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Radicidation for Elimination of Salmonellae in Frog Legs

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ABSTRACT

The D_{10} values of three *Salmonella* spp. often encountered in frog legs, i.e., *S. typhimurium, S. enteritidis* and *S. newport*, were found to be between 18 to 30 krad when cells were irradiated at 0-2°C in 0.1 M phosphate buffer, pH 7.0. The radiation sensitivities of these *Salmonella* spp. increased only marginally when cells were irradiated in frog leg homogenate. The doses of radiation required for eradication of this pathogen in fresh and frozen frog legs were 300 and 400 krad, respectively.

India is the world's largest producer of frog legs, the annual production being about 3,500 tons. Most of the production is exported to the U.S.A. and France. In recent years, a major problem facing the frog leg industry is the frequent contamination of these samples with *Salmonella*, owing to inadequacy of the conventional method (treatment with 500 ppm chlorine water) used for elimination of this enteric pathogen. Studies in our laboratory have revealed the efficacy of utilizing gamma radiation doses in the range of 0.1 - 0.25 Mrad for extending the shelf-life of seafoods through selective destruction of spoilage bacteria (*3-5*). Other investigators have reported (*6,10*) the use of radiation doses in the range of 0.3 - 1.0 Mrad for eradicating salmonellae in egg products, horse meat, desiccated coconut and animal feeds. Attempts were, therefore, made to develop a radicidation process for eliminating salmonellae from fresh and frozen frog legs. The present communication reports on the sensitivity of three species of *Salmonella* to gamma radiation in buffer and in frog leg homogenate, and the development of a radicidation process for destruction of salmonellae in fresh and frozen frog legs.

MATERIALS AND METHODS

Microorganisms

Salmonella typhimurium, Salmonella newport and *Salmonella enteritidis* originally isolated from frog legs, were used in this study.

Preparation of cell suspensions

The three *Salmonella* spp. were grown individually in nutrient broth (Difco) for 18 h, centrifuged, washed with 0.1 M phosphate buffer, pH 7.0, and suspended in the same buffer at levels of approx. 10^8 cells/ml.

Preparation of frog leg homogenates

Portions (25 g) consisting of muscle were removed from at least 4-5 frog leg samples and placed in 225 ml of lactose broth. The samples were blended for 90 s at high speed (approx. 14,000 rpm) in a Waring Blendor and the homogenates were poured in sterile flasks. The *Salmonella* count of these homogenates was assessed by the most probable number (MPN) method (*1*).

Radiation sensitivity studies

Buffered cell suspensions of *S. typhimurium, S. newport* and *S. enteritidis* were irradiated in ice in a Gamma Cell 220 (AECL, Canada) at doses ranging from 50 to 250 krad and a dose rate of 180 krad/h. In another set of experiments, cell suspensions of the above three *Salmonella* spp. were added to heat-sterilized frog leg homogenate (10%) at a level of 1×10^7 cells/ml, dispensed in test tubes (15 × 2 cm), placed in a beaker containing crushed ice and irradiated at 0-2°C at doses ranging from 50 to 300 krad. In a third set of experiments, heat-sterilized frog leg homogenate inoculated with *S. typhimurium* and *S. enteritidis* (1×10^7 cells/ml) were frozen in liquid nitrogen and then irradiated under frozen (dry ice) conditions (-40°C) at doses ranging from 50 to 500 krad. In the above three sets of experiments, the survivors were recovered on plate count agar after incubating plates at 37°C for 48 h.

Irradiation of frog legs

Frog legs were packed in sterile polyethylene bags (gauge 200) and irradiated in either a fresh condition (26-28°C) or a frozen condition (using dry ice) at doses ranging from 100 to 400 krad. After irradiation, samples were stored at -20°C for 4 months and evaluated for aerobic plate count, *Salmonella* count and organoleptic score at 4-week intervals.

Organoleptic evaluation

Sensory scores were assessed by five trained taste panelists using the hedonic scale of Miyauchi et al. (*7*).

Artificial contamination of frog legs

Frog legs were placed four at a time in a sterilized beaker containing 1 L of a 1×10^8 cells/ml suspension of *S. typhimurium* in phosphate buffer. After 3 h the frog legs, which contained 10^4 - 10^5 cells/g, were removed aseptically, packed in sterile polyethylene bags, and irradiated at doses ranging from 100 to 400 krad as described above. Fresh *S. typhimurium* cell suspensions were used for each dip treatment. To confirm that *S. typhimurium* was completely eliminated from the frog legs after irradiation of fresh or frozen samples at 300 or 400 krad, the samples were preenriched in lactose broth for 48 h, followed by selective enrichment in selenite-cystine broth for 24 h. Portions from the selective enrichment medium were plated on bismuth sulfite agar, brilliant green agar and *Salmonella-Shigella* agar.

Aerobic plate count

For determination of aerobic plate counts, portions of the sample that were blended in lactose broth were serially diluted in saline and 0.1 ml of the appropriate serial dilutions were streaked on prepoured plates of plate count agar. These plates were incubated at 37°C for 48 h.

For quantitation of salmonellae by the most probable number (MPN) method, 1-ml portions of frog leg homogenates in lactose broth were serially diluted and 1 ml each of the decimal dilutions were added to each of three separate tubes of selenite-cystine broth. This medium was incubated for 48 h at 37°C, then streaked on brilliant green agar to determine the number of positive tubes. The *Salmonella* count was estimated from a table of most probable numbers (*1*).

RESULTS AND DISCUSSION

Data on the radiation sensitivity of *S. typhimurium*, *S. newport* and *S. enteritidis* in phosphate buffer are shown in Fig. 1. The survival curves of all three isolates are exponential in nature with D_{10} values, i.e., the doses required for 90% inactivation, ranging from 18 to 30 krad. None of the survival curves shows the 'tailing effect' reported by Quinn et al. (*8*) for several *Salmonella* spp. The D_{10} values of these isolates in frog leg homogenate varied from 22 to 38 krad, the homogenate offering only marginal protection to the salmonellae during irradiation.

Irradiation in the frozen condition offered distinct protection to *S. typhimurium* and *S. enteritidis* as can be seen by the increase in the D_{10} value of *S. typhimurium* from 35 to 50 krad and of *S. enteritidis* from 40 to 50 krad (Fig. 2).

The effect of various doses of radiation on the aerobic plate count as well as the *Salmonella* count in fresh samples of frog legs is shown in Table 1. In five different experiments, it was observed that the initial counts of total bacteria and *Salmonella* ranged between 4.8×10^5 - 4×10^7 and 2.3×10^2 - 1.5×10^3, respectively. In general, higher aerobic plate counts correlated with higher *Salmonella* counts. In all cases it was observed that a dose of 300 krad was effective in totally eliminating *Salmonella*, although the aerobic plate counts were still quite significant (10^2 to 10^4/g).

Results of studies on the effect of irradiation of frog legs in the frozen condition are also shown in Table 1. Frozen samples had a similar degree of *Salmonella* contamination (initial counts) as fresh frog legs, but the aerobic plate counts of the former were less by about two orders of magnitude. It was observed that complete elimination of *Salmonella* in the frozen samples required a dose of 400 krad. No growth was observed on any of the selective agar plates. Similar results were also obtained from inoculation studies indicating that the high levels of *S. typhimurium* (10^4 - 10^5 cells/g) used in these

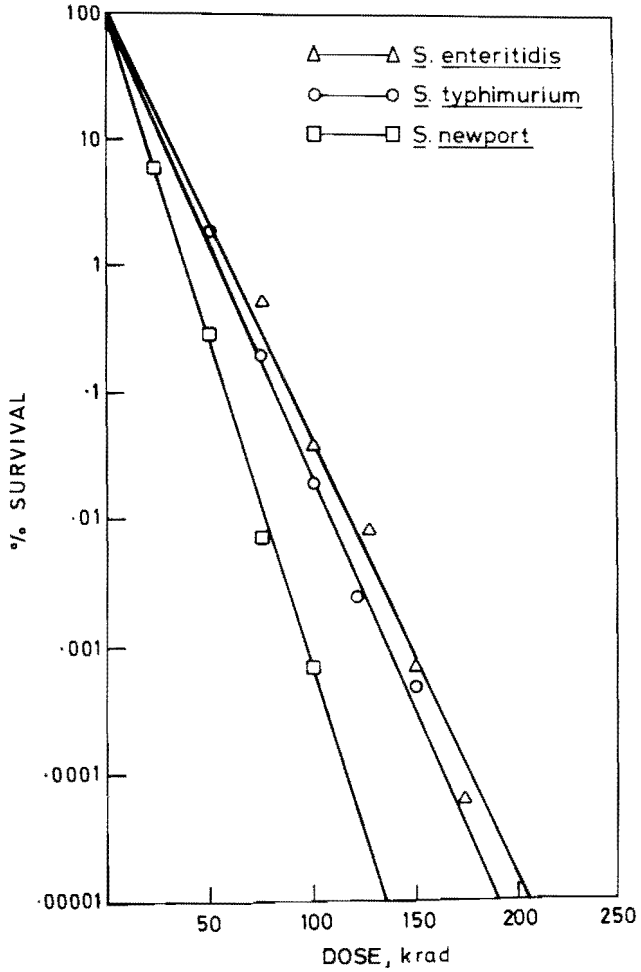

Figure 1. Salmonella *spp. cultures were suspended* (1×10^7 *cells/ml) in 0.1 M phosphate buffer, pH 7.2, and irradiated with gamme rays. Survivors were enumerated on plate count agar.*

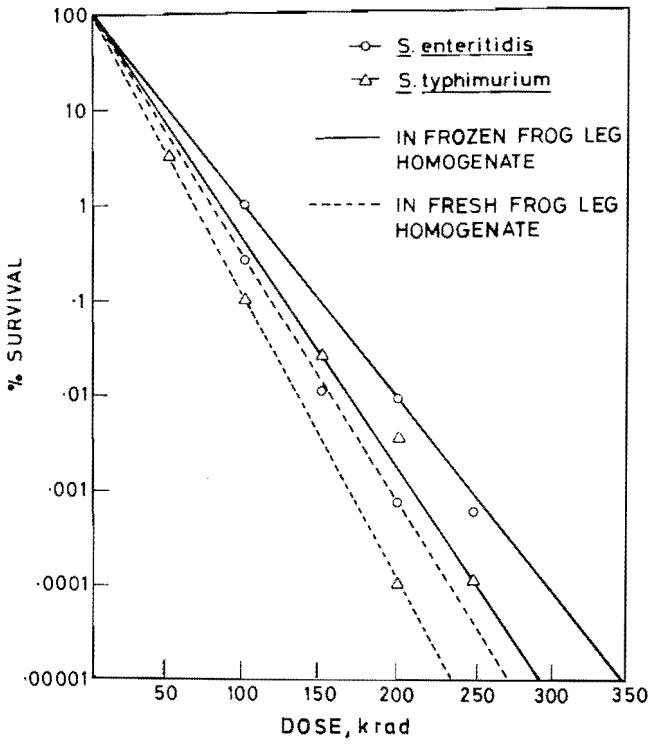

Figure 2. S. enteritidis *and* S. typhimurium *were suspended in frog leg homogenate* (1×10^7 *cells/ml in 10% homogenate) and irradiated in the fresh or frozen condition. Survivors were enumerated on plate count agar.*

TABLE 1. *Irradiation of frog legs in the fresh and frozen state.*[a]

Treatment (krad)		Aerobic plate count (per g)[b]	*Salmonella* count (per g)
Control	Fresh	$6.7 \times 10^6 \pm 0.826$	$4.3 \times 10^2 \pm 1.127$
	Frozen	$4.3 \times 10^4 \pm 0.73$	$1.5 \times 10^2 \pm 0.56$
100	Fresh	$5.6 \times 10^5 \pm 1.00$	$3.9 \times 10^1 \pm 0.740$
	Frozen	$3.8 \times 10^3 \pm 1.32$	$7.5 \times 10^1 \pm 1.50$
200	Fresh	$3.7 \times 10^4 \pm 1.33$	$7 \times 10^1 \pm 1.240$
	Frozen	$1.2 \times 10^2 \pm 1.68$	$3.9 \times 10^1 \pm 1.43$
300	Fresh	$2.8 \times 10^3 \pm 1.03$	nil
	Frozen	$3 \times 10^1 \pm 0.94$	4 ± 1.22
400	Frozen	nil	nil

[a]Frog legs were packaged in presterilized polyethylene bags and irradiated in the fresh or frozen condition at different doses.
[b]Results are the average of five individual experiments.

TABLE 2. *Storage studies of irradiated frozen frog legs.*[a]

Storage period	Treatment	Aerobic plate count (per g)	*Salmonella* count (per g)	Organoleptic scores
Initial	Control	4.0×10^4	1.5×10^2	9.0
	100 krad	1.2×10^4	1.1×10^2	9.0
	200 krad	5.0×10^3	7.0×10^1	8.5
	300 krad	4.8×10^1	2.0×10^1	8.5
	400 krad	1.5×10^1	nil	8.0
4 weeks	Control	1.5×10^4	1.1×10^2	8.5
	100 krad	4.3×10^3	7.5×10^1	8.5
	200 krad	1.2×10^2	6.4×10^1	8.0
	300 krad	1.5×10^1	7	8.0
	400 krad	nil	nil	8.0
8 weeks	Control	1.8×10^3	9.3×10^1	7.5
	100 krad	2.4×10^2	2.8×10^1	7.5
	200 krad	5.5×10^1	7	7.0
	300 krad	1.5×10^1	nil	7.5
	400 krad	nil	nil	7.5
12 weeks	Control	8.0×10^2	6.4×10^1	6.5
	100 krad	1.2×10^2	2.0×10^1	6.5
	200 krad	5.0×10^1	3	7.0
	300 krad	1.2×10^1	nil	7.5
	400 krad	nil	nil	7.5
16 weeks	Control	6.0×10^2	6.4×10^1	6.0
	100 krad	9.0×10^1	1.1×10^1	6.0
	200 krad	4.5×10^1	3	6.0
	300 krad	7	nil	7.0
	400 krad	nil	nil	7.0

[a]Packaged frozen frog legs were irradiated at different doses in the frozen condition and stored at -20°C for 16 weeks.

experiments were totally inactivated by a dose of 400 krad.

Results of storage studies indicate that frozen frog legs subjected to a dose of 400 krad were free from *Salmonella* during the entire storage period (Table 2). Although sensory ratings decreased with storage, the rate of quality deterioration of the unirradiated frozen frog legs was more rapid than that of the irradiated samples. Thus, whereas the unirradiated frozen samples were slightly preferred to the irradiated ones for up to 4 weeks of storage, the reverse was true beyond 12 weeks of post-irradiation storage (Table 2). It is pertinent to mention that fresh frog legs subjected in the iced condition to a dose of 300 krad were eliminated of *Salmonella* but had lower sensory ratings than their frozen counterparts. Hence, it is advisable to irradiate frog legs under frozen conditions.

Complex media are known to protect bacteria against irradiation. Quinn et al. (*8*) showed that the D_{10} values of several *Salmonella* spp. were four to five times higher when cells were irradiated in crab meat than in buffer. Such protection by natural food components would, in practice, necessitate the use of much higher doses for the elimination of *Salmonella* from foods. However, the present studies show that the typical *Salmonella* spp. normally encountered in frog legs were almost equally sensitive to gamma irradiation whether irradiated in buffer or frog leg homogenate, thereby requiring reasonably low doses for eradication of salmonellae from frog legs. The increased D_{10} values of *Salmonella* spp. irradiated in frozen frog leg homogenates could arise from decreased mobility of the free radicals produced, as has been shown by Stapleton and Edington (*9*). The practical significance of this observation is the requirement of higher doses for radicidation of frozen frog legs as compared with their fresh counterparts.

The above findings establish the technological feasibility of a radicidation process for the control of *Salmonella* in frozen frog legs without deteriorating the quality of the product.

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