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ORIGINAL ARTICLE

Effect of gamma radiation on heat shock protein expression of four foodborne pathogens

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Abstract

Aims: The effects of gamma radiation on three heat shock proteins (Hsps) (GroEL, DnaK and GroES) synthesis in two Gram-negative (*Escherichia coli* and *Salmonella* serotype Typhimurium) and two Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) bacteria were investigated.
Methods and Results: The bacterial strains were treated with three radiation doses to induce cell damage, to obtain a viable but nonculturable state, and to cause cell death. Western blot analysis and quantification of Hsps in bacteria were performed immediately after irradiation treatment. In the four foodborne pathogens, GroEL was strongly induced by gamma rays in a dose-dependent manner, confirming the involvement of this protein in the cellular response to the stress generated by ionizing radiation. In addition, it was found that *E. coli* exposed to gamma radiation showed a significantly induction of DnaK and GroES proteins when compared with nonirradiated bacteria, whereas a GroES slight induction and a DnaK inhibition were observed in *Salm.* Typhimurium.
Conclusions: The gamma rays influence the synthesis of Hsps in foodborne pathogen in a way that critically depends on the radiation dose.
Significance and Impact of the Study: The study of stress response to several radiation doses was undertaken to elucidate how bacteria can survive in harsh conditions and cope with gamma radiation used to control foodborne pathogens and to characterize their adaptative response to this treatment.

Introduction

All organisms respond to environmental stresses by a rapid and transient acceleration in the rate of synthesis of a group of proteins collectively called as heat shock proteins (Hsps) (Kvint *et al.* 2003). These stresses include heat shock, cold shock, osmotic shock, starvation, amino acid analogues, antibiotics, heavy metals, ultraviolet radiation, gamma radiation and alcohols. The cellular response to stress represented at the molecular level by the induced synthesis of Hsps protects cells and organisms against oxidative stress and often prevents cell death (Park *et al.* 2000). While prolonged exposure to conditions of extreme stress is harmful and can lead to cell and tissue death, induction of Hsps synthesis can result in stress tolerance. The molecular genetics of the heat shock response has been most extensively studied in *Escherichia coli* (Yura *et al.* 2000). Until now, more than 30 proteins associated with the physiological response to stress have been identified. Classical Hsps are the molecular chaperones (e.g. DnaK, GroEL, GroES and their cohorts). These proteins play a role in protein folding, assembly, repair and prevention of aggregation under stress and nonstress conditions. The chaperones act with proteases to maintain quality control of cellular proteins (Gottesman *et al.* 1997).

Gamma radiation processing of foods is being investigated in order to ensure the microbial safety and to extend the shelf life of food while avoiding the adverse effects associated with thermal processing (Barbosa-Canovas *et al.*

1997). ^{60}Cobalt produces electromagnetic gamma rays, which are similar to visible light but have higher energy. During radiation treatment, DNA molecules are heavily damaged, preventing them from functioning normally. Ionizing radiation is a physical agent that targets DNA molecules either via direct interaction or via production of free radicals and reactive oxygen species (ROS). Bacterial cells exposed to radiation will normally react in different ways: arrest cell cycle progression and repair of DNA lesions (Lee *et al.* 2001). As a result, the micro-organisms that have been affected are no longer capable of dividing (Urbain 1986). However, in eukaryotic cells, DnaK is shown to be involved in ionizing radiation-activated processes, probably because of ROS production, and DnaK induction can lead to the development of a radio-adaptative response (Park *et al.* 2000). As gamma radiation is among the most potentially harmful environmental factors, it appears reasonable to question whether Hsps are involved in protection of bacteria from the deleterious effect of gamma radiation. It is under this aspect that has turned our interest towards investigation of Hsps reaction and its significance in the response of pathogenic bacteria to gamma radiation.

The study of *E. coli, Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes* response to increasing radiation doses was undertaken to elucidate how these bacteria can survive in harsh conditions and cope with gamma radiation used to control foodborne pathogens and to characterize their adaptative response to this treatment. DnaK, GroEL and GroES expressions in response to irradiation with gamma rays at different doses were thus studied to confirm Hsps induction by ionizing radiation.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli O157:H7 strain EDL933, *Salm.* Typhimurium SL1344 strains (Institut National de la Recherche Scientifique, Institut Armand Frappier, Laval, Québec, Canada), *Staph. aureus* ATCC 29213 (American Type Culture Collection, Rockville, MD, USA) and *L. monocytogenes* HPB 2812 1/2a (Health Canada, St-Hyacinthe, Québec, Canada) were individually subcultured [1·0% (v/v)] in brain–heart infusion (BHI; Difco, Becton Dickinson, Sparks, MD, USA) broth at 37°C from the stock cultures maintained at −80°C in BHI containing 10% glycerol. One millilitre of each culture was incubated through two successive incubations of 24 h at 37°C in BHI broth (9 ml) to obtain working cultures containing approx. 10^9 CFU ml^{-1}. The cultures were centrifuged at 1300 ***g*** for 15 min and washed with sterile NaCl 0·85% (w/v) and then resuspended in BHI broth (500 ml) and incubated at 37°C for 24 h under stirring. After the last incubation of 24 h, the bacterial cultures were irradiated according to suitable dose. In order to obtain a viable but nonculturable (VBNC) state after irradiation (Rigsbee *et al.* 1997), cell cultures of *E. coli*, *Salm.* Typhimurium and *Staph. aureus* were then incubated at 37°C for 5 days, 24 h and 5 days, respectively, to permit the restoration of metabolic activity.

Irradiation

Preliminary tests were conducted to determine the VBNC and lethal doses of irradiation for each bacterium in TSB (Caillet *et al.* 2005; Caillet and Lacroix 2006). The bacterial cultures of *E. coli* (500 ml) were irradiated with doses of 0·36–0·44 kGy (mean, 0·40 kGy) to create damaged cells, 1·07–1·15 kGy (mean, 1·1 kGy) to obtain cells in the VBNC state and 1·26–1·34 kGy (mean, 1·3 kGy) to kill cells (Caillet *et al.* 2005). The bacterial cultures of *Salm.* Typhimurium (500 ml) were irradiated with doses of 0·71–0·79 kGy (mean, 0·75 kGy) to create damaged cells, 2·46–2·54 kGy (mean, 2·5 kGy) to obtain cells in the VBNC state and 3·46–3·54 kGy (mean, 3·5 kGy) to kill cells. The bacterial cultures of *Staph. aureus* (500 ml) were irradiated with doses of 1·16–1·24 kGy (mean, 1·20 kGy) to create damaged cells, 2·86–2·94 kGy (mean, 2·90 kGy) to obtain cells in the VBNC state and 3·46–3·54 kGy (mean, 3·50 kGy) to kill cells. The bacterial cultures of *L. monocytogenes* (500 ml) were irradiated with doses of 1·17–1·23 kGy (mean, 1·2 kGy) to create damaged cells and 3·46–3·54 kGy (mean, 3·5 kGy) to kill cells (Caillet and Lacroix 2006). A UC-15B irradiator (MDS Nordion International Inc., Kanata, Ontario, Canada) equipped with ^{60}Cobalt source was used to deliver radiation at a dose rate of 4·6 kGy h^{-1}. This irradiator was certified by the National Institute of Standards and Technology (Gaithersburg, MD, USA), and the dose rate was established using a correction for decay of source. Amber Perspex 3042D (Atomic Energy Research Establishment, Harwell, Oxfordshire, UK) were used to validate the dose distributions. The irradiation treatment was carried out at the Canadian Irradiation Centre (Laval, Québec, Canada) at room temperature (20°C).

Protein extraction

Immediately after irradiation or after the restoration of metabolic activity for VBNC state, 500 ml of each bacterial culture were quickly chilled in an ice/ethanol bath until the temperature dropped below 10°C. Cells were harvested by centrifugation for 15 min at 8670 ***g*** at 4°C and the resulting pellet was washed in 10 ml of phosphate

buffer saline (NaCl, 1·37 mol l^{-1}; KCl, 2·7 mmol l^{-1}; KH_2PO_4, 1·7 mmol l^{-1}; HNa_2PO_4, 0·1 mol l^{-1}; pH 7·4) at the same temperature. The bacterial pellets were then resuspended with 5 ml of buffer lysis [50 mmol l^{-1} Tris-HCl (pH 7·5); 0·1 mol l^{-1} NaCl; 0·5 mmol l^{-1} phenylmethanesulfonyl fluoride (PMSF); 1 mg ml^{-1} iodoacetamide] and cell walls were then broken using a cell disrupter (FastPREP, model FP 120, Qbiogene Inc., Carlsbad, CA, USA) containing glass beads (0·2 mm) and regulated at speed 6 for 30 s (2 × 30 s, with a 30-s waiting period between the operations) at 4°C. The suspension was centrifuged at 2000 ***g*** for 10 min to remove the glass beads and the unbroken cells. Then, the resulting supernatant, containing the proteins, was harvested and 5 ml of Tris-HCl 50 mmol l^{-1} and 0·1 mol l^{-1} NaCl were added. Finally, the suspensions were stored at −20°C.

Protein dosage

To determine the concentration of protein extracted in the four bacteria, the protein quantification was carried out on a 250-μl aliquot of the suspension using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's specifications. Absorbance was read at 562 nm with a DMS 100S spectrophotometer (Varian Canada Inc., Mississauga, ON, Canada). Standard curve of BSA was used.

Polyacrylamide gel electrophoresis and Western blotting (immunoblotting)

An aliquot of the suspension (1 mg of protein per ml) was diluted with LDS sample buffer (1×) and sample reducing agent (1×) (NuPage, Invitrogen Inc., Burlington, ON, Canada) to obtain 50 μg of total protein per well. Proteins contained in the bacterial cell extracts were separated by using sodium dodecyl sulfate–10% polyacrylamide gels in a Xcell SureLock System (Invitrogen Inc.) with the molecular size standard (MultiMark Multi-Colored Standard, Invitrogen Inc.) containing proteins of 185, 94, 52, 31, 19, 17, 11, 6 and 3 kDa. The migration of the gels was carried out at 200 V during 45 min and the colouration of the gel was carried out using Coomassie blue (Simply Blue SafeStain, Invitrogen Inc.). After electrophoresis, the proteins extracted from the four bacteria were transferred onto a nitrocellulose membrane Hybond™-ECL™ (Amersham Biosciences, Oakville, ON, Canada), and processed for immunoblotting in a Xcell II Blot Module (Invitrogen Inc.). Proteins were electroblotted at 30 V for 1 h. The membrane was blocked with 3% skim milk for 1 h at room temperature. Blots were then incubated with either GroEL rabbit polyclonal antibody (Sigma-Aldrich, Oakville, ON, Canada) conjugated with peroxidase, DnaK mouse monoclonal antibody (Stressgen, Victoria, BC, Canada) and GroES rabbit polyclonal antibody (Stressgen) raised against corresponding proteins produced by *E. coli*. For DnaK and GroES proteins, immunocomplexes were incubated with sheep anti-mouse peroxidase and donkey anti-rabbit peroxidase (Stressgen), respectively. Immunocomplexes were then visualized with kit ECL Western blotting analysis system (Amersham Biosciences). The films (Konica Minolta, Mississauga, ON, Canada) are then exposed on the membrane in a darkroom and developed with a mini-med/90 X-ray film processor (AFP Imaging Corporation, Elmsford, NY, USA). The resulting bands were analysed and quantified with an Alpha Imager™ IS-3400 (Alpha Innotech Corporation, San Leandro, CA, USA) for Windows 2000 using the standard curves. Results were expressed as micrograms of Hsps per mg of total proteins. A standard curve of 100, 50, 25 and 12·5 ng per well; 100, 50, 25 and 12·5 ng per well; 15, 10, 5 and 2 ng per well was carried out for GroEL, DnaK and GroES, respectively, with *E. coli* and a standard curve of 100, 50, 25 and 12·5 ng per well; 300, 150, 75 and 30 ng per well; 50, 25, 15 and 7 ng per well was carried out for GroEL, DnaK and GroES, respectively, with *Salm.* Typhimurium. A standard curve of 100, 50, 25 and 12·5 ng per well was carried out for GroEL with *Staph. aureus* and *L. monocytogenes.* Antibodies against *E. coli* proteins failed to recognize DnaK and GroES in *L. monocytogenes* and *Staph. aureus*.

Statistical analysis

An analysis of variance and Duncan's multiple range tests were employed to analyse the effects of irradiation on GroES, GroEL and DnaK concentration. Differences between means were considered significant at $P \leq 0{\cdot}05$. Stat-Packets Statistical Analysis software (SPSS Base 10.0, SPSS, Inc., Chicago, IL, USA) was used for the analysis. For all response variables, analysis was performed in duplicates and the experiments were repeated three times.

Results

Escherichia coli O157 : H7 cells were irradiated with different doses of gamma rays (0·4, 1·1 and 1·3 kGy) and subjected to GroEL, DnaK and GroES detection by Western blotting. Representative blots of Hsps levels in *E. coli* are shown in Fig. 1a. Quantification of GroEL, DnaK and GroES in cells nonirradiated and irradiated is shown in Fig. 1b. Results obtained with GroEL showed that protein expression in control was lower than in irradiated cells. GroEL concentration in control was 0·019 μg mg^{-1} of total proteins and significantly increased ($P \leq 0{\cdot}05$) in samples irradiated. At 0·4 kGy, GroEL concentration was

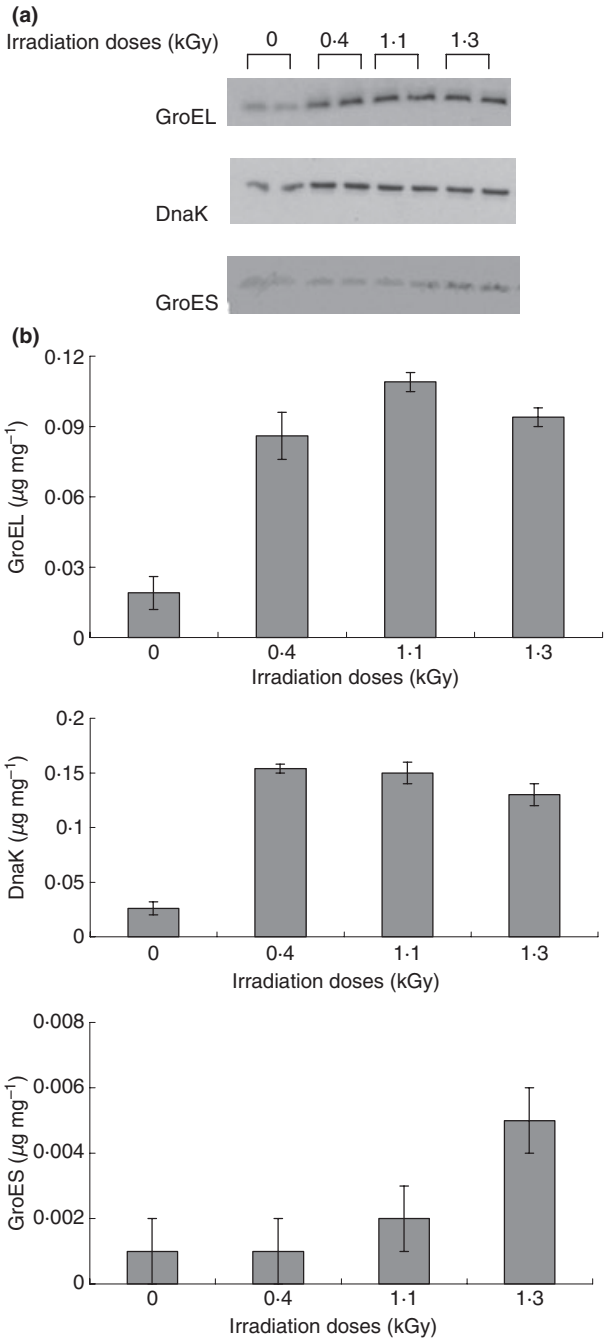

Figure 1 (a) Western blot analysis and (b) quantification of GroEL, DnaK and GroES in *Escherichia coli* O157 : H7 after gamma irradiation. Hsps levels in controls and irradiated samples at 0·4, 1·1 and 1·3 kGy are compared and shown in representative blots. Two replicates of each sample were tested. With regard to the quantification, results were expressed as micrograms of Hsps per mg of total proteins.

0·086 μg mg^{-1}, which represented a 4·52-fold increase in the expression of protein. The maximum induction was reached at 1·1 kGy with 0·109 μg mg^{-1}, representing a 5·73-fold increase in the GroEL expression. At 1·3 kGy, the GroEL level was 0·094 μg mg^{-1}, representing a 4·94-fold increase in the protein expression. Radiation treatment significantly increased ($P \leq 0{\cdot}05$) the DnaK expression regardless of the dose used. DnaK expression was 0·026 μg mg^{-1} in control and reached the maximum in samples irradiated at 0·4 and 1·1 kGy with 0·154 and 0·150 μg mg^{-1}, representing a 5·92- and 5·77-fold increase in the protein expression, respectively. At 1·3 kGy, the DnaK level was significantly lower ($P \leq 0{\cdot}05$) with 0·130 μg mg^{-1} than those in the other samples irradiated. This concentration represented a fivefold increase in the DnaK expression compared with control. The quantification showed that the GroES concentration was very low in *E. coli* O157:H7 cells. GroES expression was 0·001 μg mg^{-1} in control and 0·4-kGy sample. However, the highest radiation doses affected the GroES concentration. At 1·1 kGy, GroES concentration was 0·002 μg mg^{-1} and a fivefold increase ($P \leq 0{\cdot}05$) in the GroES expression was observed in 1·3-kGy sample with a value of 0·005 μg mg^{-1}.

Effect of gamma radiation at different doses (0·75, 2·5 and 3·5 kGy) on GroEL, DnaK and GroES expression in *Salm.* Typhimurium SL1344 is shown in Fig. 2a. Quantification of GroEL, DnaK and GroES in cells nonirradiated and irradiated is shown in Fig. 2b. GroEL concentration was 1·060 μg mg^{-1} in control. At 0·75 kGy, GroEL expression was not affected by the irradiation, as its concentration (0·915 μg mg^{-1}) was comparable with that of the control. On the contrary, a significant increase ($P \leq 0{\cdot}05$) in the GroEL expression was observed at 2·5 and 3·5 kGy with a value of 2·802 and 3·857 μg mg^{-1}, respectively. These concentrations were 2·64 and 3·63 times higher than that of the control. Radiation treatment significantly decreased ($P \leq 0{\cdot}05$) the DnaK expression regardless of the dose used. DnaK expression was 5·158 μg mg^{-1} in control and between 4·087 and 4·274 μg mg^{-1} in irradiated samples. An average 1·24-fold decrease in the DnaK level was observed in irradiated samples compared with control, but no significant difference ($P > 0{\cdot}05$) was observed between irradiated samples. The results showed that the GroES expression was affected differently depending on the radiation dose. GroES expression was 0·660 μg mg^{-1} in control and reached the maximum in samples irradiated at 0·75 kGy with 0·821 μg mg^{-1}, representing a 1·24-fold increase in the protein expression. However, a low concentration of GroES (0·323 μg mg^{-1}) was found at 2·5 kGy, representing a 2·06-fold decrease in the GroES level compared with the control, while GroES expression seemed not affected at 3·5 kGy, as its concentration (0·612 μg mg^{-1}) was comparable with that of the control.

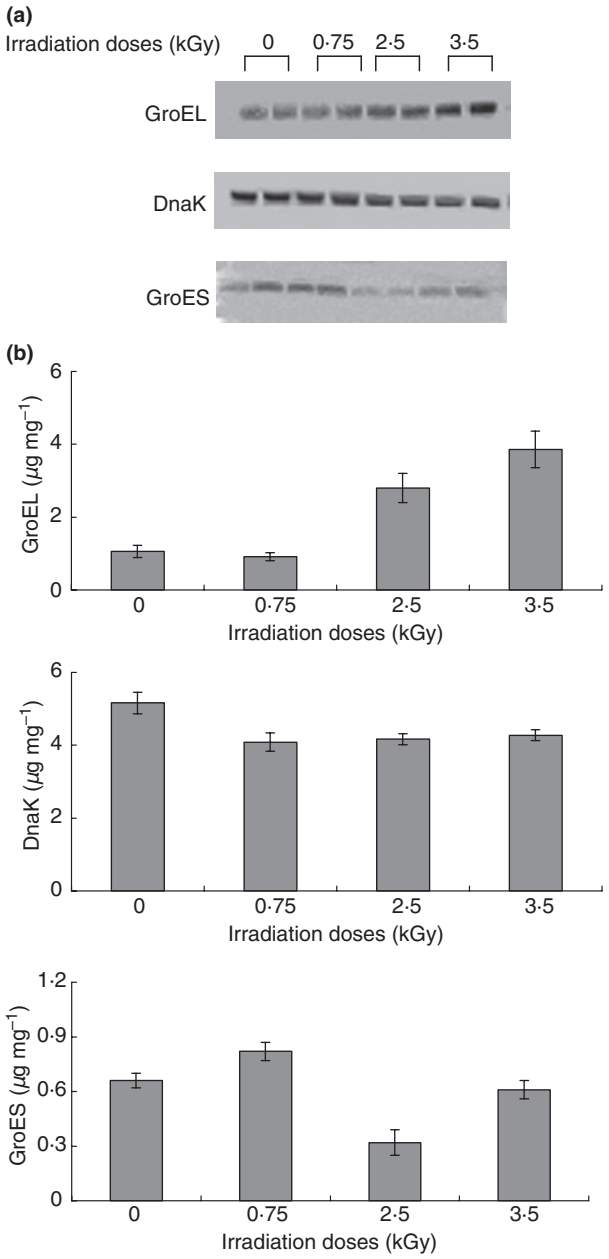

Figure 2 (a) Western blot analysis and (b) quantification of GroEL, DnaK and GroES in *Salmonella* Typhimurium SL1344 after gamma irradiation. Hsps levels in controls and irradiated samples at 0·75, 2·5 and 3·5 kGy are compared and shown in representative blots. Two replicates of each sample were tested. With regard to the quantification, results were expressed as micrograms of Hsps per mg of total proteins.

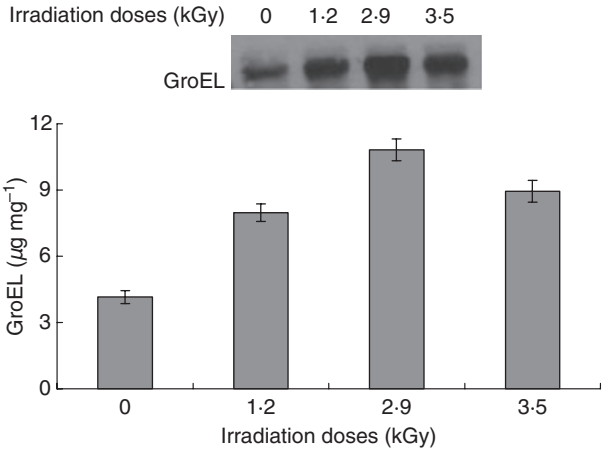

Figure 3 Western blot analysis and quantification of GroEL in *Staphylococcus aureus* ATCC 29213 after gamma irradiation. GroEL levels in control and irradiated samples at 1·2, 2·9 and 3·5 kGy are compared and shown in representative blots. With regard to the quantification, results were expressed as micrograms of Hsps per mg of total proteins.

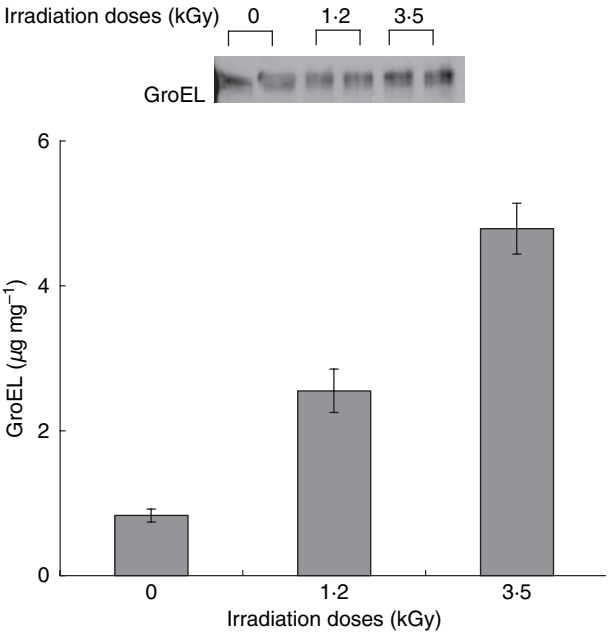

Figure 4 Western blot analysis and quantification of GroEL in *Listeria monocytogenes* HPB 2812 1/2a after gamma irradiation. GroEL levels in control and irradiated samples at 1·2 and 3·5 kGy are compared and shown in representative blots. With regard to the quantification, results were expressed as micrograms of Hsps per mg of total proteins.

Western blot analysis and quantification of GroEL in *Staph. aureus* ATCC 29213 and *L. monocytogenes* HPB 2812 1/2a after gamma irradiation are presented in Figs 3 and 4, respectively. Results showed that radiation treatment significantly increased ($P \leq 0{\cdot}05$) the protein expression in both bacteria. In *Staph. aureus*, GroEL expression was 4·152 μg mg^{-1} in control, whereas GroEL level in samples irradiated at 1·2, 2·9 and 3·5 kGy reached 7·981, 10·820 and 8·945 μg mg^{-1}, representing 1·92-, 2·60- and

2·15-fold increase in the protein expression, respectively. In *L. monocytogenes*, GroEL expression was 0·83 μg mg^{-1} in control and reached 2·555 and 4·797 μg mg^{-1} in 1·2- and 3·5-kGy samples, respectively. These concentrations were 3·07 and 5·77 times higher than that of the control, respectively.

Discussion

Several studies have shown that at least two families of Hsps, the DnaK and GroEL homologues, are intimately involved in protein processing and were in fact expressed constitutively in cells under normal conditions (Ellis and van der Vies 1991; Boutibonnes *et al.* 1993). We have shown that the basal level of DnaK, GroEL and GroES proteins was extremely variable according to the bacterial strain. In *Salm.* Typhimurium, these three proteins showed a very high basal level compared with *E. coli*. GroEL, DnaK and GroES concentrations were 56, 198 and 660 times higher in *Salm.* Typhimurium than that of *E. coli,* respectively. In addition, GroEL basal level in *L. monocytogenes* and *Staph. aureus* was 47 and 218 times higher than that of *E. coli,* respectively.

The Hsps cytoprotective function is not fully understood, although these proteins act as molecular chaperones or modulators of intracellular levels of ROS. Recently, Hsps have been proposed to play a significant role in DNA repair after UV or γ-ray irradiation (Park *et al.* 2000; Calini *et al.* 2003). There is evidence that the stress response is important for intracellular survival of foodborne pathogens; however, no previous studies have been conducted in order to identify and quantify stress proteins induced by gamma radiation in these microorganisms. In this study, we observed that in the four foodborne pathogens, GroEL was strongly induced by gamma rays in a dose-dependent manner confirming the involvement of this protein in the cellular response to stress which is induced by ionizing radiation (Fig. 5). However, the GroEL protein was more induced in *E. coli* and *L. monocytogenes* than in *Salm.* Typhimurium and *Staph. aureus,* which have synthesized this protein at a higher basal level than *E. coli* or *L. monocytogenes* and the higher baseline of synthesis could obscure the relatively smaller increase of synthesis after exposure in an hostile environment. A similar trend was observed in *Enterococcus faecalis* where GroEL has showed a higher basal level than in *E. coli* and a weaker increase after heat shock (Boutibonnes *et al.* 1993). In addition, results revealed in *E. coli* that gamma rays triggered a significant induction of synthesis of DnaK and GroES proteins, and hence these proteins are also involved in the response to stress generated by gamma radiation (Fig. 5). Maximum

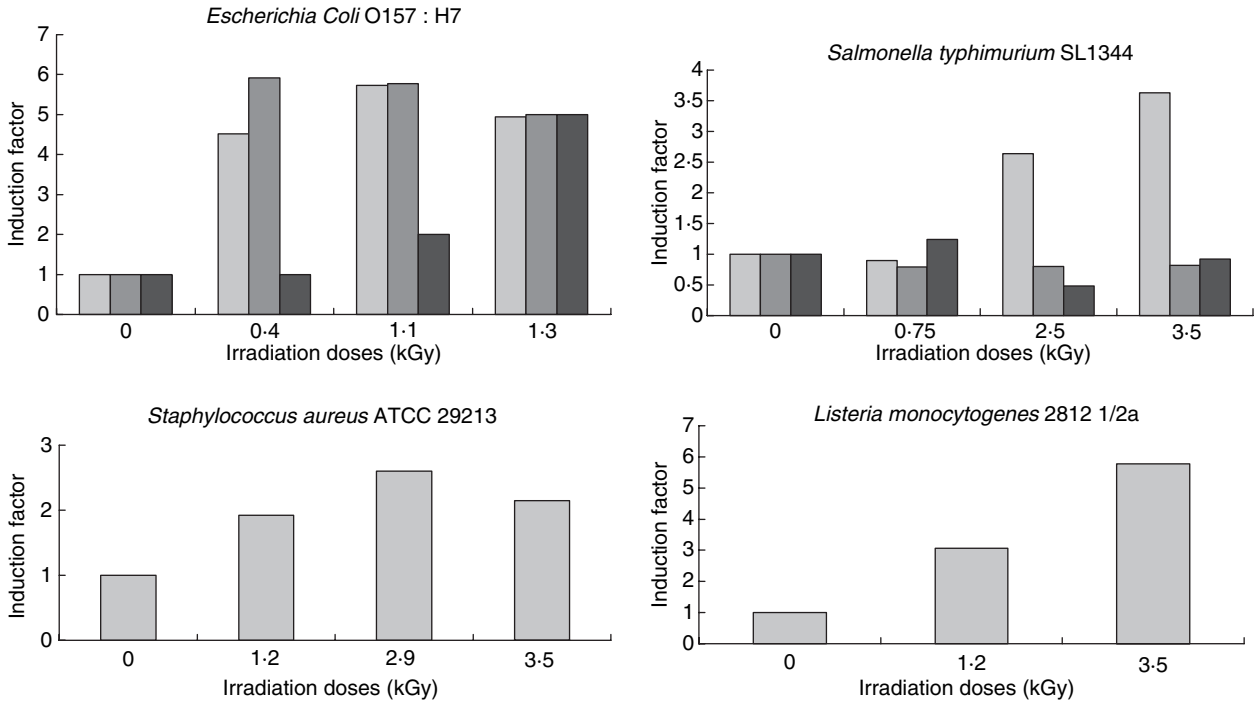

Figure 5 Induction profile of Hsps after gamma-radiation treatment of four foodborne pathogens. The induction factor is calculated as the ratio of the mean quantification value for each irradiated cell to the mean quantification value for corresponding control. Figure caption: (□) GroEL, (▒) DnaK and (■) GroES.

induction of DnaK and GroES correlated with irradiation dose and showed stronger expression when higher doses were used. Verbenko and Kalinin (1995) showed that several Hsps were induced by gamma rays in *E. coli* K-12 in which the SOS responses are constitutively expressed, and an interaction of the two stress systems in bacterial cells is possible. In addition, DnaK has been reported to be induced by gamma rays in eukaryotic cells (Sierra-Rivera *et al.* 1993) to induce radioresistance (Park *et al.* 2000) and to prevent radiation-induced cell death (Lee *et al.* 2001). According to Calini *et al.* (2003), DnaK overexpression acts as a radioprotective mechanism towards the first event of DNA damage and increases long-term viability.

The results showed different levels of induction between proteins in *Salm.* Typhimurium (Fig. 5). GroEL protein was strongly induced, whereas GroES expression was very low and DnaK expression was repressed. In the few bacterial strains studied in literature (*E. coli, Lactobacillus*), GroEL and GroES genes are organized into operon (Arsène *et al.* 2000; Lim *et al.* 2001). The genetic organization of *Salm.* Typhimurium should probably be similar, and the differential induction that was observed for GroES and GroEL proteins suggests a post-transcriptional regulation mechanism. A regulation of GroESL mRNAs has already been observed in *Agrobacterium tumefaciens* (Segal and Ron 1998). In *Salm.* Typhi, heat shock treatment induced an inhibition of DnaK expression, while its basal level was very high (Tang *et al.* 1997). A study is needed to clarify the inhibition of DnaK expression by gamma rays; however, according to Calini *et al.* (2003) DnaK level could be a function of time and irradiation dose.

The lethal dose used in our study is the lowest radiation dose required to inhibit completely the growth of the four bacteria (i.e. the minimum inhibitory dose). Although all the cells were killed at this radiation dose, we observed a substantial increase in the synthesis of the three Hsps proteins in *E. coli* and GroEL protein in the three other bacteria. This may be related to the fact that Hsps induction occurs very rapidly, and that it is detectable even after 5–10 s of exposure in a hostile environment (Tang *et al.* 1997). Irradiator was used to deliver radiation at a dose rate of 4·6 kGy h^{-1} and, therefore, the treatment lasted several minutes to reach the lethal dose.

Bacteria in the VBNC state have a system of repair specific to each strain that enables them to survive and multiply after a recovery period (Olivier *et al.* 1991). Irradiation at 1·1 kGy followed by 5 days of incubation allowed *E. coli* O157 : H7 to reach the VBNC state (Caillet *et al.* 2005), while irradiation at 2·5 and 2·9 kGy followed by 24 h and 5 days of incubation allowed *Salm.* Typhimurium and *Staph. aureus* to restore of metabolic activity, respectively (unpublished data). However, no radiation dose has succeeded in restoring the metabolic activity of *L. monocytogenes* (Caillet and Lacroix 2006). The high Hsps level observed in irradiated bacteria to obtain cells in the VBNC state could be the determining factor of cell survival during the recovery period.

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