieved compared with those in carrots and lettuce.

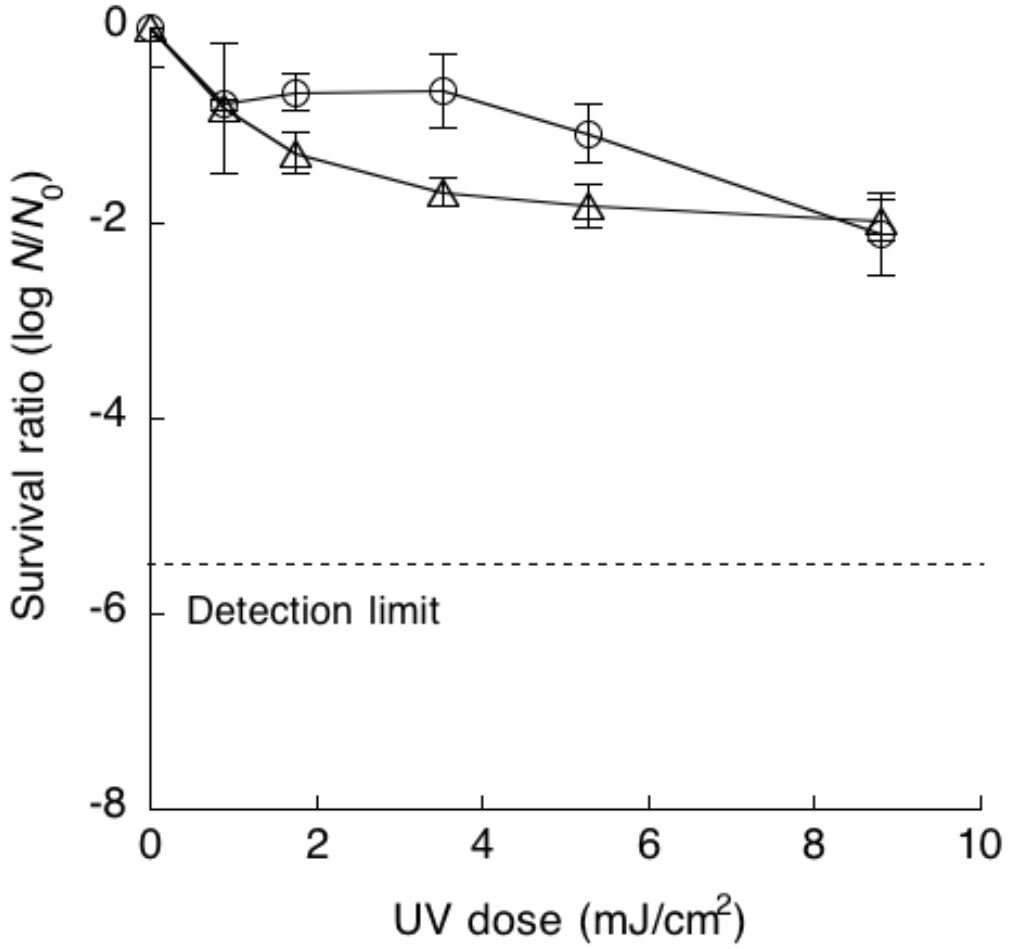


Fig. 5-3. The changes in the viable bacterial cell counts on lettuce (○) and cherry tomato (△) by UVC-LED at 0.013 mW/cm^2. The error bars represent mean values with standard deviation (n = 3).

Firmness and color were not significantly changed by the UV-C irradiation by UVC-LED

at 10 mJ/cm^2 (Fig. 5-3-5-6). Kim et al. (2013) investigated the changes in color values and texture parameters of lettuce under two-sided UV lamp treatment at UV intensity of 6.80 mW/cm^2. They found that there were not significant ($P > 0.05$) difference from those of non-treated samples up to 20.40 kJ/m^2. However, when the irradiation dose increased to 40.80 kJ/m^2, the L* values and firmness parameter of lettuce greatly decreased while a* and b* values significantly ($P < 0.05$) increased. UVC irradiation by UVC-LED may also influence on food quality by such a higher irradiation dose.

Doyle and Erickson (2008) stated that interventions applied to produce should inactivate pathogens by at least 3 logs. In the present study, UVC irradiation by using a UVC-LED allowed to reduce the *E. coli* ATCC25922 inoculated on tomato and lettuce by 2 log-cycles without significant negative impact on the quality of those vegetables. Thus, using UVC-LED could contribute to reduce the number of food poisoning although it does not certainly ensure the microbiological safety of fresh vegetables. As possible practical use, we would be able to inactivate pathogens on the surface of raw food by installing UVC-LEDs on the wall of refrigerator in the future development.

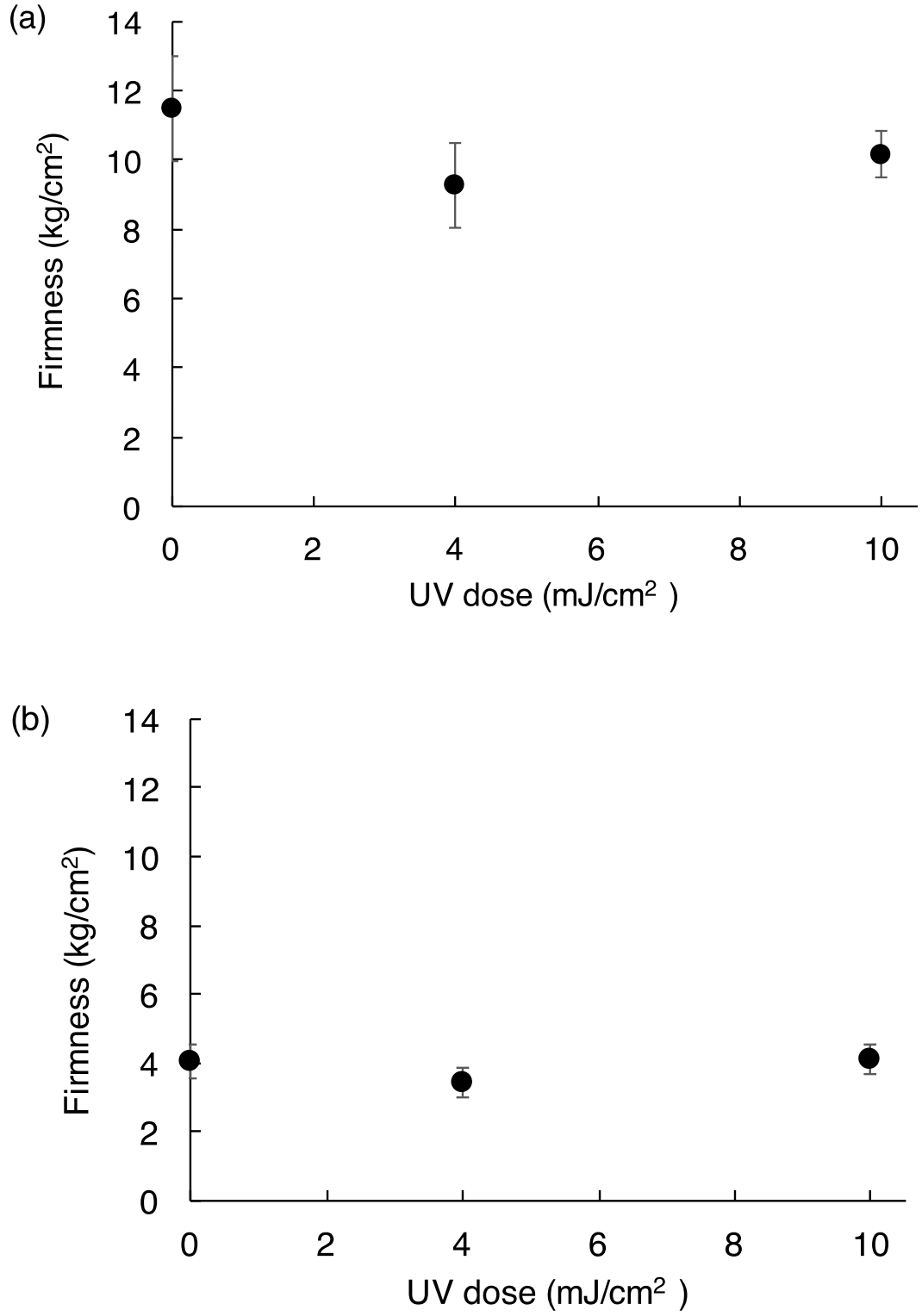


Fig. 5-4. The changes of firmness of cherry tomato (a) and lettuce (b) by UVC-LED at 0.013 mW/cm^2. The error bars represent mean values with standard deviation (n = 5).

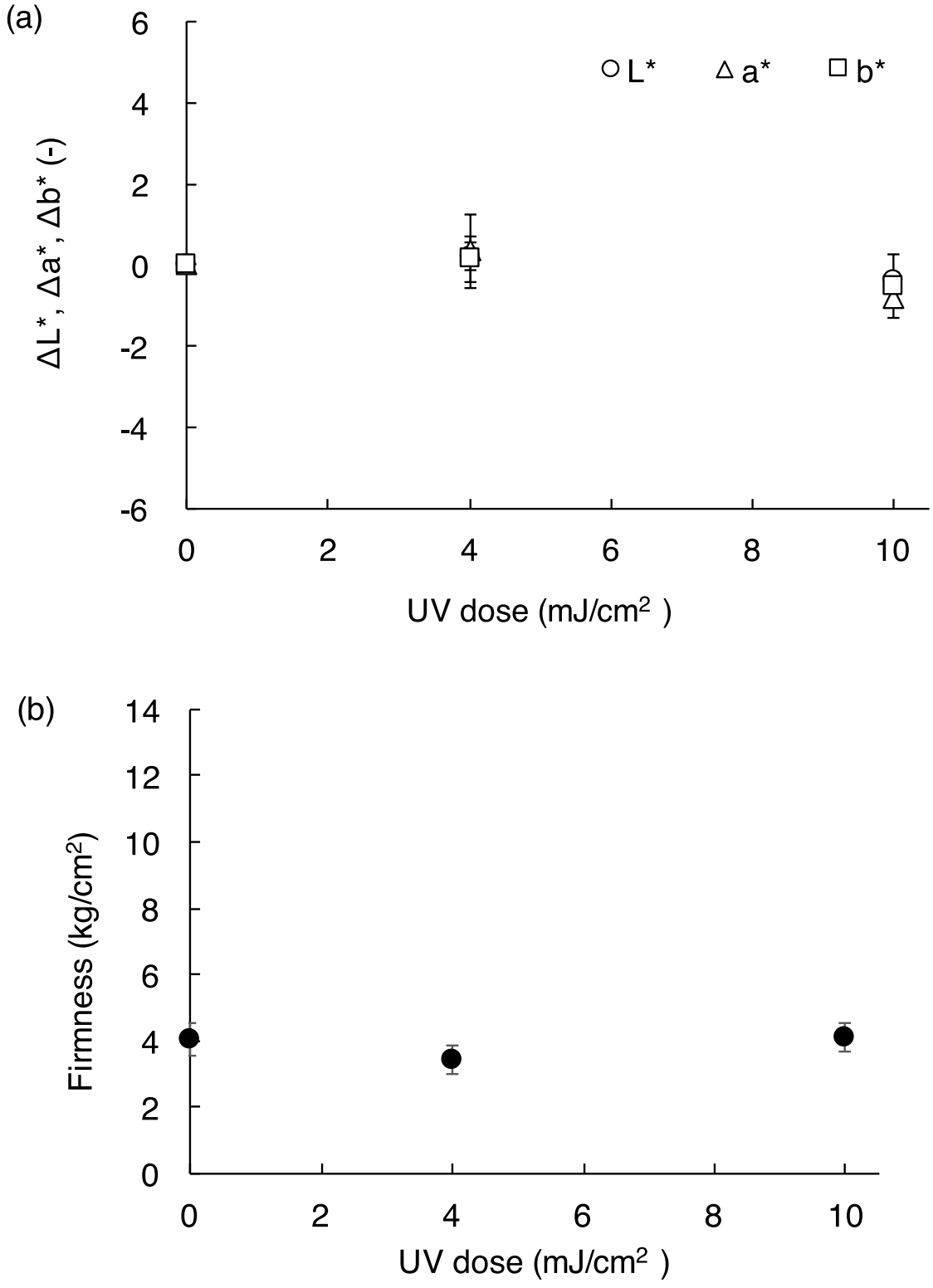


Fig. 5-5. Changes in the color of cherry tomato (a) and lettuce (b) by UVC-LED at 0.013 mW/cm^2. The error bars represent mean values with standard deviation (n = 3).

Chapter 6 Summary

6. Summary

In the present study, we investigated non-thermal microbial effect by ohmic heating and UVC-LED.

In chapter 2, we investigated. the non-thermal effects of Ohmic heating on *Bacillus subtilis* spores in a sodium chloride aqueous solution at 101°C (i.e., the boiling point) as well as the effects of electric field intensity and frequency during Ohmic heating. The inactivation effect on *B. subtilis* was greater for all electric field conditions (5, 10, and 20 V/cm) than for conventional external heating. In particular, 20 V/cm showed a significantly higher inactivation effect ($P < 0.05$) on *B. subtilis* than those of CH at 8, 10, 12, 14, and 16 min. However, there were no significant differences ($P > 0.05$) in survival kinetics between 20 kHz, 40 kHz, and, 60 kHz; *B. subtilis* spores were inactivated more efficiently as the frequency increased. B. subtilis spores were almost completely inactivated at 14−16 min for the 60 kHz treatment, but spores were still alive at 20 kHz and 40 kHz for the same treatment times. These results demonstrated that Ohmic heating inactivates *B. subtilis* spores more effectively than conventional heating. Ohmic heating conditions with high electric field intensities and high frequencies resulted in efficient *B. subtilis* spore inactivation

In chapter 3, we evaluated the applicability of an ultraviolet C light-emitting diode (UVC-LED) for microbial inactivation in ice. The effects of UV intensity and UV dose of the UVC-LED on *Escherichia coli* ATCC 25922 and the effects of the UVC-LED compared with a conventional UV lamp on distilled water and ice cubes were investigated to evaluate the

performance of the UVC-LED. Finally, we assessed the effects of the UVC-LED on pathogens such as *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in an ice cubes. The results indicated that the microbial inactivation effect of the UVC-LED depended on the UV dose at all UV intensity conditions in distilled water and ice, and the UVC-LED could more efficiently inactivate *E. coli* ATCC 25922 in distilled water and ice than the UV lamp. Furthermore, UVC-LED irradiation reduced the viable numbers of pathogen by 6–7 log cycles under 160 mJ/cm^2, although the bactericidal effect was slightly dependent on the type of bacteria. *L. monocytogenes* in ice was relatively more sensitive to UVC irradiation than *E. coli* O157:H7, and *S.* Typhimurium. These results demonstrated that UVC-LED irradiation could contribute to assuring the safety of ice in the food industry.

In chapter 4, to maximize the characteristics of UVC-LED, we examined the effect of multiple UVC-LEDs and the different irradiation angles of UVC on objective ice cube. Furthermore, we developed a bench-scale test apparatus that is installed eight UVC-LED modules placing in different angles and height. The bactericidal results indicated that increase in the number of applying UVC-LED modules and increase in the directions of irradiation tended to efficiently inactivate pathogenic bacteria in ice. In particular, irradiation by two UVC-LED from two different directions showed significantly ($P < 0.05$) greater log-cycle reductions of *E. coli* O157:H7 at 3.75, 18.8, and 56.3 mJ/cm^2 and *S.* Typhimurium at 18.8 mJ/cm^2 than those of the irradiation from same direction. However, there was no significant difference ($P > 0.05$) in inactivation of *L. monocytogenes* between the irradiation procedures. The similar bactericidal effect was found in the case of using three UVC-LEDs, while there

were no significant differences ($P > 0.05$) regardless of the irradiation directions and kind of bacteria. All the pathogenic bacteria in an ice cube were completely inactivated (> 6.2 log CFU/mL) within 20 min irradiation by the developed bench scale test UVC-LED apparatus under -30˚C environment. The results obtained in the present study would illustrate the effective usage of UVC-LED for optimizing the bacterial inactivation by UVC irradiation.

In chapter 6, we investigated the effect of UVC-LED on bacteria in vegetable and on the quality of vegetable, simultaneously. As the result, The UV-C irradiation by UVC-LED with 10 mJ/cm^2 reduced the number of *E. coli* inoculated on tomatoes and lettuce by 2 log-cycles without significant negative impact on the quality of those vegetables. Thus, using UVC-LED could contribute to reduction of food poisoning although it does not certainly ensure the safety of fresh vegetables.

These insights would contribute to reduction of thermal degradation of the food and disinfection of the food which are not suitable for heating.

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