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Inactivation of *Escherichia coli* O157 and *Salmonella* Enteritidis in raw beef liver by gamma irradiation

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ABSTRACT

Irradiation of ground beef and beef liver inoculated with *Escherichia coli* O157 466 and DT66 and *Salmonella* Enteritidis 3313 were performed with gamma rays from cobalt-60 at refrigerated and frozen temperatures under air- and vacuum-packaged conditions. Results showed that D₁₀ values for all pathogens in frozen beef liver were higher than those in frozen ground beef samples, with significant differences observed between the D₁₀ values of *E. coli* O157 466 and *S. Enteritidis* 3313 under air-packaged conditions, as well as in *E. coli* O157 DT66 and *S. Enteritidis* 3313 under vacuum-packaged conditions. To verify effective bacterial inactivation under high bacterial-contamination levels (10⁵–10⁷ CFU/g), survival/death interfaces of *E. coli* O157 DT66 and *S. Enteritidis* 3313 inoculated in beef liver under vacuum-packaged and frozen conditions were constructed, with results suggesting that doses from 5.3 kGy to 5.5 kGy and 8.2 kGy–8.5 kGy would be sufficient to kill 10⁵ CFU/g of *E. coli* O157 and *S. Enteritidis* 3313, respectively, at a 95%–99% predicted confidence interval. These results suggested that food matrixes containing high amounts of antioxidants (such as beef liver) and treated under frozen and vacuum-packaged conditions require additional consideration and evaluation for applications of irradiation treatment.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella* spp. are naturally occurring bacteria that are pathogenic to humans and can be found within the gastrointestinal tract of cattle (Schilling et al., 2009). These pathogenic bacteria are a public health concern and considered major foodborne pathogens requiring regulation in the beef meat-marketing industry. In April 2011, an EHEC outbreak caused by “*yu-khoe*” (raw minced beef with raw egg yolk) in Japan resulted in hospitalization of 181 people and five deaths (Urakami, 2011). Additionally, since July 2012, the serving of “*reba-sashi*” (sashimi-style of raw beef liver) in restaurants has been prohibited in Japan due to high contamination rates associated with coliform bacteria, including EHEC (Shinagawa, 2012), that cannot be completely eradicated by chemical treatment. It is difficult to effectively reduce the bacterial risk in these

types of uncooked foods, because they cannot undergo heat treatment.

The use of gamma irradiation treatment can effectively kill foodborne pathogens in meat and extend its shelf life in the absence of a heating process. This method has been accepted in > 34 countries for commercial food processing and preservation (Loaharanu, 2000). Irradiation has been studied extensively for use in improving the safety of meat products. The United States Food and Drug Administration (FDA) approved irradiation for poultry and red meats in order to control foodborne pathogens and extend product shelf life (Kwon et al., 2008), and irradiation at up to 4.5 kGy and 7.0 kGy of fresh and frozen red meat, respectively, has been approved in the United States. (US FDA, 1997). The primary mechanism of foodborne-pathogen inactivation by irradiation involves the breakage of chemical bonds within the DNA of the cells (Urbain, 1986), as well as alteration of membrane permeability and other cellular functions that contribute to cell death. Additionally,

Abbreviations: BPW, buffered peptone water; EHEC, enterohemorrhagic *Escherichia coli*; FDA, Food and Drug Administration; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VRBG, violet red bile glucose

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bacterial cells can be damaged by radicals formed from the lysis of water molecules in food and from reactions with oxygen (Lopez-Gonzalez et al., 1999).

Several studies reported the effectiveness of irradiation treatment to reduce the risk of EHEC and *Salmonella* spp. in meat and ready-to-eat foods. Specifically, the D_{10} value range for ground beef was determined to range from 0.305 kGy to 0.307 kGy for *E. coli* O157 and from 0.745 kGy to 0.800 kGy for *Salmonella* spp. under frozen conditions (Clavero et al., 1994). In our previous study, irradiation of ground beef and beef liver inoculated with *Campylobacter jejuni* was performed by gamma rays from a cobalt-60 source (Kawasaki et al., 2018), revealing D_{10} values for *C. jejuni* in beef liver higher than those in ground beef under each irradiation condition. Moreover, under frozen conditions, a significant difference in D_{10} value was observed between ground beef and beef liver; therefore, irradiation efficacy on each pathogen in beef liver needs to be determined, because the D_{10} value data for ground beef could not be extrapolated. Therefore, the present study compared variations in the irradiation sensitivity of *E. coli* O157 and *Salmonella* spp. in ground beef and beef liver at refrigerated and frozen temperatures and under aerobic and anaerobic conditions. Additionally, we assessed the appropriate irradiation dose necessary for complete elimination of bacteria at high inoculation levels (10^5 – 10^7 CFU/g) in beef liver samples.

2. Materials and methods

2.1. Sample preparation

Whole raw beef liver and raw ground beef were obtained from a slaughterhouse center (Shibaura, Tokyo) and local supermarket. The samples were transported to the laboratory under refrigerated conditions (4 °C), and whole raw beef liver and raw ground beef were divided under sterile conditions into 25 g portions, packaged in gas-impenetrable laminate film bags (300 mm × 200 mm, total thickness 112 μm, ceramic deposited PET/PE, gas transmission rates for oxygen 0.5 mL/m²·day·atm; Mitsubishi Chemical Industry Co. Ltd., Tokyo, Japan), and stored in a freezer at –80 °C until use.

2.2. Bacterial strains and preparation of bacterial cultures

Enterohemorrhagic verotoxin-producing *E. coli* O157 (stx-1 and -2) strain 466 isolated from a clinical sample, non-verotoxin-producing *E. coli* O157 strain DT66 isolated from bovine feces, and *Salmonella* Enteritidis 3313 were used in this study. Bacterial strains were grown overnight at 37 °C with rotary shaking in 30 mL of trypticase soy broth (BBL, Cockeysville, MD, USA). Enriched culture broth was transferred to centrifuge tubes, and the cells were collected by centrifugation for 5 min at 4000 g at 4 °C. Bacterial cell pellets were washed with phosphate-buffered saline (PBS) and suspended in 8 mL of PBS, with the obtained bacterial suspension containing ~ 10^9 CFU/mL.

2.3. Inoculation and packaging

Inoculum (100 μL; 20 μL × 5 positions; ~ 10^9 CFU/mL) was injected into raw beef liver and raw ground beef (25 g each) using a plastic medical syringe with a 21G stainless steel needle (TERUMO Co. Ltd., Tokyo, Japan). The bacterial concentration of each sample was ~7 log CFU/g. Inoculated samples were immediately packaged or vacuum packaged in gas-impenetrable laminate film bags (see section 2.1.) and stored at 0 °C or –80 °C for 2 h before irradiation.

2.4. Irradiation conditions

To determine the D_{10} value, *E. coli* O157-inoculated and -packaged samples were irradiated at 0 kGy–1.2 kGy with ice (0 °C) and at 0 kGy–3.0 kGy with dry ice (–80 °C), and *S. Enteritidis*-inoculated and

-packaged samples were irradiated at 0 kGy–3.0 kGy with ice and at 0 kGy–5.0 kGy with dry ice with gamma rays from a cobalt-60 source in a self-shielded irradiator (Gamma Cell-220; Nordion International Inc., Kanata, Ontario, Canada) at Food Research Institute, NARO. The dose rate was ~3 kGy/h. An alanine pellet dosimeter (Bruker Biospin, Ltd., Rheinstetten, Germany) was attached to the surface of the plastic bag, and absorbed doses were determined using an electron-spin paramagnetic spectrophotometer (Bruker EMX; Bruker Biospin, Ltd.).

2.5. Microbiological analysis

Irradiation samples (25 g) were immediately transferred to a sterile plastic stomacher bag (StomaFilter; GSI Creos, Tokyo, Japan) with 225 mL of buffered peptone water (BPW; ISO 6579; Merck, Darmstadt, Germany) and pummeled in a stomacher (Pro-media SH-IIM; ELMEX, Tokyo, Japan) for 1 min. Serial decimal dilutions were prepared and spread on violet red bile glucose (VRBG; Oxoid, Basingstoke, UK) agar plates using a spiral plater instrument (EDDY JET; IUL, Barcelona, Spain). After incubation for 24 h at 35 °C, *E. coli* O157 and *Salmonella* colonies were counted, and the colonies checked using immuno-chromatography *E. coli* O157 and *Salmonella* detection kits (Singlepath *E. coli* O157 and Singlepath *Salmonella*; Merck). At least five colonies were picked from the VRBG plates, with all of these colonies checked to ensure positivity for both *E. coli* O157 or *Salmonella*.

2.6. Determination of D_{10} value and statistical analysis

Bacterial radiosensitization under combined-treatment conditions was evaluated by linear regression. All experiments were performed in triplicate, and D_{10} values from radiation were calculated as the inverse of the slope derived from the inactivation curves. Each condition used for the inactivation curves was compared by testing for differences between two linear regression slopes ($p < 0.05$). Predicted confidence intervals (95–99%) were determined from each inactivation curve, with these analyses performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

2.7. Determination of *E. coli* O157 and *Salmonella* survival or death following irradiation treatment

Liver samples (25 g) inoculated with high levels *E. coli* O157 DT66 (10^4 – 10^7 CFU/g) or *S. Enteritidis* 3313 (10^5 – 10^7 CFU/g) were vacuum packed and gamma-irradiated at a gamma room in The National Institutes for Quantum and Radiological Science and Technology, Takasaki (No. 7 cell; dose rate: 2.5 kGy/h). After irradiation, *E. coli* O157 or *Salmonella* survival or death was determined in beef liver samples. Samples (25 g; $n = 5$) were enriched with 225 mL of BPW at 35 °C for 24 h. Enriched samples were streaked on VRBG agar plates, and colonies were confirmed using immuno-chromatography *E. coli* O157 and *Salmonella* detection kits (Merck).

3. Results and discussion

Numerous experimental studies have been conducted to estimate pathogenic bacterial radiosensitivity in ground beef; however, except for a report focusing on *Campylobacter* (Kawasaki et al., 2018), none have reported on the radiosensitivity of pathogenic bacteria in beef livers. In the present study, the effect of irradiation on *E. coli* O157 and *Salmonella* spp. in beef liver air- and vacuum-packaged at 0 °C and –80 °C, respectively, was investigated. Table 1 shows the radiation D_{10} values of *E. coli* O157 and *S. Enteritidis* in ground beef and beef liver under various atmosphere conditions (air and vacuum) at refrigeration temperature (0 °C). The D_{10} values of *E. coli* O157 466 and DT66 and *S. Enteritidis* strains were observed from 0.25 kGy to 0.33 kGy and at 0.58 kGy, respectively, in ground beef under air-packaged conditions at refrigeration temperature. Similarly, Clavero et al. (1994) described D_{10}

Table 1 D_{10} values of *E. coli* O157 and *S. Enteritidis* strains in ground beef and beef liver under refrigerated conditions.

Strain		Matrix	Air			Vacuum		
<i>E. coli</i> O157	466	Ground beef	0.25 ± 0.01	$R^2 = 0.97$	A*	NT		
		Beef liver	0.26 ± 0.01	$R^2 = 0.99$	A**	NT		
	DT66	Ground beef	0.33 ± 0.02	$R^2 = 0.85$	Aa*	0.35 ± 0.05	$R^2 = 0.94$	Aa
		Beef liver	0.34 ± 0.02	$R^2 = 0.96$	Aa**	0.41 ± 0.01	$R^2 = 0.96$	Ab
<i>S. Enteritidis</i>	3313	Ground beef	0.58 ± 0.03	$R^2 = 0.96$	Aa	0.60 ± 0.03	$R^2 = 0.96$	Aa
		Beef liver	0.65 ± 0.06	$R^2 = 0.97$	Aa	0.67 ± 0.08	$R^2 = 0.95$	Aa

Values are mean ± standard deviation. Within each row and column for each individual strain, means with different uppercase and lowercase letters, respectively, are significantly different ($p < 0.05$).

*, **: significant differences between *E. coli* O157 466 and DT66 strains ($p < 0.05$).

Table 2 D_{10} values for *E. coli* O157 and *S. Enteritidis* strains in ground beef and beef liver under frozen conditions.

Strains		Matrix	Air			Vacuum		
<i>E. coli</i> O157	466	Ground beef	0.51 ± 0.05	$R^2 = 0.97$	A*	NT		
		Beef liver	0.76 ± 0.07	$R^2 = 0.96$	B	NT		
	DT66	Ground beef	0.69 ± 0.01	$R^2 = 0.96$	Aa*	0.78 ± 0.07	$R^2 = 0.97$	Aa
		Beef liver	0.85 ± 0.11	$R^2 = 0.95$	Aa	0.95 ± 0.08	$R^2 = 0.98$	Bb
<i>S. Enteritidis</i>	3313	Ground beef	1.00 ± 0.01	$R^2 = 0.97$	Aa	1.03 ± 0.10	$R^2 = 0.95$	Aa
		Beef liver	1.38 ± 0.07	$R^2 = 0.96$	Bb	1.47 ± 0.12	$R^2 = 0.96$	Bb

Values are mean ± standard deviation. Within each row and column for each individual strain, means with different uppercase and lowercase letters, respectively, are significantly different ($p < 0.05$).

*: significant differences between *E. coli* O157 466 and DT66 strains ($p < 0.05$).

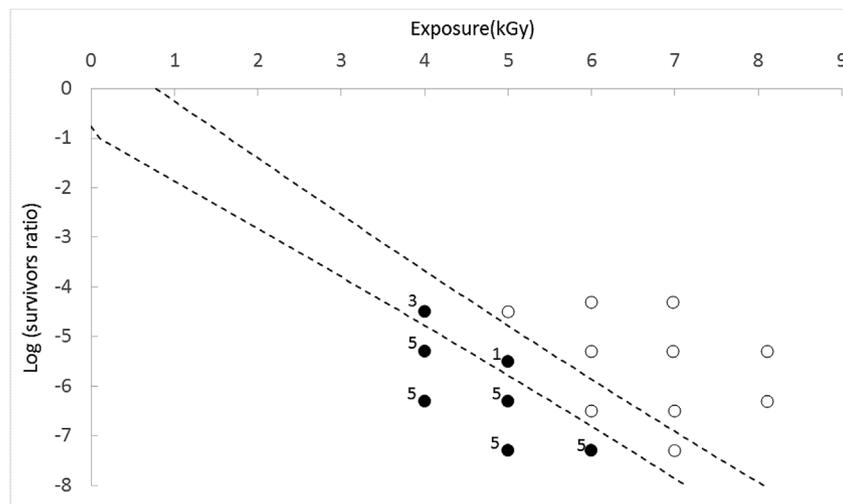


Fig. 1. Survival/death interface for *E. coli* O157 DT66-inoculated, vacuum-packaged beef liver after gamma irradiation at frozen temperature ($-80\text{ }^{\circ}\text{C}$). The solid line indicates 95% predicted confidence intervals calculated from survival plots of *E. coli* DT66 under these conditions. Closed or open circle showed survival or death after enrichment, and numbers describe bacterial positivity found in five samples.

values from 0.241 kGy to 0.251 kGy and from 0.621 kGy to 0.661 kGy in low- and high-fat beef, respectively, under refrigerated conditions. Additionally, we observed differences in D_{10} values between *E. coli* 466 isolated from a clinical sample and strain DT66 isolated from bovine feces in ground beef and beef liver maintained at $0\text{ }^{\circ}\text{C}$ under air-packaged conditions (0.25 vs. 0.33 kGy; and 0.26 vs. 0.34 kGy, respectively). By contrast, the D_{10} values of *E. coli* O157 and *S. Enteritidis* in beef liver were observed from 0.26 kGy to 0.34 kGy and 0.65 kGy, respectively. These results showed that the D_{10} values of *E. coli* and *S. Enteritidis* in beef liver were slightly higher than those in ground beef samples, although there were no significant differences observed among ground beef and beef liver samples under air-packaged condition at refrigeration temperature. The D_{10} values of *E. coli* DT66 and *S. Enteritidis* under vacuum-packaged conditions were observed at 0.35 kGy and 0.60 kGy in ground beef and 0.41 kGy and 0.67 kGy in beef liver, respectively, with a significant difference in irradiation sensitivity observed in beef liver inoculated with *E. coli* DT66 between air- and vacuum-packaged

conditions.

Table 2 shows the D_{10} values of *E. coli* O157 and *S. Enteritidis* in ground beef and beef liver under various atmosphere conditions (air and vacuum) at frozen temperature ($-80\text{ }^{\circ}\text{C}$). D_{10} values are generally higher at freezing temperatures than at refrigeration temperatures, and here, significant differences were observed in all samples between refrigerated and frozen conditions. In frozen conditions, the D_{10} values in beef liver were higher than those in ground beef samples. Additionally, we observed significant differences between the D_{10} values of *E. coli* O157 466 and *S. Enteritidis* under air-packaged conditions (0.51 vs. 0.76 kGy; and 1.00 vs. 1.38 kGy, respectively), as well as between *E. coli* O157 DT66 and *S. Enteritidis* under vacuum-packaged conditions (0.78 vs. 0.95 kGy; and 1.03 vs. 1.47 kGy, respectively). Moreover, a significant difference in the D_{10} value in *E. coli* O157 DT66 was observed in beef liver between air- and vacuum-packaged conditions (0.85 vs. 0.95 kGy). In general, irradiation is carried out at $4\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$ that reflecting refrigeration or frozen conditions. Our experiments were done

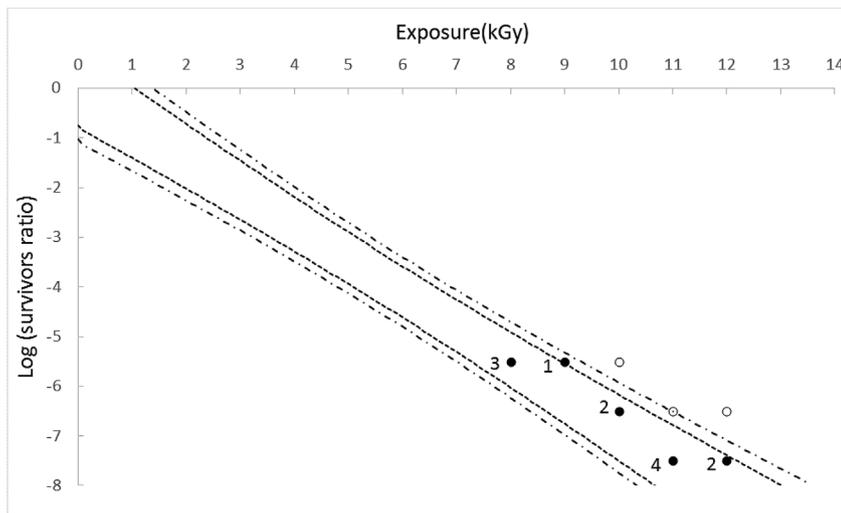


Fig. 2. Survival/death interface for *S. Enteritidis* 3313-inoculated, vacuum-packaged beef liver after gamma-irradiation at frozen temperature ($-80\text{ }^{\circ}\text{C}$). Solid and scattered lines indicate 95% and 99% predicted confidence intervals, respectively, calculated from survival plots of *S. Enteritidis* 3313 under these conditions. Closed or open circle showed survival or death after enrichment, and numbers describe bacterial positivity found in five samples.

at 0 or $-80\text{ }^{\circ}\text{C}$, lower than those general refrigeration and frozen temperature in order to avoid underestimation during actual irradiation condition.

During inactivation of pathogenic bacteria in food matrixes by irradiation, radicals can form from the lysis of water molecules in food resulting from reactions with oxygen, with these radicals capable of breaking chemical bonds in DNA and resulting in bacterial-cell inactivation (Lopez-Gonzalez et al., 1999). Additionally, alterations in membrane permeability and other cellular functions can contribute to cell death (von Sonntag, 1987). Compared with ground beef, beef-liver matrix contains high levels of antioxidants, such as β -carotene and retinol (USDA, 2013; Darwish et al., 2016). We speculated that the existence of high concentrations of antioxidants in beef liver would eliminate reactive oxygen species (ROS) formed during the irradiation process, which damaged bacterial DNA. Additionally, it is likely that the vacuum-packaged conditions of beef liver samples played a role in maintaining low levels of ROS. This suggests that when radicals are not produced, the efficiency of bacterial-inactivation processes will be decreased.

To verify the effectiveness of bacterial inactivation by irradiation under high levels of bacterial contamination (10^5 – 10^7 CFU/g), the survival/death interfaces of *E. coli* O157 DT66 and *S. Enteritidis* 3313 inoculated in beef liver under vacuum-packaged and frozen conditions were evaluated (Figs. 1 and 2). All inoculated samples exposed to radiation, except for one sample inoculated with *S. Enteritidis* 3313, at $> 95\%$ of the predicted confidence estimates according to irradiation inactivation curves for *E. coli* O157 DT66 and *S. Enteritidis* 3313 were negative for bacteria. Additionally, all irradiated samples were negative for bacteria following irradiation doses $> 99\%$ of the predicted confidence estimates. These results suggested that an applied dose from 5.3 kGy to 5.5 kGy and from 8.2 kGy to 8.5 kGy was sufficient to kill 10^5 CFU/g of *E. coli* O157 and *S. Enteritidis*, respectively, at 95%–99% predicted confidence intervals.

For food distribution, freezing and vacuum- or modified-atmosphere-packaging techniques are generally used to maintain food quality. Moreover, application of irradiation to eliminate bacteria in food products requires the maintenance of low temperatures and control of oxygen exposure in order to prevent lipid oxidation in food matrixes. However, it is difficult to achieve conditions enabling 5-log reductions in *Salmonella* levels in beef liver, given that the FDA (US FDA, 1997) permits irradiation treatment of only < 7.0 kGy in order to preserve meat products. Our results suggest that food matrixes containing high amounts of antioxidants and being treated under frozen and vacuum-packaged conditions require additional consideration and evaluation for applications of irradiation treatment.

4. Conclusions

These results showed that the D_{10} value of beef liver was higher than that of ground beef under different irradiation conditions, and that significant differences in D_{10} values under frozen conditions were observed. Further consideration and evaluation is necessary to determine the appropriate application of irradiation treatment of food matrixes with a porous structure that have been frozen and contain high amounts of antioxidants.

Conflicts of interest

The authors declare no conflicts of interest.

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