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X-ray irradiation inactivation of *Escherichia coli* O157:H7, *Salmonella enterica* Serovar Typhimurium, and *Listeria monocytogenes* on sliced cheese and its bactericidal mechanisms

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A B S T R A C T

In the last two decades several foodborne disease outbreaks associated with cheese products were reported. The objective of this study was to investigate the efficacy of X-ray irradiation for the inactivation of foodborne pathogens on sliced cheese and to elucidate the underlying mechanisms of the lethal effect. In addition, the effect of the X-ray irradiation on product quality was determined. A mixed culture containing *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* was inoculated on the surfaces of cheese slices. The inoculated samples were re-packaged and treated with 0, 0.2, 0.4, 0.6, and 0.8 kGy of X-ray radiation. Approximately 5 log reductions in the viability of the three pathogens on samples were achieved at an irradiation dose of 0.6 kGy. Furthermore, the color values (L^*, a^*, and b^*) and texture parameters of sliced cheeses were not altered significantly (all $P > 0.05$) after treatment at the maximum dose of 0.8 kGy. Various fluorescence staining methods were utilized to analyze the bactericidal mechanisms. The analyses confirmed that levels of depolarization of cell membranes, generation of reactive oxygen species, and intracellular enzyme inactivation were strongly related to the trends of microbial inactivation. The results of the present study suggest that X-ray irradiation may be an innovative antimicrobial intervention for various post-packaged dairy food products.

1. Introduction

In the dairy industry, cheeses have become a major consumer product. More than one-third of the milk produced in the United States is used to make cheese (Velasco et al., 2016). *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are important pathogens of concern to the dairy industry. In 2017, eight people were infected with *L. monocytogenes* in four US states after consuming cheese. All eight people were hospitalized and, two died (CDC, 2017). The commercial cheeses made with pasteurized milk between from October 2006 through February 2007 also caused listeriosis outbreak in Germany (Koch et al., 2010). In 2010, cheeses contaminated with *E. coli* O157:H7 caused an outbreak of infections in five US states. Fifteen persons became ill and required hospitalization and one developed hemolytic uremic syndrome (HUS) (CDC, 2010). In the US, there was a recent *E. coli* O157:H7 outbreak which delivered by cheddar cheeses and caused 7 illnesses and 1 hospitalization (CDC, 2016). Outbreaks of, salmonellosis associated with cheese consumption occurred in Canada and the US (Ha et al., 2017; Kim et al., 2016). In 2008, the outbreaks of *Salmonella enterica* traced to cheddar cheeses, resulting in 70 illnesses in the US (CDC, 2008).

In the process of production, cheeses can be contaminated by pathogens after pasteurization, cutting, packaging, and during transport. Silva et al. (2003) isolated *L. monocytogenes* in pasteurized milk samples, drainage, wooden shelves, the floor of the cheese refrigeration room, and finished cheese samples in a cheese processing facility. Therefore, additional control methods are needed to inactivate pathogens on cheese surfaces after the packaging step. A non-thermal technique can protect foods from contamination with pathogens while maintaining the nutritional and sensory characteristics of the food products, and thus has been increasingly applied to food processing (Ha et al., 2016). To inactivate pathogens on cheeses, several new non-thermal processing have been evaluated, including dielectric barrier discharge plasma (Lee et al., 2012; Yong et al., 2015) and high pressure (Evert-Arriagada et al., 2012). However, dielectric barrier discharge plasma treatment can change the color, flavor, odor, and acceptability of sliced cheese (Lee et al., 2012; Yong et al., 2015). The high pressure treatment can change sensory characteristics of cheese, such as color

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and firmness (Evert-Arriagada et al., 2012).

Ionizing radiation is an efficient non-thermal pathogen control treatment. According to a 1980 joint study report issued by the Food and Agriculture Organization, International Atomic Energy Agency, and the World Health Organization (FAO/IAEA/WHO), ionizing radiation of any food up to a dose level of 10 kGy presents no toxicological hazard and does not cause nutritional or microbiological problems. In 1992, a joint study group report by the WHO and the International Organisation of Consumers Unions (IOCU; now Consumers International) reaffirmed the stability of irradiated foods. A 1999 WHO report described that irradiated foods are safe and have no toxicological risks. Among the forms of ionizing radiation, X-rays obtained by bombarding a metal target, such as tantalum, with high velocity electrons has continued to receive attention as an attractive alternative to gamma-ray or E-beam irradiation (Jung et al., 2015; Mahmoud, 2009). X-rays have the advantages of higher penetration power than E-beams and absence of harmful radioactive sources, such as Cobalt-60 or Cesium-137 associated with gamma-rays (Janatpour et al., 2005; Jeong et al., 2010; Song et al., 2016). Moreover, the rising costs of radioactive sources (Jung et al., 2015) and negative consumer perception of gamma-ray irradiated foods (Deliza et al., 2010) are driving the demand for new ionizing radiation sources.

Recently, several studies reported that treatment with X-rays is effective in inactivating pathogens without affecting the quality of the treated foods. Mahmoud (2009) reported that X-ray treatment significantly ($P < 0.05$) reduced the content of viable *Cronobacter* to less than detectable limits (ca. 10^8–10^9 log reduction) in skim milk, low-fat milk, and whole-fat milk. X-ray inactivation of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella enterica*, and *Shigella flexneri* on spinach leaves was investigated by Mahmoud et al. (2010), who concluded that this irradiation did not significantly affect the color of spinach leaves, while reducing the viable populations of the pathogens to less than their detectable limits. In addition, the effectiveness of pathogen inactivation and changes in quality factor in foods irradiated with X-rays were not significantly different when compared to those of other ionizing radiation such as gamma-rays and E-beams (Jung et al., 2015; Song et al., 2016). However, to the best of our knowledge, there are no published data on the inactivation of pathogens by X-ray treatment on packaged cheese products. Furthermore, no previous study has identified the mechanisms of bacterial inactivation by X-ray radiation.

The study investigated the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on packaged sliced cheese using X-ray irradiation, evaluated the change of quality factors following X-ray treatment, and sought to elucidate the mechanism of the bacterial inactivation.

2. Materials and methods

2.1. Bacterial strains

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104), and *L. monocytogenes* (ATCC 15313, ATCC 19111, and ATCC 19115), were obtained from the bacterial culture collection of Hankyong National University (Anseong, South Korea) and were used in the experiments. All strains were stored frozen at −80 °C in tryptic soy broth (TSB; MB Cell, Los Angeles, CA, USA) containing 20% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; MB Cell), incubated at 37 °C for 24 h, and stored at 4 °C. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured individually in 5 ml of TSB with 0.6% yeast extract (YE; Difco, Sparks, MD, USA) at 37 °C for 24 h, followed by centrifugation (3200 rpm for 20 min at 4 °C), and washing three times with 0.2% sterile peptone water (PW; Difco). The final pellets were resuspended in 9 ml of PW, corresponding to approximately 10^7 to 10^8 colony forming units (CFU)/ml. Subsequently, suspended pellets of each strain of the three pathogenic species were combined to produce mixed culture cocktails (nine strains total). These cocktails, consisting of a final concentration of approximately 10^7 CFU/ml, were used for the inactivation study. To analyze the mechanism of inactivation, each final three strain pellets of *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* was resuspended in 15 ml of phosphate-buffered saline (PBS; 0.1 M), respectively.

2.2. Sample preparation and inoculation

Sliced cheddar cheese (approximately 85 × 85 × 2 mm) was purchased from a local grocery store (Anseong, South Korea) and stored at refrigerator temperature (4 °C). Sliced cheeses were screened for the presence of tested pathogens before conducting the experiments. For inoculation, 0.1 ml of the mixed culture cocktail described above was applied to the surface of each sliced cheese piece with a micropipette and spread by means of a sterile glass spreader for 1 min for even distribution of pathogens. The inoculated samples were dried for 3 min inside a biosafety hood without the fan running and were repackaged with their own plastic films (polyethylene). National Institute of Standards and Technology reported that the thickness of polyethylene and polypropylene materials within 10 mm did not affect the penetration ability of X-rays (Jung et al., 2015). One sliced cheese (approximately 25 g with an inoculum level of $10^{5–6}$ CFU/sample) was used with each experimental batch.

2.3. Irradiation and dosimetry

A model CP-160 Cabinet X-Radiator System (Faxitron Inc., Tucson, AZ, USA) housed at the irradiation processing facility (Korea Atomic Energy Research Institute, Jeong-eup, South Korea) was used for the experiments. The MXR-160 X-ray tube (Comet, Flamatt, Switzerland) consisted of a stationary anode, tungsten target, metal ceramic with 0.8 mm beryllium inherent filtration, and a 0.5 mm copper additional filter. Irradiator operation conditions were at 160 kV and 10 mA. All samples were irradiated vertical to the incident radiation. The inoculated cheese samples were placed at the same position and distance inside the exposure chamber and received radiation doses of 0, 0.2, 0.4, 0.6, and 0.8 kGy. Each applied radiation dose was adjusted by treatment time. The subsequent experiments were conducted in our laboratory (Hankyong National University, Anseong, South Korea) after moving from the institution. To study the inactivation mechanism, 3 ml of bacterial cell suspension of each species was poured in individual wells of a sterile 12-well flat-bottom polystyrene plate (SPL Inc., Seoul, South Korea) and was irradiated with X-rays at a dose of 0, 0.4, or 0.8 kGy under identical conditions. It is an independent experiment using separately grown cells. The volume of the cell suspension (3 ml) and the treatment doses (0.4 and 0.8 kGy) were selected after preliminary experiments were performed.

The absorbed doses were measured by alanine dosimeters (ES 200-2106/E2044562; Bruker Biospin GmbH, Rheinstetten, Germany) and an e-scan alanine dosimeter reader (Bruker Biospin GmbH). Four alanine dosimeters were attached to the top of samples for each radiation dose and the doses of the mechanisms study were verified by attaching a dosimeter to the top of the 12-well flat-bottom polystyrene plate.

2.4. Bacterial enumeration

After X-ray irradiation, the sliced cheese samples were transferred into sterile stomacher bags (Labphas, Inc., Sainte-Julie, Quebec, Canada) containing 100 ml of PW, and homogenized for 2 min using a stomacher (HBM-400B; HBM Biomed, Tianjin, China). Then, 1 ml aliquots of each sample were 10-fold serially diluted in 9 ml of PW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; MB Cell), xylose lysine desoxycholate agar (XLD; MB Cell), and Oxford agar base (OAB; MB Cell) with Bacto Oxford antimicrobial supplement (MB Cell) were used as the selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. All agar media were incubated at 37 °C for 24 h and typical colonies were counted. To confirm the identity of the pathogens, colonies were randomly selected from the enumeration plates and subjected to biochemical and serological tests. These tests consisted of the *E. coli* O157:H7 latex agglutination assay (RIM; Remel, Lenexa, KS, USA), the *Salmonella* latex agglutination assay (Oxoid, Ogdensberg, NY, USA), and the API *Listeria* test (bioMérieux, Inc., St. Louis, MO, USA) for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

2.5. Enumeration of injured cells

The liquid repair method was used to enumerate injured cells of the three pathogens (Hara-Kudo et al., 2000; Wu, 2008). Injured cells are easily resuscitated on nonselective broth or liquid medium in < 2 h, and the liquid medium repair method is simpler and faster than solid agar overlay method, and it also has no lack of accuracy (Ha and Kang, 2014). One milliliter aliquots of irradiated samples were 10-fold serially diluted in 9 ml of TSB with 0.6% YE, and incubated at 25 °C for 2 h to allow injured cells to resuscitate. Following the recovery step, 0.1 ml of diluent was spread-plated onto each selective medium. All agar plates were incubated for 22 h at 37 °C and typical colonies were counted.

2.6. Membrane potential assay

To investigate changes in cell membrane potential after X-ray treatment, the accumulation of bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Eugene, OR, USA) was measured. Bacteria cells were resuspended in PBS to an optical density at 600 nm (OD_{600}) of approximately 0.3. Suspensions of *L. monocytogenes* were incubated with 0.5 μg $DiBAC_4(3)$/ml at room temperature for 2 min in the dark. Suspensions of *E. coli* O157:H7 and *S.* Typhimurium were incubated with 2.5 μg/ml $DiBAC_4(3)$ with 4 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 15 min in the dark. For positive controls, which were considered to have complete cell membrane depolarization, cells were heat-shocked at 55 °C for 15 min. After incubation, each dyed sample was centrifuged at 3200 rpm for 20 min and washed twice with PBS to remove excess dye. The cell pellet was resuspended in PBS, and fluorescence was measured using a spectrofluorometer (Spectra Max Gemini XS; Molecular Devices, CA, USA) at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence values from untreated cells were subtracted from those of treated cells, and the data were normalized against the OD_{600} of the cell suspensions. Each value was expressed as a ratio to that of the positive control.

2.7. Detection of reactive oxygen species (ROS)

The fluorescent dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate ($CM\text{-}H_2DCFDA$; Invitrogen, Carlsbad, CA, USA) was used to quantitatively assess ROS to the pathogen cells induced by X-ray radiation. *E. coli* O157:H7, *S.* Typhimurium, and *Listeria monocytogenes* cells were resuspended in PBS to adjust the OD_{600} to approximately 0.3. Each suspension of bacteria cells was incubated with $CM\text{-}H_2DCFDA$ at a final concentration of 5 μM for 15 min at 37 °C. After incubation, cells were collected by centrifugation 3200 rpm for 20 min and washed twice with PBS and fluorescence measured with a spectrofluorometer at excitation and emission wavelengths of 495 and 520 nm, respectively. The obtained fluorescence data from untreated and treated cells were normalized for OD_{600} of the cell suspensions.

2.8. Enzymatic activity assay

In order to determine intracellular enzyme damage to pathogen cells, 5(6)-carboxyfluorescein diacetate (cFDA; Sigma-Aldrich) was used. For measurement of cFDA values, *E. coli* O157:H7, *S.* Typhimurium, or *Listeria monocytogenes* cells adjusted to an OD_{680} of approximately 0.2 in PBS. Each cell suspension was incubated with cFDA at a final concentration of 50 μM for 15 min at 37 °C. After incubation, cells were collected by centrifugation at 3200 rpm for 20 min and washed twice with PBS to remove excessive cFDA. The fluorescence measured with a spectrofluorometer at excitation and emission wavelengths of 492 and 517 nm, respectively. The cFDA fluorescence values for each bacteria cell suspension were normalized with the OD_{680} of the cell suspensions, and data obtained for untreated cells were subtracted from those for treated cells.

2.9. Measurement of product quality

To determine the effect of X-ray treatment on the color of sliced cheese surfaces, a model CR400 colorimeter (Minolta Co., Osaka, Japan) was used to measure the color changes of treated samples. The color attributes were quantified from the values of L^*, a^*, and b^*, and measured at random locations on sample. L^*, a^*, and b^* values indicate color lightness, redness, and yellowness of the sample, respectively. All measurements were performed in triplicate.

In order to analyze the change in texture of the X-ray treated sliced cheese, a model CT3-10k texture analyzer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) was used with a model TA10 cylindrical probe (Brookfield Engineering Laboratories, Inc.) 35 mm in length with a sharp edge. Two stacked slices (45 by 45 mm) were placed onto the press holder and the probe was moved down at a rate of 2 mm/s. The maximum force required to press the sample was recorded using Texture Pro CT software (version 1.2; Brookfield Engineering Laboratories, Inc.). The peak force required to shear the samples was utilized as an indicator of hardness. All experiments were replicated three times.

Sensory evaluation was carried out to determine how specific attributes (flavor and overall acceptability) varied over X-ray-treated samples when compared to a non-treated control. The panelists consisted of 13 members (5 men and 8 women; age range 22–26) from the Department of Food and Biotechnology, Hankyong National University, and scores were obtained by rating the sensory attributes using the 7-point hedonic scales: flavor and overall acceptability (7, very good; 6, good; 5, below good/above fair; 4, fair; 3, below fair/above poor; 2, poor; 1, very poor). Samples, labeled with 3-digit random numbers, were placed on white paper plates and served. The presentation order was randomized, and the panelists rinsed their mouth with water before tasting each sample.

2.10. Statistical analysis

All experiments were repeated three times with duplicate samples. Data were analyzed by Duncan's multiple-range test of a statistical analysis system (SAS Institute, Cary, NC, USA). A P-value of < 0.05 indicated a significant difference.

3. Results

3.1. Inactivation of pathogenic bacteria by X-ray treatment

Table 1 shows the actual measured doses obtained using alanine dosimeters. The actual irradiated doses displayed the same trends as target doses (Table 1). Reductions in the viable counts of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on sliced cheese surfaces during X-ray treatment are summarized in Table 2. The bactericidal effect of X-rays increased with increasing the X-ray dose. Treatment at a dose of 0.2 kGy resulted in 2.47, 1.93, and 2.28 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, while 4.08, 3.28, and 3.70 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, were achieved by X-ray

Table 1
Actual measured dose for X-ray irradiation[a].

Target dose (kGy)	Actual measured dose (kGy)
	X-ray
0.2	0.22 ± 0.02
0.4	0.43 ± 0.04
0.6	0.63 ± 0.05
0.8	0.84 ± 0.09

[a] Data represent mean ± standard deviation from three replications.

treatment with a dose of 0.4 kGy. The viable populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were all reduced to less than the detectable limit (< 0.7 log CFU/g) by X-ray treatments of 0.6 and 0.8 kGy. The D_{10}-values for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were ca. 0.08, 0.10, and 0.08 kGy, respectively. Within the range of possible enumeration (0.2 and 0.4 kGy), the degree of inactivation of *E. coli* O157:H7 was higher than that of *S.* Typhimurium or *L. monocytogenes*.

3.2. Resuscitation of injured cells

Levels of sublethally injured *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on sliced cheese surfaces following X-ray irradiation are presented in Table 2. Taking the difference between inactivation of samples subjected to injured cell recovery methods and those plated directly on selective media revealed the presence of ≥1.15 and ≥0.61 log CFU/g of injured *S.* Typhimurium cells after irradiation at 0.6 and 0.8 kGy, respectively. Following irradiation of the inoculated sliced cheese with X-ray doses of 0.6 and 0.8 kGy, no injured cells were detected for *E. coli* O157:H7, respectively. For *L. monocytogenes*, ≥0.30 log CFU/g of and no injured cells were observed after 0.6 and 0.8 kGy treatments, respectively. Significant ($P < 0.05$) recovery of injured *S.* Typhimurium cells was observed by the resuscitation step (liquid broth recovery method) compared to direct plating on selective agar (XLD). However, as for *E. coli* O157:H7 and *L. monocytogenes*, no statistically significant ($P > 0.05$) differences were evident between the levels of the surviving cells during all X-ray treatments.

3.3. Depolarization of cell membrane

In order to measure changes of the cell membrane potential, treated bacteria were stained with the membrane sensitive probe $DiBAC_4(3)$. Data on the accumulation of $DiBAC_4(3)$ in *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after X-ray irradiation are shown in Table 3. Fluorescence values of treated *S.* Typhimurium and *L. monocytogenes* were significantly ($P < 0.05$) increased when compared with untreated cells. However, there were no significant ($P > 0.05$) differences between 0.4 and 0.8 kGy treatment doses. For *E. coli* O157:H7, although $DiBAC_4(3)$ accumulation values increased with increasing X-ray doses, statistically significant ($P > 0.05$) differences were not observed (Table 3). Based on overall result pattern, it could be concluded that the X-ray irradiation induced cell membrane depolarization of the three pathogenic bacteria.

Table 3
Ratio of $DiBAC_4(3)$ accumulation values in treated bacteria to that in the positive control after X-ray treatment.[a]

Treatment dose (kGy)	$DiBAC_4(3)$ percentage (%)		
	Gram negative		Gram positive
	E. coli O157:H7	*S.* Typhimurium	*L. monocytogenes*
0	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00 ± 0.00 A
0.4	0.08 ± 0.08 A	0.24 ± 0.17 B	0.22 ± 0.06 B
0.8	0.11 ± 0.07 A	0.28 ± 0.09 B	0.32 ± 0.17 B

[a] Data represent mean ± standard deviation from three replications. Different letters within the same bacteria column indicate significant difference ($P < 0.05$). Normalized data were obtained by subtracting fluorescence values of untreated cells from those of treated cells and dividing by the positive control value and expressing this value as a percentage as follows: $DiBAC_4(3)$ percentage = (fluorescence value after treatment − fluorescence value of non-treated) / (OD_{600} · fluorescence value of positive control).

3.4. Generation of ROS

To test if ROS were involved in the X-ray inactivation mechanisms, treated bacteria were stained with the fluorescent dye CM-H_2DCFDA to detect intracellular ROS. As shown in Table 4, for all three pathogens, fluorescence values of X-ray treated cells were significantly increased ($P < 0.05$) compared to untreated cells. ROS generation values of *E. coli* O157:H7 and *L. monocytogenes* increased with increasing X-ray doses. However, in case of *S.* Typhimurium, there was no significant ($P > 0.05$) difference in the ROS generation value between the 0.4 and 0.8 kGy doses (Table 4). The data indicated that X-ray irradiation resulted in increasing generation of ROS in the bacterial cells.

3.5. Determination of esterase activity

To quantitatively analyze the loss of intracellular esterase activity, X-ray-treated bacteria were stained with the fluorescent dye cFDA. The cFDA conversion values following X-ray treatment for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* are shown in Table 5. Overall, the cFDA conversion values in the irradiated pathogens were significantly increased ($P < 0.05$) compared to the controls. Therefore, X-ray irradiation could induce perturbation of enzymatic activity to the pathogens. Among the three pathogens, *E. coli* O157:H7 showed the lowest conversion values of cFDA. In case of *L. monocytogenes*, although the cFDA conversion values were slightly increased in accordance with

Table 2
Log reductions of surviving cells and cells including injured *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on sliced cheese surfaces treated with X-ray.[a]

Treatment dose (kGy)	Log reduction [$\log_{10}(N_0/N)$] by organism and selection medium					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
	SA	SAR	SA	SAR	SA	SAR
0	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
0.2	2.47 ± 0.66 Ba	2.78 ± 0.02 Ba	1.93 ± 0.28 Ba	1.48 ± 0.79 Ba	2.28 ± 0.11 Ba	2.30 ± 0.50 Ba
0.4	4.08 ± 0.45 Ca	4.16 ± 0.15 Ca	3.28 ± 0.49 Ca	2.75 ± 0.50 Ca	3.70 ± 0.35 Ca	3.49 ± 0.52 BCa
0.6	> 5.45 ± 0.14 Da	> 5.77 ± 0.89 Da	> 4.47 ± 0.04 Da	3.32 ± 0.21 CDb	> 4.99 ± 0.36 Da	4.69 ± 1.47 CDa
0.8	> 5.45 ± 0.14 Da	> 5.77 ± 0.89 Da	> 4.47 ± 0.04 Da	3.86 ± 0.37 Db	> 4.99 ± 0.36 Da	> 5.14 ± 0.84 Da

[a] Data represent means ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$). SA, plating directly on selective agar; SAR, plating on selective agar preceded by a resuscitation step. > reductions were calculated based on the initial counts and the detection limit (0.7 log CFU/g).

Table 4
ROS generation values of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* inferred from the ROS detection assay by using CM-H_2DCFDA.[a]

Treatment dose (kGy)	Microorganism		
	E. coli O157:H7	*S.* Typhimurium	*L. monocytogenes*
0	461.04 ± 40.58 A	324.79 ± 41.17 A	590.06 ± 28.20 A
0.4	501.00 ± 24.17 AB	403.10 ± 13.21 B	640.75 ± 67.77 AB
0.8	563.69 ± 33.28 B	404.40 ± 35.99 B	678.79 ± 9.72 B

[a] Data represent mean ± standard deviation from three replications. Different letters within the same pathogen column indicate significant difference ($P < 0.05$). Fluorescence values were normalized against the OD_{600} of the cell suspensions.

Table 5
Levels of intracellular enzyme inactivation in X-ray treated cells inferred from cFDA conversion tests.[a]

Treatment dose (kGy)	Microorganism		
	E. coli O157:H7	*S.* Typhimurium	*L. monocytogenes*
0	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00 ± 0.00 A
0.4	75.17 ± 26.29 AB	566.86 ± 272.37 B	661.26 ± 233.7 B
0.8	108.16 ± 83.04 B	1461.56 ± 105.99 C	897.09 ± 192.14 B

[a] Data represent mean ± standard deviation from three replications. Different letters within the same pathogen column indicate significant difference ($P < 0.05$). The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} as follows: cFDA conversion value = |(fluorescence value after treatment − fluorescence value of non-treated) / OD_{680}|.

the prolonged treatment dose, statistically significant differences were not observed between 0.4 and 0.8 kGy treatment intervals ($P > 0.05$).

3.6. Effect of X-ray treatment on product quality

The color, texture parameters, and sensory attributes of the sliced cheeses after X-ray treatment are presented in Table 6. The L^*, a^*, and b^* values were not significantly different ($P > 0.05$) from those of untreated samples. Although the maximum load values of the texture measurements were slightly decreased, statistically significant differences were not detected during the entire treatment interval ($P > 0.05$). Also, there were no significant ($P > 0.05$) differences among all tested samples scored by the hedonic scale for flavor and overall acceptability, indicating that X-ray for 0.8 kGy did not significantly alter the sensory quality of sliced cheese products. Thus, X-ray irradiation of up to 0.8 kGy did not significantly alter the quality of the sliced cheese products (Table 6).

4. Discussion

Common packaged ready-to-eat (RTE) foods, such as sliced cheeses have been implicated in outbreaks of foodborne pathogens (Coillie et al., 2004). Cross-contamination occurs frequently during processing as slicing, cut, and packing (Lianou and Sofos, 2007; Rød et al., 2012). RTE foods are consumed directly, without a final sterilization step (Guenther et al., 2009; Ha et al., 2017). An additional superficial antimicrobial intervention step may become essential in order to control pathogens on RTE foods. A bactericidal process must be effective in reducing microorganisms while not resulting in unacceptable changes in food product quality. In this regard, various novel non-thermal irradiation treatments involving pulsed ultraviolet light and E-beam have been evaluated for the control of pathogenic microorganisms on RTE foods (Wambura and Verghese, 2011; Zhu et al., 2004). However, pulsed ultraviolet light easily deteriorates the quality of sliced ham, particularly color values (Wambura and Verghese, 2011). E-beam irradiation influences the odor and flavor of RTE turkey breast rolls (Zhu et al., 2004) and, unlike X-rays, has low penetrability (Farkasa and Mohácsi-Farkasb, 2011; Tahergorabi et al., 2012). Aymerich et al. (2008) reported that E-beams have a penetration depth of approximately 8–10 cm, while X-ray penetrates approximately 80–100 cm, at the same dose of 4 kGy. Thus, E-beams are limited to the treatment of the surface of food or thin layered foods. On the other hand, X-ray irradiation presently reduced the populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in packaged sliced cheeses to less than detection limits (< 0.7 log CFU/g; Tables 2 and 6). Furthermore, after treatment with the maximum dose of 0.8 kGy, color values (L^*, a^*, and b^*) and maximum load values of samples were not significantly different from the control ($P > 0.05$). Additionally, no gross alterations in sensory attributes (flavor and overall acceptability) of PE-packaged cheese products were detected after 0.8 kGy of X-ray treatment (Table 6). These results indicate that the X-ray treatment was effective in reducing the number of pathogens on film-packaged sliced cheeses while maintaining product quality. Hence, X-ray treatment could become an attractive solution to the decontamination of other packaged RTE food products.

Sublethally injured cells can be a threat to food safety as their noninjured counterparts, because they are able to repair themselves and regain all their normal properties including virulence under suitable conditions. Thus, the significance of injured pathogens should not be ignored (Chen et al., 2013; Wu, 2008). In this study, the extent to which sublethally injured pathogens survived after X-ray treatment was assessed using the liquid repair method. No significant ($P > 0.05$) recovery levels of *E. coli* O157:H7 and *L. monocytogenes* injured cells were observed throughout treatment (Table 2). This indicates that X-ray effectively inactivated *E. coli* O157:H7 and *L. monocytogenes* on sliced cheeses without generating many injured cells capable of repair and growth. For *S.* Typhimurium, significant ($P < 0.05$) repair of injured cells was detected (Table 2). Similar increases of sublethally injured *S.* Typhimurium after ionizing radiation (E-beam) exposure have been previously reported (Sarjeant et al., 2005; Tesfai et al., 2011). In the present study, *S.* Typhimurium exposed to X-rays also displayed significant radio-resistances, consistent with the previous E-beam researches.

The common types of ionizing radiation used for foods are gamma-ray or E-beam irradiation. The main bactericidal mechanisms of

Table 6
Surface color values, maximum load values for quantifying texture, and sensory attributes of sliced cheese treated with X-ray irradiation.[a]

Treatment dose (kGy)	Color value for parameter			Maximum load (g)	Sensory attribute score	
	L^*	a^*	b^*		Flavor	Overall
0	82.52 ± 0.17 A	10.85 ± 0.66 A	43.54 ± 0.59 A	2727 ± 185.21 A	5.83 ± 0.51 A	5.68 ± 0.46 A
0.4	82.55 ± 0.34 A	10.76 ± 0.20 A	43.83 ± 0.13 A	2721 ± 108.04 A	5.90 ± 0.45 A	5.75 ± 0.65 A
0.8	82.00 ± 0.33 A	10.73 ± 0.22 A	43.38 ± 0.09 A	2696 ± 122.02 A	5.80 ± 0.50 A	5.55 ± 0.58 A

[a] Data represent means ± standard deviations from three replications. Means with the same letters within each column are not significantly different ($P > 0.05$). L^*, lightness; a^*, redness; b^*, yellowness. Sensory attributes are from panelist scorecard analysis on a 7-point hedonic scale, where 7 is very good and 1 is very poor. n = 13.

ionizing radiation are known as microbial DNA damage and free radicals activity (Black and Jaczynski, 2008; Lado and Yousef, 2002; López-González et al., 1999). However, the underlying inactivation mechanisms of X-ray treatment are not well understood. In the present study, we focused on other factors except for the DNA damage revealed in many previous ionizing radiation studies. Accordingly, the critical inactivation mechanisms of X-ray irradiation were investigated based on the cell membrane potential, generation of ROS, and intracellular enzyme activity (esterase) (Tables 3–5).

When cell activity declines, membrane depolarization occurs and homeostasis activity that normally maintains different concentrations of ions inside and outside of the cell is disrupted (Ha et al., 2017). Although the reduction of membrane potential does not indicate cell inactivation, it plays an important role in cell physiology. Hence, membrane potential is strongly related to membrane damage, because membrane depolarization develops prior to structural membrane damage (Kim et al., 2017). $DiBAC_4(3)$ accumulates when the dye crosses the cell membrane as a result of membrane depolarization (Rezaeinejad and Ivanov, 2011; Sánchez et al., 2010). For this reason, the degree of depolarization of cell membranes can be measured by the fluorescence value of the dye that accumulates inside cells. As shown in Table 3, X-ray treatment changed cell membrane potentials of the treated pathogenic bacteria. Depolarization of cell membranes may be one of the mechanisms related to the X-ray inactivation.

ROS including superoxide (O_2^-) and hydroxyl radicals (OH·) are created when ionizing radiation, such as X-rays, splits water molecules within the food products or the resident bacteria (Chen et al., 2010; Esnault et al., 2010; Niemira and Solomon, 2005). CM-H_2DCFDA can penetrate the cell membrane and is hydrolyzed in the cytosol to form the dichlorofluorescin (DCFH) carboxylate anion, which is oxidized to 2′,7′-dichlorofluorescein (DCF) by ROS (Wojtala et al., 2014). Therefore, the generation of ROS can be determined by measuring the fluorescence value of DCF. In the present study, significant ROS generation observed following X-ray irradiation was evident in all three pathogens ($P < 0.05$; Table 4). These radicals can cause lethal damage to biomolecules including DNA and membrane lipid (Chen et al., 2010; Dong et al., 2015). Accordingly, it may be inferred that X-ray treatment induces ROS generation and then adversely affects cell membrane integrity as a secondary form of damage. This inference agrees with the previously studied inactivation mechanism of ionizing radiation (Black and Jaczynski, 2008).

We also measured the loss of enzymatic activity inside the pathogenic bacteria. The cFDA dye can freely diffuse across cell membranes and is converted by nonspecific esterases into carboxyfluorescein (cF) (Ha et al., 2017; Majeed et al., 2015). Therefore, fluorescence values of cF can be indicator of both intracellular esterase activity and metabolic activity (Ritz et al., 2001). As shown in Table 5, the loss of esterase activity was observed in all X-ray-treated pathogens. The collective results, which include cell membrane depolarization, generation of ROS, and disruption of enzymatic activity, might be related to the main inactivation mechanisms of X-ray irradiation.

In conclusion, the results of the present study indicate that X-ray irradiation is suitable for the reduction of microbial contamination of packaged sliced cheeses by the aforementioned mechanisms without affecting product quality. The degree of microbial contamination that can occur during the slicing and packaging steps greatly influences the quality of the end products. Therefore, preventing cross-contamination is very important in RTE food production. In this sense, X-ray treatment can be applied to dairy products and also various packaged foodstuffs as a new post-processing antimicrobial technology. Although the lab-scale instrument used in the current study was a low capacity batch type, X-ray irradiation on an industrial scale for post-packaging microbial decontamination can be performed on a continuous basis with a high-energy apparatus.

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