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ガンマ線と電子線照射による嫌気性菌と通性嫌気性菌の生存への影響

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Effects of Gamma Ray and Electron-Beam Irradiations on Survival of Anaerobic and Facultatively Anaerobic Bacteria

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An extension of the approval for food irradiation is desired due to the increase in the incidence of food poisoning in the world. One anaerobic (*Clostridium perfringens*) and four facultatively anaerobic (*Bacillus cereus, Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Enteritidis) bacteria irradiated with gamma ray or electron beam (E-beam) were tested in terms of survival on agar under packaging atmosphere. Using pouch pack, effects of two irradiations on survival of anaerobic and facultatively anaerobic bacteria were evaluated comparatively. E-beam irradiation was more effective than gamma ray irradiation in decreasing the D_{10} value of *B. cereus* at 4 ℃, slightly more effective in that of *E. coli* O157, and similarly effective in that of the other three bacteria at 4 ℃. The gamma irradiation of the bacteria without incubation at 4 ℃ before irradiation was more effective than that of the bacteria with incubation overnight at 4 ℃ before irradiation in decreasing the D10 values of these bacteria (*B. cereus, E. coli* O157, and *L. monocytogenes*). Furthermore, ground beef patties inoculated with bacteria were irradiated with 1 kGy by E-beam (5 MeV) at 4 ℃. The inoculated bacteria in the 1-9 mm beef patties were killed by 1 kGy E-beam irradiation and some bacteria in more than 9 mm beef patties were not killed by the irradiation.

Keywords: gamma ray irradiation, Electron-beam irradiation, anaerobic bacteria, facultatively anaerobic bacteria

Introduction

Due to increasing concerns on food safety, the Food and Drug Administration, USA, has recently approved meat irradiation[1]. This approval has attracted attention of food-processing industries and consumers worldwide. One of the major targets of this approval is the enterohaemorrhagic *Escherichia coli* O157:H7 (*E. coli* O157), the causative agent of several outbreaks in the USA[2]. Irradiation causes radical formation[3] followed breakage of the chemical bonds of DNA molecules[4], and consumption oxygen and water. Oxygen reduction is more eminent in inner part of irradiated food with electron beam (E-beam) than those with gamma ray. Oxygen demand of bacteria affects their survival in food. Therefore, depth-survival relationship of actual food is not predictable by dose-depth curve, which is provided by American Standard and Testing Materials (ASTM).

Gamma ray and E-beam irradiations are major methods of food irradiation[5]. We report herein the effects of these methods on the survival of five types of bacteria. Furthermore, E-beam irradiation of ground beef patties inoculated with bacteria is discussed.

Materials and Methods

1) Irradiation of pouch packs

The bacteria strains used were *Bacillus cereus* (IFO3001, spore-former), *E. coli* O157 (non-verotoxin type), *Listeria monocytogenes* [AZ-97035, Scot A(4b)], *Salmonella* Enteritidis (PT4, E930448), and *Clostridium perfringens* (NCTC 8798). *C. perfringens* is anaerobic. The other four bacteria used are facultatively anaerobic.

Preparations of bacteria samples were performed as follows. Overnight cultures of the bacteria (excluding *B. cereus*) at 36 ℃ were diluted with physiological saline solution to 10^{-7} concentration. The overnight culture

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medium used for *E. coli* O157, *L. monocytogenes*, and S. Enteritidis was trypto-soy broth, and that for *C. perfringens* was Gifu anaerobic medium (GAM, Nissui, Tokyo). *B. cereus* was prepared as follows. After culturing *B. cereus* on a trypto-soy agar plate (TSA, Nissui) overnight at 36 ℃, the plate was further incubated for a few more days at room temperature. From the colonies on the plate, a bacterial suspension was prepared in pasteurized physiological saline solution, heated at 70 ℃ for 20 min, and then immediately cooled. The suspension was stored in a 0.5-ml micro-tube at -20 ℃ prior to use. Before use, the stock solution was diluted to 10^{-6} concentration.

TSA was used as the culture medium for *B. cereus, E. coli* O157, *L. monocytogenes*, and *S.* Enteritidis. Clostridia Count Agar (Nissui) was used as the culture medium for *C. perfringens*.

Sample preparations (three samples at the dose level) in pouch packs were performed as follows. Ten ml of the bacterial suspension and 15 ml of the sterilized medium at approximately 50 ℃ were mixed in a pouch pack (pouch pack for culture of anaerobic bacteria, polyethylene terephthalates, thickness 30 μm plus polyethylene, thickness 20 μm, Sakami Ika, Osaka). The mixtures were sealed by a heat sealer and were left to stand overnight at 4 ℃. These cells were resting cells, when they were irradiated. The thickness and the diameter of the pouch packs were 5 mm and 94 mm, respectively, when the packs were filled with the above-mentioned mixture. The number of bacteria just before irradiation was represented by colony number of un-irradiated bacteria pack. Each bacterium grew up in the agar in the pouch pack to form a colony by incubating overnight at 36 ℃.

For the studies on the effect of gamma ray irradiation on bacterial survival without pre-incubation at 4 ℃ (as growing cells), freshly prepared packs were also irradiated at room temperature. Other conditions were the same as those described above.

Gamma ray irradiation (Source ^{60}Co at 2 kGy/h) and E-beam irradiation (5 MeV, 36 MGy/h) with pre-incubation at 4 ℃ were performed at 4 ℃ in pouch pack (slightly permeable to air) without big air bubble. On the other hand, samples without pre-incubation at 4 ℃ were irradiated at room temperature. The doses applied were approximately 0, 0.5, 1.0, and 1.5 kGy. The levels of applied doses of E-beam were controlled by cart speed.

Dosimetry was performed according to the methods of Miyahara et al.[6]. The absorbed dose was measured using a Gammachrome YR PMMA dosimeter (AEA, UK) for gamma ray irradiation and Radiachromic dosimeter (Far West Technology, USA) for E-beam irradiation. Each dosimeter of calibration was performed by the National Physics Laboratory, UK, and was traceable to the source. The power of E-beam was measured by the method of ASTM[7]. For measurement of absorbed dose of gamma irradiation, dosimetry was performed on the irradiated surface on each sample. For that of E-beam, dosimetry was performed at three points on a cart surface at each dose level.

Determinations of bacterial survival were performed as follows. Irradiated samples were cultured overnight at 36 ℃. The effects of irradiation on bacterial survival were measured by determining the colony count in media. Cell concentration in the bacterial suspension was expressed as CFU (colony-forming unit(s)).

2) Irradiation of ground beef

Sample preparations of ground beef patties with bacteria for E-beam irradiation were performed as follows. Ground beef patties (10 g, 52 mm in diameter and 3 mm in thickness) inoculated with bacteria (*E. coli* O157, *L. monocytogenes*, and *C. perfringens*) were wrapped. They were arranged in a pile (3 mm thickness of each patty) and were irradiated at 1 kGy with 5 MeV E-beam at 4 ℃. The inoculation was carried out as follows. An overnight-culture diluted with physiological saline solution was used for inoculation. Each concentration of bacteria was calculated by plating, culturing overnight at 36 ℃, and measuring colonies (TSA for *E. coli* O157 and *Listeria* under aerobic conditions, Clostridium welchii agar (CW agar) (Eiken, Tokyo) under anaerobic conditions). The inoculation doses were 3700, 600, and 165 cells, respectively. A 5-mm-diameter paper tab was put on each patty and permeated with each bacteria 50 μl solution. Three sets of 9 folded patties with bacteria were irradiated and CFU were showed as the mean value of three data. The ground beef patties were kept at 4 ℃ during preparation, overnight pre-incubation, irradiation and overnight post-incubation.

Detections of bacteria in irradiated beef patties were performed as follows. The patties were homogenized in 90 ml physiological saline solution using a homogenizer. Ten ml of the homogenate and 15 ml of the sterilized medium at approximately 50 ℃ were mixed in a pouch pack, and the mixture was incubated overnight at 36 ℃. CHROMagar O157 TAM (CHROMagar, Paris), PALCAM agar (Merck, Darmstadt), and Clostridia Count Agar were

used for the detection of *E. coli* O157, *L. monocytogenes* and *C. perfringens*, respectively. These selective media showed the colonies colored violet, black, and black, respectively. Colonies that grew in the medium in the pouch pack were counted and used as a measure of bacterial survival.

Dosimetry of absorbed dose of E-beam was measured on each surface of beef patty. Details about dosimetry of E-beam described above. Absorbed dose of each patty, the mean dose was calculated by absorbed doses on each surface of two patties.

Statistical analyses were performed as follows. The means of triplicate pack counts and population reduction data were analyzed using T-test, and D_{10} values were calculated by fitting the regression line, respectively. D_{10} value is the amount of radiation required to reduce the population of a specific bacterium by 90% (1 $\log_{10}$ cycle) under the stated conditions[8].

Results and Discussion

The effects of the gamma ray and E-beam irradiations were compared with the dose-population plots of each bacterium as shown in Fig. 1. The population of *B. cereus* with E-beam irradiation was reduced more sharply with dose increase than that with gamma irradiation. Although the population of bacteria (*E. coli* O157, 1741.7 CFU/10 ml) in the sample for E-beam irradiation was larger than that (878.0 CFU/10 ml) for gamma irradiation before irradiation, the population in sample irradiated by E-beam was less than that by gamma ray at any dose we examined. This means that E-beam irradiation reduced the bacteria population more effectively than gamma irradiation but without any statistical difference. As to *L. monocytogenes*, S. Enteritidis, and *C. perfringens*, we could not conclude which irradiation was exceeding by the data of Fig.1. The smaller reductions of Bacteria (*B. cereus*, *E. coli* O157, S. Enteritidis, and *C. perfringens*) were observed at low dose (0.5 kGy) by gamma-irradiation than those by E-beam.

The effects of these irradiations were also compared by D_{10} values. The results are shown in Table 1. When *B. cereus* were incubated overnight at 4 ℃ before irradiation, E-beam irradiation was significantly more effective than gamma-ray irradiation in decreasing the D_{10} value. There were statistically significant differences between the means of CFU with gamma ray and E-beam irradiations (Table 1). These results are accord with the conclusions of following reports. Tallentire reported that E-beam irradiation of *B. megaterium* was more effective than gamma irradiation at anaerobic condition[9]. Sekiguchi et al. also reported that gamma irradiation at anaerobic condition was less effective than E-beam irradiation to *B. pumilus* and *B. globigii*[10]. However, 90% cells of *B. cereus* in the pouch were heat-unstable (60 ℃, 10 min). It meant that these cells were not spore type. We need to perform further studies why gamma irradiation was not effective to the Bacillus in anaerobic condition. Other bacteria did not show remarkable differences of the efficacy between two kinds of irradiations.

Without incubation at 4 ℃, D_{10} values of three kinds of bacteria (*B. cereus*, *E. coli* O157 and *L. monocytogenes*) were reduced by gamma ray irradiation comparing to that of bacteria with incubation overnight at 4 ℃, as shown in Table 1. When bacterial suspensions were prepared in the pouch packs with hot media (50 ℃), all the bacteria might appear to be in the log phase of growth. On the other hand, these bacteria in the pouch packs incubated overnight at 4 ℃ might be resting cells. Therefore, the cell cycle of bacteria during irradiation appears to be crucial for the effects of irradiation on bacterial survival.

In order to examine depth-survival relation in food sample, ground beef patties were inoculated with bacteria and were irradiated with 1 kGy E-beam. Bacteria in the patties homogenates were cultured in a pouch pack. The results are shown in Fig. 2. The range (depth where dose is equivalent to that at the surface) is 9.8 mm.

In the first, second, and third patties, the irradiation effect on bacterial survival rate was evident. In the first patty, no survival bacteria were found. In the second and third patties, few survivors were observed. The absorbed doses at the center of 1st, 2nd and 3rd patties were 1.03, 1.13, and 1.18 kGy, respectively. These patties were sufficient to reduce most of bacteria population, respectively. The absorbed doses at the center of 4th, 5th and 6th patties were 0.95, 0.41 and 0.05 kGy, respectively. As shown in Fig. 2, the populations of *E. coli* O157 and *L. monocytogenes* in the fourth patty were 1757 and 140 CFU/10 g, respectively. The mean CFU were larger than those expected by the D_{10} values of them and the absorbed doses in the fourth patty. In the fourth patty (boundary between irradiated and un-irradiated or below effective dose), many black colonies appeared in the Clostridia pouch pack. Although all black colonies that appeared in Clostridia Count Agar were not necessarily anaerobic bacteria, these many black colonies (5037 black colonies) were not only the inoculated anaerobic bacteria

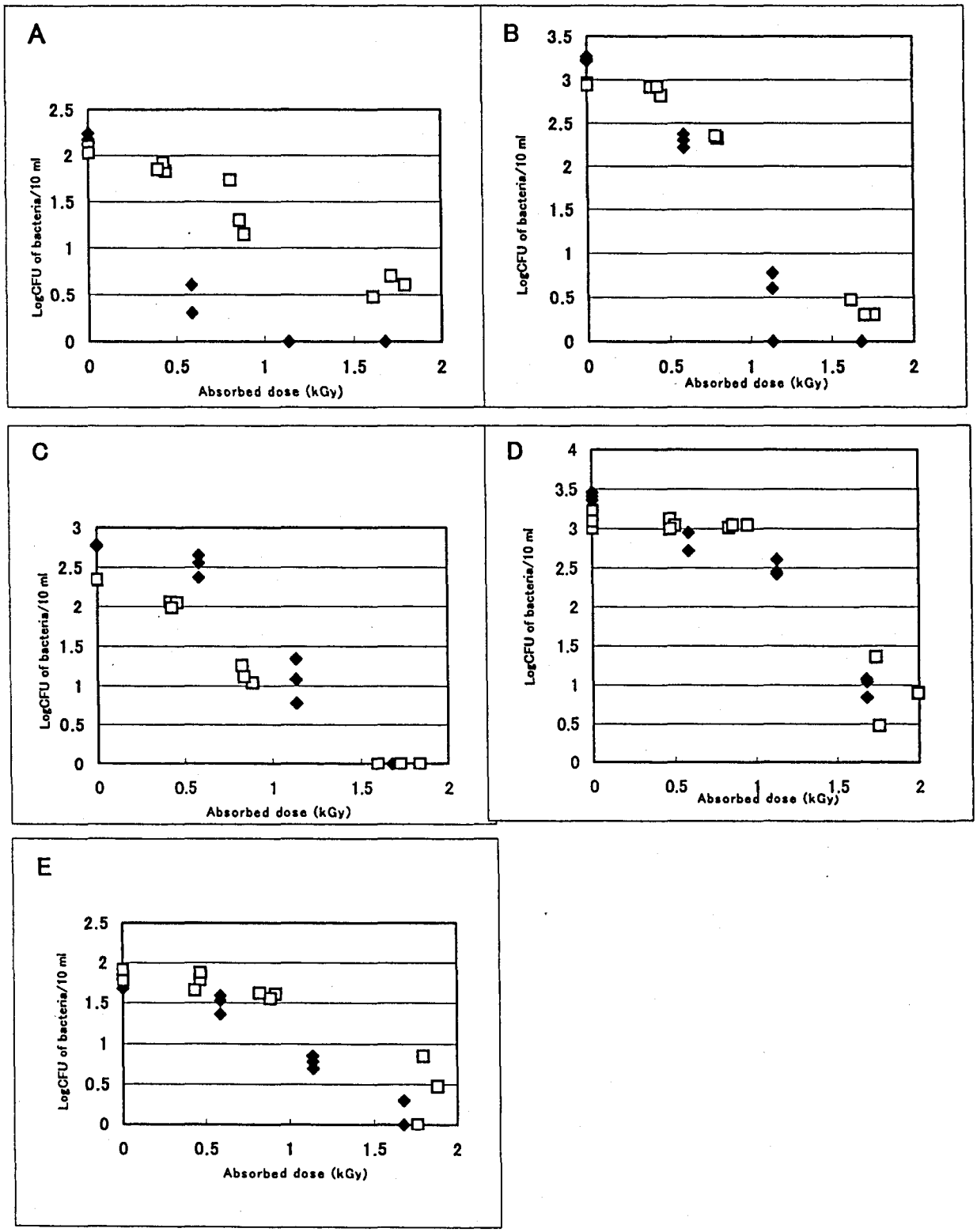

Fig. 1. Reduction of bacterial populations by irradiation

These samples were incubated overnight at 4 ℃ before irradiation. Populations of bacteria are shown as □(gamma irradiation) and ◆(E-beam irradiation). To facilitate log analysis, CFU values of zero were assigned a values of 0.001. A: Irradiation of *B. cereus*. B: Irradiation of *E. coli* O157 NVT. C: Irradiation of *L. monocytogenes*. D: Irradiation of *S.* Enteritidis. E: Irradiation of *C. perfringens*.

Legend to TABLE 1. Comparison of D10 (kGy) of pathogenic bacgteria with gamma-ray or E-beam irradiation and with or without incubation before gamma-ray irradiation at 4 ℃.

a) D10 (kGy) values were calculated by fitting the regression line. b) Bacteria incubated overnight at 36 ℃, packed in pouch pack, incubated overnight at 4 ℃ and irradiated c) Bacteria incubated overnight at 36 ℃, packed in pouch pack and irradiated soon. d) Statistically significant difference ($\alpha > 0.05$) between the mean of CFU with gamma-ray irradiation and that with E-beam at the similar dose.

Bacterium	With incubation overnight at 4 °C before irradiation[b]		Without incubation at 4 °C before irradiation[c]
	Gamma ray irradiation	E-beam irradiation	Gamma ray irradiation
B. cereus	1.12[a]	0.33*[d]	0.47
E. coli O157	0.5	0.48	0.22
L. monocytogenes	0.47	0.43	0.32
S. Enteritidis	0.46	0.36	0.53
C. perfringens	0.98	0.78	1.68

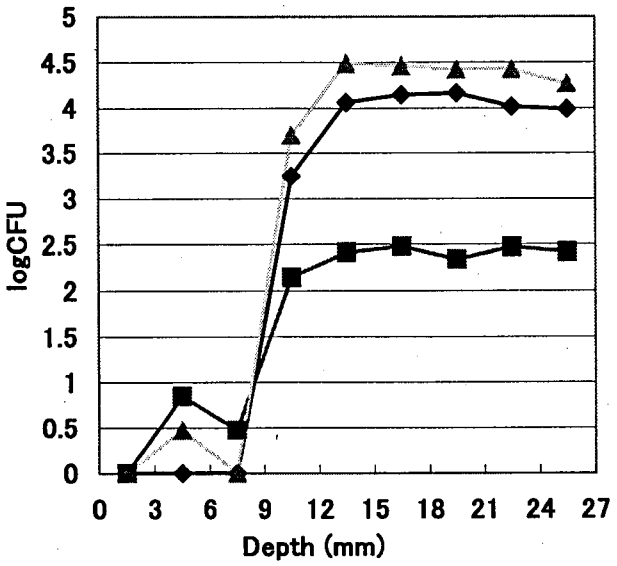

Fig. 2. Depth vs. CFU profiles in ground beef patties

Ground beef patties (10 g, 52 mm in diameter and 3 mm in thickness) inoculated with bacteria [◆ *E. coli* O157, (3700 cells), ■ *L. monocytogenes*, (600 cells), and ▲ *C. perfringens*, (165 cells)] were wrapped with Saran wrap. They were arranged in a pile (3 mm thickness of each patty, 9 piles thick) and were irradiated at 1 kGy with 5 MeV E-beam at 4 ℃. After irradiation, the numbers of bacteria in the patties were detected as colored colonies in selective agar after cultivation overnight.

(165 cells inoculated), but also originally present ones (resident bacteria) in the beef patty. At the bottom of the fourth patty, absorbed dose (0.73 kGy) were insufficient to reduce the population of *C. perfringens*. But these results could not be expected enough from the D_{10} value in Table 1 and the absorbed dose. After irradiation, the action of the repairing enzyme requires induction time at a particular temperature in survival bacteria[11]. The long incubation period at 4 ℃ may facilitate the recovery and growth of anaerobic bacteria. Therefore, storage condition of food after irradiation affects survival of contaminated bacteria in food.

In conclusion, comparing gamma-ray irradiation, E-beam irradiation of the pouch packs was remarkably effective in reducing the survival rates of *B. cereus*, slightly in that of *E. coli* O157, and even in that of *L.*

monocytogenes, *S.* Enteritidis and *C. perfringens* with incubation overnight at 4 ℃ before irradiation. With gamma irradiation, growing bacteria without incubation at 4 ℃ before irradiation were more easily damaged than resting cells with incubation overnight at 4 ℃ before irradiation. E-beam irradiation (1 kGy, 36 MGy/h, 5 MeV) of ground beef patties inoculated with bacteria decreased bacterial survival rate in a certain area (0-9 mm thickness of beef patty). In the boundary area (9-12 mm), however, bacteria sensitive to irradiation were decreased, while population of resistant bacteria inoculated and originally present increased. From these results, in order to ensure that food is sufficiently irradiated with E-beam, absorbed dose and anaerobic bacterial growth should be checked both on the surface and at the bottom of sample.

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