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Radiation sensitivity of *Acinetobacter* spp. and their redicidation for preservation of meat at low temperature

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Abstract

In recent years, interest has been growing to preserve food including meat applying gamma radiation recommended by International Atomic Energy Agency to extend the shelf-life of meat retaining the organoleptic conditions as it is. In view of this point the present study aims to check the sensitivity of *Acinetobacter* spp. isolated from meat to gamma radiations. Seven species of *Acinetobacter* viz. *A. lwoffii* M1; *A. baumannii* M8; *A. calcoaceticus* M19; *A. junii* M20; *A. johnsonnii* M23; *A. haemolyticus* M27 and *A. radioresistens* M25 isolated from meat were exposed to gamma radiation at the dose level of 0.1 to 10 KGy. The D_{10} value of *Acinetobacter* was found highest 1.25 KGy in *A. radioresistens* M25, which was 4 to 8 times higher than other genospecies of *Acinetobacter*. *Acinetobacter radioresistens* M25 contains one plasmid of 45 Kb. The radicidation dose of 4 KGy gamma radiations was found to be sufficient to eliminate the natural contamination of meat and contamination by *Acinetobacter*. To eliminate radiation resistant *Acinetobacter* contamination a dose of 4 to 5 KGy was required. Development of the radicidation process for preservation of meat to eliminate *Acinetobacter* as contaminants at low temperature is one of the new and interesting phenomena. Attempts of finding the appropriate radicidation dose for preservation of meat at low temperature will open up new avenues for commercial preservation of meat.

Introduction

Meats contain a broad spectrum of microorganisms arising from the carcass and the processing operations involve equipment and human contact. Fresh meat provides a favorable environment for microorganisms, which constitute public health and organoleptic spoilage problems. To eliminate these problems, various processing techniques have been investigated. Gamma irradiation is an example of a potential commercial process to extend shelf life of meat and aid in public health protection[1]. Some members of the microflora of commercially processed red meat are sensitive to gamma radiation whereas others are quite resistant to comparable doses[2]. It has been reported that the radiation resistant bacteria from meat, made a general classification with minimal characterization of these bacteria as *Moraxella-Acinetobacter* group[3].

There are many reports for the radioresistance of Gram-positive bacteria[4-6]. However, there are a few reports for that of Gram-negative bacteria[5,7]. A radioresistance strain of *Acinetobacter* F0-1 has been isolated from the tampon's cotton sterilized by 3 Mrad irradiation of cobalt-60 γ-ray[8]. It was reported that *Acinetobacter* has a good radiation resistance and for this reason they are part of the aerobic microflora of some irradiated food[3,5,9]. The radiation resistant vegetative bacteria *Acinetobacter-Moraxella* has been isolated and characterized from beef and their radiation resistance ranged from D_{10} values of 273 to 2039 krad[9]. On the basis of radio-resistance pattern of *Acinetobacter* Nishimura et al.[10] proposed a new species *Acinetobacter radioresistens*. There have been a few reports about the sensitivity of *Acinetobacter* strains to gamma rays[5,7,9]. However, there are no detailed taxonomic characters. Ino and Nishimura[11] examined the radiation sensitivity of a few strains of *Acinetobacter calcoaceicus* and reported that they were radiation sensitive. The similar findings were also observed by Kairiyama et al[8,12].

It has been studied the efficacy of utilizing irradiation doses (range 1 to 3 KGy) to extend the shelf-life meats at 0 to 2^0C through selective destruction of spoilage bacteria[13,14]. In view of the

approval of United States Food and Drug Administration for irradiation of meat[15] the present work was undertaken to study the response of *Acinetobacter* to gamma radiation isolated from meat and to develop the radicidation process using gamma radiation for preservation of meat at low temperature eliminating *Acinetobacter*.

Materials and Methods

Isolation and biotyping of Acinetobacter: Seven *Acinetobacter* strains were isolated from meat by enrichment of respective samples in Baumann's enrichment medium' and selected for gamma radiation sensitivity test[16,17]. Identification of these isolates up to genus level was carried out as per the chromosomal DNA transformation assay[18-20]. Delineation of the isolates into various species was performed as per the biochemical scheme recommended by Bouvet and Grimont[21,22,] Gennari[23] and with some additional tests. The reconfirmation of the speciation was done by API 20 NE system[24].

Bacterial strains and culture media used: Seven strains of *Acinetobacter* spp. consists of *A. calcoaceticus* M19, *A. baumannii* M8, *A. lwoffii* M1, *A. haemolyticus* M27, *A. junii* M20, *A. johnsonii* M23 & *A. radioresistens* M25, were used in the present study. The control strains used included *Acinetobacter radioresistens* FO-1^T (IAM 13186^T), *A. radioresistens* G82012 (IAM 13188), *A. radioresistens* G82076 (IAM 13187) and non-resistant bacteria *A. calcoaceticus* BD413 trp E27, *A. lwoffii* MTCC 496, *A. calcoaceticus* MTCC127, *A. calcoaceticus* MTCC1271, *E.coli* K12 HB101& *E. coli* DH5α. Nutrient agar (NA) was used for propagation of organism and subcultures were inoculated in nutrient broth. All cultures were incubated at 28°C, and since the isolates used were aerobic, vigorous shaking (200 rpm) was employed to enhance the growth.

Growth rate determination: Growth rates of cultures were determined by both viable counts with nutrient agar and with turbidity measurements. The turbidity of cultures was measured at a wavelength of 560 nm. The cultures were diluted in 0.2 mM phosphate buffer (pH 7.2) to give an absorbance less than 0.04. The absorbance readings were then multiplied by the dilution factor to calculate the true absorbance. Maximum growth rates for the exponential phase of growth of each culture were estimated by the method of least squares, and the coefficients were used to calculate cell-doubling times[25].

Irradiation: A ^{60}Co isotope group gamma chamber 900 irradiator of the Department of Nuclear Chemistry, Pune University was used for gamma irradiation. Cultures in test tube (15/40 mm) were either irradiated at ambient air temperature (25°C) or quick-frozen in dry ice acetone and kept at –28°C during irradiation by utilizing dry ice in the sample carrier. The various doses used for gamma irradiation were 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 KGy at the rate 1.3 Krad/min. During any delay before or after irradiation, the frozen cultures were held at –20°C.

Survival of Acinetobacter spp. to gamma radiation: After immediate irradiation each irradiated cultures were spread out on nutrient agar plate by spread plate technique for total viable count (0.2 mM phosphate buffer, pH 7.2 was used for serial dilution of the cultures). Inoculated nutrient agar plates were incubated at 28°C up to 4 days to allow recovery and growth of the irradiated cells for determination of survival rates to gamma radiation and its D_{10} values. Surviving fractions were calculated by dividing irradiated counts by the corresponding non-irradiated control counts and the experiments were done at three conditions (a) after immediate expose to gamma radiation, (b) values from cultures preserved at 28°C for 15 days and (c) values from cultures preserved at 4°C for 15 days after exposing gamma radiation.

Bacteriological status (quantitative) of unirradiated and irradiated meat: Total aerobic count of fresh meat and irradiated meat (of different doses: 2, 4, 6 and 8 KGy) was done as per method described by Saha and Chopade[17]. The meat samples were frozen at –20°C for 1 hour just before irradiation.

Development of radicidation process by artificially inoculated pack studies: Radicidation process was developed for eradicating *Acinetobacter* spp. from meats. For this process meat samples (each 50 g chopped meat) were collected aseptically from local market of Pune city. Each 50 g quantity of meat was packed in sterile polythene bag and irradiated at individual doses of 2.0, 4.0, 6.0 and 8.0 KGy under frozen condition, using dry ice in cobalt^{60}-package irradiator at a dose rate of 1.3 Krad/min. Unirradiated meat samples were used as control. *Acinetobacters* and other standard cultures were grown individually in nutrient broth for 18 to 24 hours at 28°C, centrifuged (5,000 rpm/6 min), washed with phosphate buffer (0.2 mM, pH 7.2) and suspended in the same buffer at levels of approximately 1.0 x 10^8 cells/mL. Cubes of processed meat (50 g) procured from local market at Pune city, were completely submerged with

suspensions of *Acinetobacters* and other standard cultures of the above mentioned levels. After 3 hours at 4-5°C, the samples were drained, packed under aerobic conditions in sterile polythene bags and frozen at –20°C prior to irradiation at above-mentioned doses. Fresh cell suspensions of organisms were used for each sample dip treatment. One part of each irradiated inoculated pack sample was preserved at 4°C and -20°C respectively for 4 weeks. One gram of each irradiated meat samples impregnated with bacterial cultures was suspended in 50 mL sterile saline (0.85%) and shaken 200 rpm at 28°C for 30 min 0, 1, 2 and 4 weeks interval. The above meat homogenates were serially diluted up to 10^{-8} in normal saline and 100 µL of the appropriate dilutions were then spread on pre-poured total plate count agar. The colony count was taken after incubation for 4 days.

Plasmid content of radiation resistant Acinetobacter: Plasmid DNA extraction of all 4 *Acinetobacter radioresistens* strains M25, F0-1, G82012 and G82076 was followed methods described by Maniatis et al[26] and Kado & Liu[27]. Plasmid band was confirmed by agarose gel electrophoresis.

Results

Seven meat isolates selected were identified to be *Acinetobacter lwoffii, A. calcoaceticus, A. baumannii, A. haemolyticus, A junii, A. johnsonii* and *A. radioresistens*. The chromosomal DNA transformation assay confirmed the generic identity of the isolates[18,19], while the biochemical scheme proposed by Bouvet and Grimont[21,22]; Gennari[23] and API 20NE microtube system helped us in delineation of the isolates into various *Acinetobacter* spp.

The growth rates of all seven *Acinetobacter* spp. were checked at 28°C with vigorous shaking at 150 rpm. The stationary phase of growth of all cultures were found after 72 hours. Based on their growth phase the cultures were chosen for irradiation during their stationary phases. Because the cultures of log growth phase were found to be more sensitive to gamma radiation as compared to cultures of stationary growth phase (data not shown).

The radiation sensitivity of *Acinetobacter* sp. to gamma radiations were shown in Table I. The D_{10} values of *Acinetobacter* strains were found to be *A. lwoffii* (M1) 0.2 KGy, *A. baumannii* (M8) 0.4 KGy, *A. calcoaceticus* (M19) 0.2 KGy and *A. calcoaceticus* BD413 trp E27 <0.2 KGy. The D_{10}

Table I: Radiation sensitivity of *Acinetobacter* genospecies isolated from meat

Bacteria	Strain number	D_{10} value[a] (Kgy)		
		A	B	C
A. lwoffii	M1	0.2	0.4	0.1
A. baumannii	M8	0.4	0.6	0.1
A. calcoaceticus	M19	0.2	0.4	0.1
A. junii	M20	0.2	0.2	0.1
A. johnsonnii	M23	0.4	0.4	0.1
A. haemolyticus	M27	0.2	0.2	0.1
A. radioresistens	M25	1.2	1.5	0.2
Standard strains:				
A. radioresistens	FO-1 (IAM 131814)	2.2[b]	ND	ND
A. radioresistens	G82012 (IAM 13186)	2.04[b]	ND	ND
A. radioresistens	G82076 (IAQM 13187)	1.25[b]	ND	ND
A. lwoffii	ATCC 15309	0.22[b]	ND	ND
A. lwoffii	MTCC 496	>0.1	<0.2	<0.1
A. lwoffii	CCM 5572 (ATCC 17987)	0.24[b]	ND	ND
A. haemolyticus	CCM 2358 (ATCC 17906, =CIP 64.3)	0.17[b]	ND	ND
A. junii	CIP 64.5= (ATCC 17908)	0.11[b]	ND	ND
A. johnsonnii	CIP 64.6= (ATCC 17909)	0.096[b]	ND	ND
A. calcoaceticus	IAM 12088 (ATCC 19606)	0.12[b]	ND	ND
A. calcoaceticus	BD413 trp E27	0.1	<0.2	<0.1
A. calcoaceticus	MTCC1271	>0.1	0.2	<0.1

[a]Measured under air-equilibrium in 0.067M phosphate buffer; [b]Values from paper of Ino and Nishimura, 1990; A: Values after immediate exposure to gamma radiation; B: Values from cultures preserved at 28° C for 15 days after exposing gamma radiation; C: Values from cultures preserved at 4°C for 15 days after exposing gamma radiation; ND: not done

Table II: Bacteriological status of irradiate and non-irradiate meat

Samples	TBC at room temperature (28°C) (cfu/g)	TBC at 4°C for 10 days (cfu/g)
Fresh meat (control)	$21.66 \times 10^8 \pm 1.24$	$6 \times 10^8 \pm 0.81$
Irradiate meat (2 Kgy*)	$2 \times 10^4 \pm 0.81$	$5 \times 10^2 \pm 0.81$
Irradiate meat (4 KGy)	$1.33 \times 10^2 \pm 0.4$	27 ± 2.9
Irradiate meat (6 KGy)	31 ± 3.7	0
Irradiate meat (8 KGy)	0	0

*1.3 Krad/min; TBC = Total bacterial count; cfu = colony forming unit

values of *A. radioresistens* M25 and *A. radioresistens* FO-1 both were found similar and the respective D_{10} values were 1.2 and 2.2 KGy (Table I). The D_{10} values of standard strains used in this study were *A. radioresistens* F0-1 (IAM 13186) 2.2, *A. radioresistens* G82012 (IAM 13188) 2.04, *A. radioresistens* G82076 (IAM 13187) 1.25, *A. lwoffii* ATCC15309 0.22, *A. lwoffii* MTCC 496 <0.1, *A. lwoffii* CCM 5572 (ATCC 17987) 0.24, *A. haemolyticus* CCM 2358 (ATCC 17906, = CIP 64.3) 0.17, *A. junii* CIP 64.5 (ATCC 17908) 0.11, *A. johnsonii* CIP 64.6 (ATCC 17909) 0.096, *A. calcoaceticus* IAM 12088 (ATCC 19606) 0.12, *A. calcoaceticus* BD413 trp E27 0.1 and *A. calcoaceticus* MTCC 1271 > 0.1 KGy (Table I).

Bactriological status (quantitative) of non-irradiated and irradiated (2; 4; 6; 7; 8 KGy) meat is shown in Table II. The non-irradiated sample contained total bacterial count (TBC) 10^8 coloni forming unit (cfu)/g both at 28°C and at 4°C for 10 days preserved meat. In irradiated meat it was found 10^4cfu/g at 0 hour at room temperature and at 2 KGy, while it was found 10^2 cfu/g at 4°C after 10 days. The bacterial count was 10^2 cfu/g at room temperature at 4 KGy, while it was 27 cfu/g at 4°C after 10 days count. At 6 KGy bacterial count was 31 cfu/g at RT and it was zero at 4°C. No growth was found at the dose level of 8 KGy (Table II).

The results of inoculated pack studies using eight *Acinetobacter* spp. in meat employing different doses of gamma radiation are presented in Figure 1 and 2. Irradiated (4 KGy) meat samples were artificially contaminated with 10^8 cells/g of *A. lwoffii* M1, *A. baumannii* M8, *A. calcoaceticus* M19, *A. junii* M20, *A. johnsonii* M23, *A. haemolyticus* M27, *A radioresistens* M25 and *A. radioresistens* F0-1 separately and applied the radiation doses for each 4 KGy and 6 KGy separately. Though the radicidation dose of 2 KGy reduced the *Acinetobacter* count more than 4 log cycle. The radicidation dose of 4 KGy could eliminate completely non-radioresistant *Acinetobacter* from meat preserved in both the temperatures 4°C and -20°C. But *Acinetobacter radioresistens* strains M25 and F0-1 could survive up to 10^2 cfu/g at the radicidation dose of 4KGy, while the strains could be eliminated completely at the radicidation dose of 6 KGy from meat preservation at both the temperatures at 4°C and -20°C.

The plasmid content of radiation resistant *Acinetobacter* revealed that each of the resistant strains was harboring a single plasmid. The size of the plasmids of all strains was found to be similar (45 Kb). The antibiotic resistance was found decrease in irradiated cells as compared to parent cells of *Acinetobacter radioresistens* strains.

Discussion

Food is vital for human existence. Conservation and preservation of food is a prerequisite for food security and it provides economic stability and self-reliance to a nation. World Health organization in 1980 clarified the position regarding the medical acceptability of irradiated foods when it said no health hazard is caused by irradiating any food up to a dose of 10 KGy or 1 Mrad and hence food treated in this way no longer need to be tested for toxicity. In comparison with heat or chemical treatment, irradiation is considered a more effective and appropriate technology to destroy food borne pathogens. An expert group constituted by FAO/ WHO/ IAEA in 1997 again affirmed the safety of doses higher than 10 KGy. In 1998 a number of food items including meat and meat products were permitted for radiation processing and the dose level was 0.3 to 0.15 KGy for fresh vegetable, 2.5 to 4 KGy for meat and meat products and 6.0 to 14.0 KGy for spices[28].

Time and temperature are important factor for the survival of irradiated *Acinetobacter* spp. Survival of *A. radioresistens* M25 was found to be 1.5 to 4 times higher than other species of *Acinetobacter* irradiated at the same conditions. No strains of *Acinetobacter* sp.could survive at 4 KGy, where 3% of *A. radioresistens* could survive at same dose and even, *A. radioresistens* M25 had survived (1.0×10^{-3}%) at 10 KGy. The percent survival of all *Acinetobacter* species were found higher, cultures preserved at 28°C for 15 days than immediate exposing gamma radiation. The D_{10}

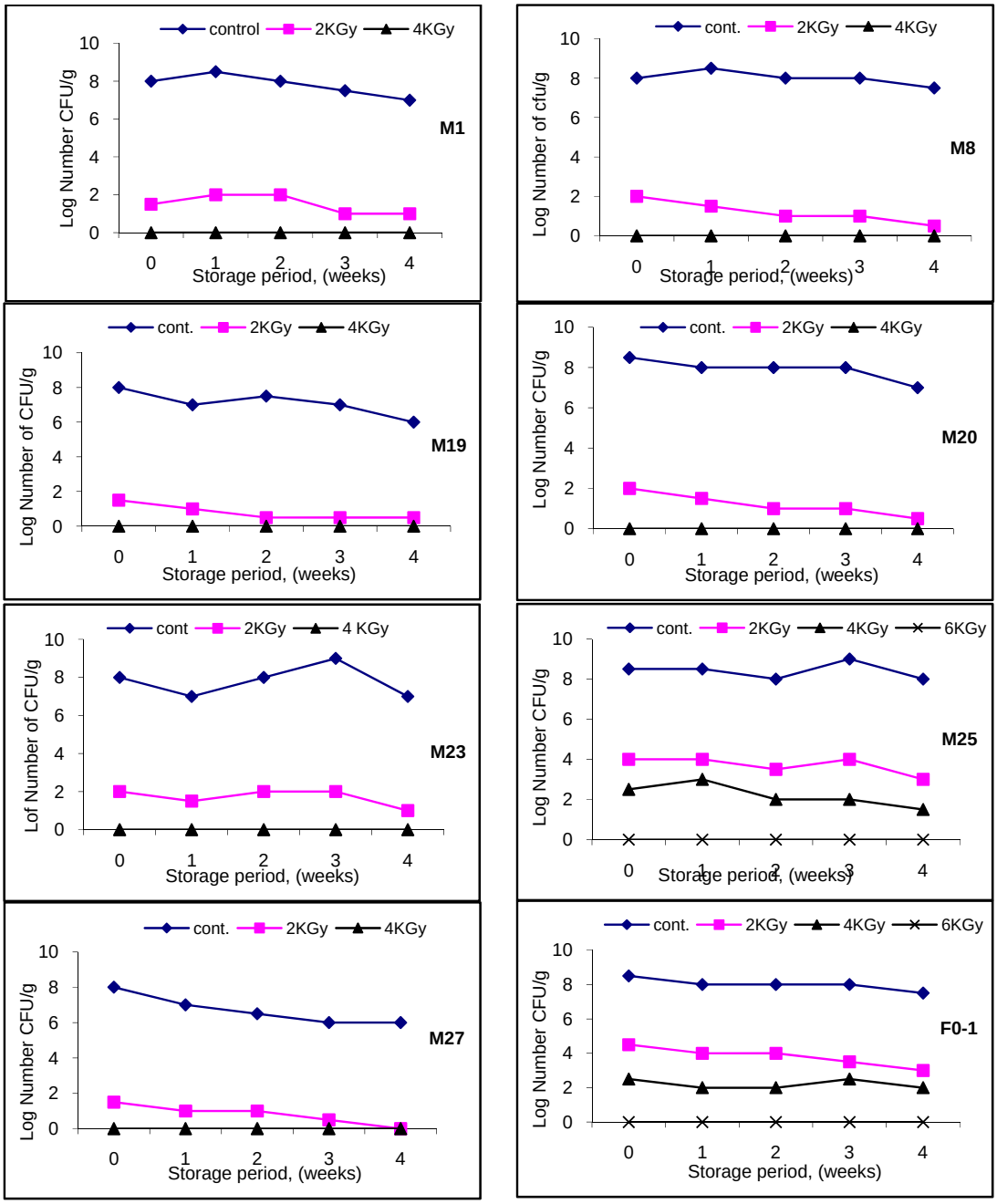

Figure 1: Effect of irradiation at 4°C on counts of *Acinetobacter* species in artificially inoculated peak studies. M1: *A. lwoffil*; M8: *A. baumannii*; M19: *A. calcoacetlcus*; M20: *A. junii*; M23: *A. johnsonnii*; M25: *A. radioresistens*; M27: *A. haemolyticus* and F0-1: *A. radioresistens*

values of *A. lwoffii* M1, *A. baumannii* M8, *A. calcoaceticus* M19, *A. junii* M20, *A. johnsonii* M23 and *A. haemolyticus* M27 were between 0.2 to 0.6 KGy, which were 2-3 times less than that of *A. radioresistens* M25 and were 1.5 to 4 times higher than those of *A. lwoffii* MTCC496, *A. calcoaceticus* BD 413 trp E27 and *A. calcoaceticus* MTCC 1271 strains irradiated under same conditions. The D_{10} values of *A. lwoffii*, *A. baumannii*, *A. calcoaceticus* and *A. radioresistens* were found 1.25 to 2 times higher in the respective irradiated cultures were preserved at 28°C for 15 days than from immediate exposing cultures to gamma radiation. A similar work has done by Keller and Maxcy[25] showed that cells of *Acinetobacter-Moraxella* sp. were recovered within 50 hours incubation at 32°C and 50 to 150 hours incubation at 18°C after 4.0 Mrads gamma radiation.

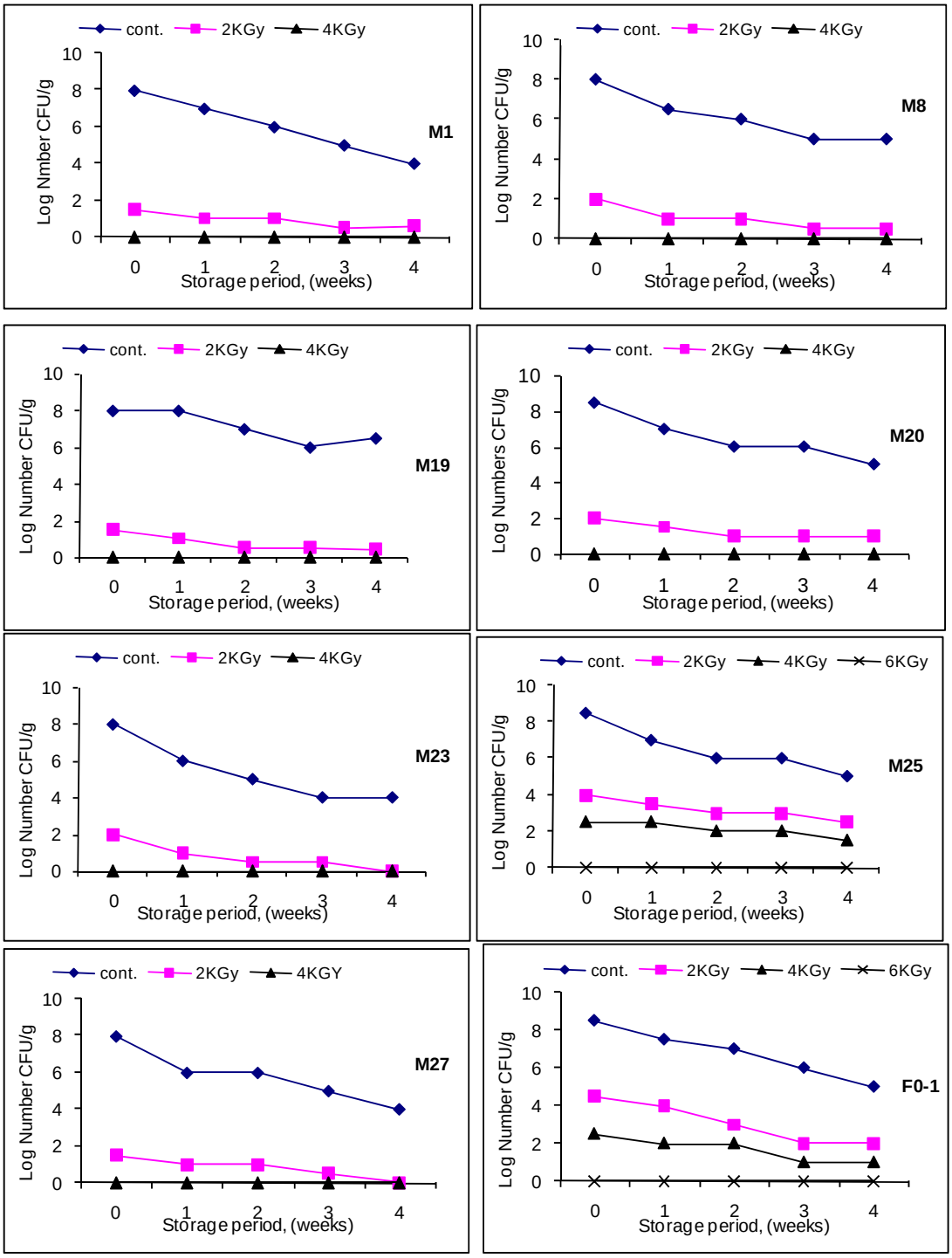

Figure 2: Effect of irradiation at 20°C on counts of *Acinetobacter* species in artificially inoculated peak studies. M1: *A. lwoffil*; M8: *A. baumannii*; M19: *A. calcoacetlcus*; M20: *A. junii*; M23: *A. johnsonnii*; M25: *A. radioresistens*; M27: *A. haemolyticus* and F0-1: *A. radioresistens*

Irradiated *Acinetobacter* cultures preserved at low temperature (4°C) were found to be sensitive in all conditions. *A. lwoffii* and *A. johnsonii* was found to be more sensitive (0.01% at 0.8 KGy) as compared to other spp. of *Acinetobacter* at these conditions. The interesting thing was that reduction of cells (cfu) of *A. baumannii* was rapid at 10 Krad, where it could survive (3.6×10^{-5}%) up to dose level of 2.0 KGy, which is found next to *A. radioresistens* M25 (1.33%). No strains of

Acinetobacter species had survived at the dose level of 4.0 KGy except *A. radioresistens* M25 (0.1%) and it could survive (8.0 10^{-3}%) at 6 KGy. It had been observed that the D_{10} values of all *Acinetobacter* species including *A. lwoffii* MTCC 496, *A. calcoaceticus* BD 413 try E27 and *A. calcoaceticus* MTCC 1271 the standard strains were between <0.1 to 0.1 KGy, which were about two times less than that of *A. radioresistens* M25 (0.2 KGy) strains irradiated and preserved under same conditions. The D_{10} values of all *Acinetobacter* species were found 2 to 8 times less the respective irradiated cultures preserved at 4°C for 15 days.

In bacteriological study of meat it was observed that total bacterial count of fresh meat and meat preserved at 4°C for 10 days was 10^8 cfu /g[23]. The radicidation dose of 2 KGy reduced the bacterial count 4 log cycles in fresh meat, where it was found to be 6 log cycles of irradiated meat preserved at 4°C for 10 days. To eliminate the bacterial flora completely higher radicidation doses (4, 6 and 8 KGy) were applied. 4 KGy reduced total aerobic counts of fresh meat by 6 log cycles, where it reduced a significant level (27 cfu/g) at low temperature preserved meat. At 6 KGy, 31 cfu/g was observed and it was zero at low temperature and no growth was found at radicidation dose of 8 KGy. It revealed that a radicidation dose of 4 KGy gamma radiations and low temperature could eliminate total aerobic count at significant level. Alur el al[29] showed that a radicidation dose of 2.5 KGy could reduce mesophilic aerobic counts of pork meat products by 2-3 log cycles.

In inoculated pack studies it was observed that 7 species of *Acinetobacter* artificially contaminated with irradiated meat survived up to 4 weeks at low temperatures (4°C) without significant reduction of cell number (cfu/g). Reduction of cell count was found to be 1 to 2 log cycles at the end of 4 weeks. Although the radicidation dose of 2 KGy reduced *Acinetobacer* counts about 6 log cycles, but it could not reduce *A. radioresistens* M25 and F0-1 more than 4 log cycles. At this radiation treatment a significant number of *A. radioresistens* M25 and F0-1 (10^3 to 10^5) had survived. However, increase in radiation treatment to 4 and 6 KGy could eliminate *A. radioresistens* strains completely in all the meat pack. It was observed that a radicidation dose of 4 KGy eliminated *A. lwoffii*, *A. calcoaceticus*, *A. baumannii*, *A. junii*, *A. johnsonii* and *A. haemolyticus* completely, where *A. radioresistens* strains reduced up to a significant level. Radicidation dose of 6 KGy eliminated *A. radioresistens* strains completely.

Inoculated pack studies at frozen temperature (-20°C), observed that there was no significant differences for reduction of *Acinetobacter*. Reduction of *Acinetobacter* counts in control experiment was found higher (2 to 4 log cycle) in frozen (-20°C) meat pack studies as compared to refrigerator (4°C) meat pack studies. But interestingly no significant difference was marked by radiation treatment between meat pack stored at 4°C and -20°C (Figure 2). This may be the cause of psychrophilic nature of *Acinetobacter*. Kamat et al[30] showed that a dose of 4 KGy under cryogenic conditions (-40°C) completely eliminated *Salmonella* from frozen frog legs, shrimps and chicken meats. However, 4 KGy of gamma radiation completely eliminated non-radioresistant strains of *Acinetobacter* and also a significant level of radioresistant strains.

In our previous work it had been shown that 0.1% potassium sorbate, 0.2% sodium benzoate, 0.1% sodium nitrite, 0.1% acetic acid and lactic acid and the combination of 0.05% sodium nitrite + 3.5% sodium chloride inhibited *Acinetobacter* growth at a significant level at 4°C[31]. The present investigation revealed that meat was also amenable to radicidation treatment for total elimination of *Acinetobacter*. A dose of 2 to 4 KGy would suffice to eliminate naturally contaminated samples and non-radioresistant *Acinetobacter*. However, 4 to 6 KGy of gamma radiation was required for meat, highly contaminated with radioresistant *Acinetobacter*.

In conclusion, it can be said that isolation of *Acinetobacter radioresistens* M25 from meat is the first report, which resistance pattern was very close to *A. radioresistens* G82076. To develop a radicidation process by using Gamma radiation for preservation of meat at low temperature a dose of 4 KGy would be suffice to eliminate naturally contamination of meat and contamination by non-radioresistant *Acinetobacter*, but 4 to 6 KGy of gamma radiations is required to eliminate radio-resistance *Acinetobacter* completely from meat.

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