

## Factors Affecting Growth and Toxin Production by *Clostridium Botulinum* Type E on Irradiated (0.3 Mrad) Chicken Skins

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### ABSTRACT

A model system (chicken skins with chicken exudate) was used to determine if *Clostridium botulinum* type E (Beluga) spores, stressed by low dose irradiation, would develop and produce toxin at abuse temperatures of 10 and 30°C in the absence of characteristic spoilage. Unstressed spores germinated, multiplied, and produced toxin on vacuum-packed chicken skins, stored at either 30 or 10°C. Cell numbers increased faster and toxin was evident sooner at 30°C than at 10°C. At 30°C, growth occurred and toxin was produced more slowly when samples were incubated aerobically than anaerobically. When samples were incubated aerobically at 10°C, no toxin was detected within a test period of 14 days. An irradiation dose of 0.3 Mrad at 5°C reduced a spore population on vacuum-sealed chicken skins by about 90%. The surviving population produced toxin at 30°C under either aerobic or anaerobic conditions, at 10°C no toxin was detected even on skins incubated anaerobically. Under the worst conditions (30°C, vacuum packed) toxin was not detected prior to characteristic spoilage caused by the natural flora surviving 0.3 Mrad.

### INTRODUCTION

LOW-DOSE (less than 1 Mrad) irradiation of poultry has been recommended for the reduction of *Salmonellae* and the extension of shelf-life when refrigerated. *Salmonellae* are recognized as the main public health hazard associated with poultry (Todd, 1980) and fresh broiler carcasses have a limited shelf-life in retail outlets (Mountney, 1976).

To date poultry, irradiated with a low-dose (< 1 Mrad), has not been approved for human consumption in the United States. However, the Netherlands and South Africa have approved the sale of chickens irradiated at  $\leq$  0.3 Mrad and 0.2 to 0.7 Mrad, respectively (Elias, 1980).

The above recommended doses are not designed to sterilize the product. Therefore, the irradiated product must be refrigerated to inhibit the multiplication of potential radiation survivors, such as *Clostridium botulinum* spores. Although the proteolytic strains of *C. botulinum* types A and B will not multiply at temperatures below 10°C (Ohye and Scott, 1953), *C. botulinum* type E multiplies and produces toxin when stored at 3.3°C (Schmidt et al., 1961). The potential for a problem with *C. botulinum* type E in low-dose irradiated fish resulted in the recommendation that such fish be stored at  $\leq$  3°C (Ingram and Farkas, 1977).

This investigation was undertaken to determine the ability of unirradiated and irradiated (stressed) *C. botulinum* type E spores, to outgrow and produce toxin on chicken skins at 30 and 10°C.

### MATERIALS & METHODS

#### Chicken skin preparation

Broiler breast skins were obtained from local supermarkets. A round piece of skin was embedded in liquid wax inside a small Petri dish so that a single surface with an area of 7 cm<sup>2</sup> was exposed. The samples were frozen and stored at -30°C. Sterile chicken skins were obtained by irradiating the skins in Petri dishes with 4.1 Mrad at -30°C and storing them frozen until experimentation.

#### Inoculation of skins

0.1 ml of the appropriate dilution of bacterial spore suspension was added to thawed sterile skins together with 0.3 ml of filter sterilized exudate. The exudate was the liquid that accumulated in the trays or absorbed in the packing paper which supported the chicken. The exudate was added to prevent drying of the skins and to simulate the real situation of broilers stored in retail outlets.

#### Test organisms

For most of the experiments, *C. botulinum* type E, strain Beluga was used, in some experiments, strains T304 and VH were used. Spore crops of each strain were produced and harvested as described by Bruch et al. (1968). The spores were frozen (in acetone-dry ice bath) in 2 ml aliquots and stored at -30°C until experimentation. Before each experiment the spores were thawed and heated to 60°C for 3 min (to inactivate vegetative cells and perhaps thereby activating the spores).

#### Counting procedures

The skin sample was removed from the wax with sterile forceps and placed in a small blender jar with 10 ml of gel-phosphate (0.07M phosphate buffer, pH 6.5, containing 0.1% gelatin) and blended for ca 1 min. To enumerate *C. botulinum* cells 1 ml of homogenate was diluted decimally in chilled, sterile 0.1% peptone. One-milliliter aliquots were inoculated into each of duplicate tubes (11 x 202 mm) containing 0.2 ml of filter-sterilized 5% NaHCO<sub>3</sub>, and 11 ml of molten TPG agar medium (5% trypticase (BBL), 0.5% peptone (Difco), 0.4% glucose (Fisher) 0.2% sodium thioglycolate (Difco), and 3% agar (BBL) at pH 7.0) was added to each tube. The tubes were stratified with 1.5-2 cm of the same medium, incubated at 30°C for 30-40 hr, and the numbers of colonies formed in the duplicate tubes were averaged.

To enumerate the natural flora of chicken skins 1 ml of homogenate was diluted decimally in chilled, sterile 0.1% peptone. One-milliliter aliquots were inoculated into each of duplicate Petri plates and molten Plate Count agar (BBL) was added. The plates were stratified with the same medium, incubated at 25°C for 72 hr, and the numbers of colonies formed in the duplicate plates were averaged.

#### Growth experiments

For anaerobic growth, skins were inoculated with the Beluga strain (10<sup>3</sup> spores per 7 cm<sup>2</sup>) and incubated at 10 and 30°C in a vacuum sealed flexible pouch. "Aerobic" growth on skins was studied with inocula of 10<sup>2</sup> and 5 x 10<sup>3</sup> spores/7 cm<sup>2</sup>. The skins were incubated in a desiccator containing water (to avoid drying of the skins during incubation).

#### Irradiation survival curves

The strains Beluga and 1304 (ca. 5 x 10<sup>6</sup> spores/7 cm<sup>2</sup>) were inoculated on chicken skins, vacuum sealed in flexible pouches, and irradiated at 5°C. Six ml of each one of the three strains of *C. botulinum* type E, containing ca 5 x 10<sup>7</sup> spores/ml were transferred to sterile pyrex tubes (16 x 150 mm), vacuum sealed, and irradiated at

-Continued on next page

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## C. BOTULINUM ON IRRADIATED CHICKEN SKINS

5°C. The D-values (dose required for 90% inactivation or one log<sub>10</sub> reduction) was obtained by linear regression of the log N/N<sub>0</sub> versus radiation dose.

### Growth after irradiation

Skins were inoculated with the strain Beluga (10<sup>4</sup>–10<sup>6</sup> spores/7 cm<sup>2</sup>), irradiated in vacuum sealed (40 mm Hg) flexible pouches with 0.3 Mrad, and stored at 10 or 30°C in the pouches. At 30°C aerobic growth after irradiation was also studied: inoculated skins were irradiated in pouches with low vacuum (610 mm Hg), irradiated (0.3 Mrad), the pouches were opened and the skins were transferred to a desiccator.

### Determination and assay of botulinum toxin

The skin samples, blended with gel-phosphate were centrifuged under refrigeration, the supernatant was trypsinized as described in the *Bacteriological Analytical Manual of Food* (1976), and 1 ml was injected intraperitoneally into a male mouse. Control toxicity was done by heating the sample as described in the Manual. The mouse LD<sub>50</sub> was determined according to the method described by Schantz and Kautter (1978).

### Growth after irradiation together with natural flora

Nonsterilized chicken skins were inoculated with *C. botulinum* type E spores (ca. 5 × 10<sup>4</sup>/7 cm<sup>2</sup>), and 0.3 ml of nonsterile chicken exudate was added. The skins were vacuum sealed in a pouch, irradiated at 5°C with 0.3 Mrad, and incubated at 30°C. The samples were checked daily for odor and toxin production.

### Flexible pouch material

The pouches were made of 0.025 mm polyiminocarpoyl (Nylon 6) as the outside layer, 0.009 mm aluminum foil as the middle layer and 0.051 mm chemically bonded polyethylene terephthalate – medium density, polyethylene (3M scotchpack 9) as the inside (Petri-dish containing) layer. The pouch was not permeable to O<sub>2</sub>.

Each experiment described in this paper was repeated at least three times, and the results shown represent the average of all experiments.

## RESULTS

### Growth experiments on radiation sterilized chicken skins

When chicken skins, inoculated with *C. botulinum* type

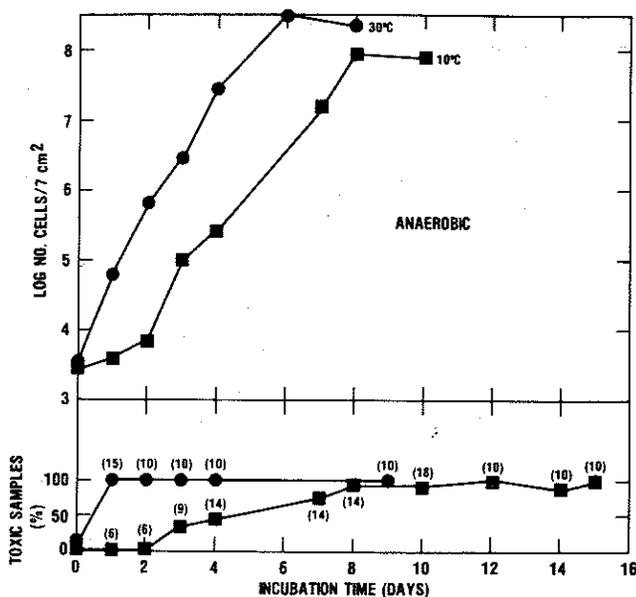


Fig. 1—Growth and toxin production of *C. botulinum* type E (Beluga) on radiation sterilized chicken skins, vacuum sealed, and incubated at 30 and 10°C. Symbols: ●, 30°C; ■, 10°C. The number in parentheses represent the number of skins tested for toxin.

E Beluga spores and irradiated, were incubated anaerobically (vacuum sealed in flexible pouches) at 30°C, cells multiplied and produced toxin within one day (Fig. 1). At 10°C there was little, if any, multiplication and toxin production within the first day. About 40% of the tested samples became toxic within 3 days and 95% of the tested samples were toxic after 8 days, when the number of cells was maximum. Occasionally a sample incubated for up to 14 days at 10°C was nontoxic.

Inoculation of chicken skins with ca 5 × 10<sup>3</sup> spores per 7 cm<sup>2</sup> followed by aerobic incubation at 30°C resulted in growth and toxin production (Fig. 2). At lower levels of inoculum (ca 10<sup>2</sup> spores/7 cm<sup>2</sup>) the production of toxin under aerobic conditions was more erratic (data not shown). Growth rate and toxin production were slower under aerobic than anaerobic conditions (Fig. 1 and 2). At 10°C under aerobic conditions there was little, if any increase in cell numbers and no toxin was detected within 14 days.

### Radiation resistance

Radiation survival curves of *C. botulinum* type E spores on radiation sterilized chicken skins were different from those of spores suspended in water (Fig. 3). The survival curves of irradiated spores on skins could be divided into 3 parts: (i) shoulder (of about 0.1 Mrad), (ii) logarithmic decline (between 0.1 to 0.6–0.7 Mrad) with a D value of 0.16 and 0.17 for Beluga and 1304, respectively, (iii) tail (from 0.7–1 Mrad). In water the survival curve had a more extensive shoulder and no tail was evident by 0.8 Mrad. The difference in irradiation resistance between the strains was small, strain 1304 appearing to be more resistant than Beluga. The survival curves for Beluga, VH and 1304 strains in water exhibited shoulders up to 0.22, 0.26 and 0.29 Mrad, respectively, followed by a logarithmic decline. The D values were 0.099, 0.103 and 0.109 Mrad for Beluga, VH and 1304, respectively.

Radiation sterilized chicken skins were inoculated with *C. botulinum* (Beluga) spores, vacuum sealed in pouches, and irradiated (5°C) with 0.3 Mrad which reduced the population by about 90%. The surviving population grew and produced toxin at 30°C (Fig. 4). About 42% of the

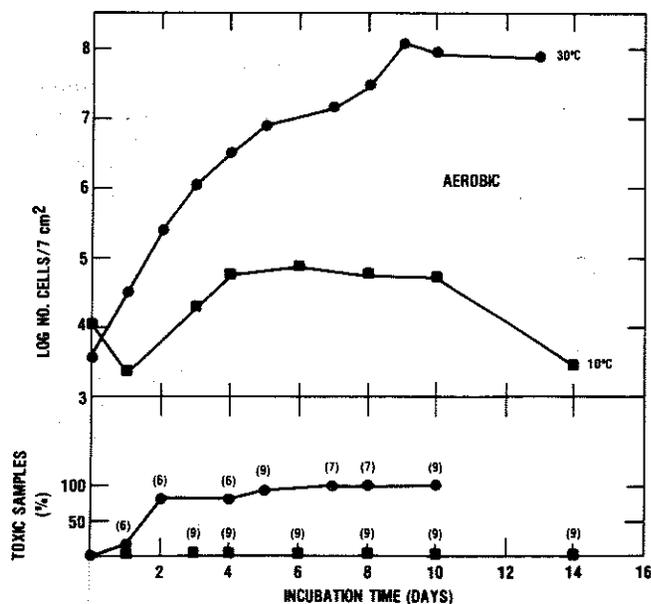


Fig. 2—Growth and toxin production of *C. botulinum* type E (Beluga) on radiation sterilized chicken skins, incubated aerobically at 30 and 10°C. Symbols: ●, 30°C; ■, 10°C. The number in parentheses represent the number of skins tested for toxin.

samples were toxic after one day and all samples were toxic within three days. The irradiation survivors also grew and produced toxin under aerobic conditions at 30°C. However, at 10°C under anaerobic (Fig. 4) or aerobic conditions (not shown) the surviving cells decreased and no toxin was detected during 18 days of storage.

#### Toxin titer on chicken skins

Experiments to determine toxin titer were carried out after maximal growth had occurred (Fig. 1 and 4) under various conditions (Table 1). Unirradiated cells produced much less toxin at 10°C than at 30°C. The highest amount of toxin was produced at 30°C after irradiation (0.3 Mrad), but no detectable toxin was found within 18 days in irradiated samples incubated at 10°C.

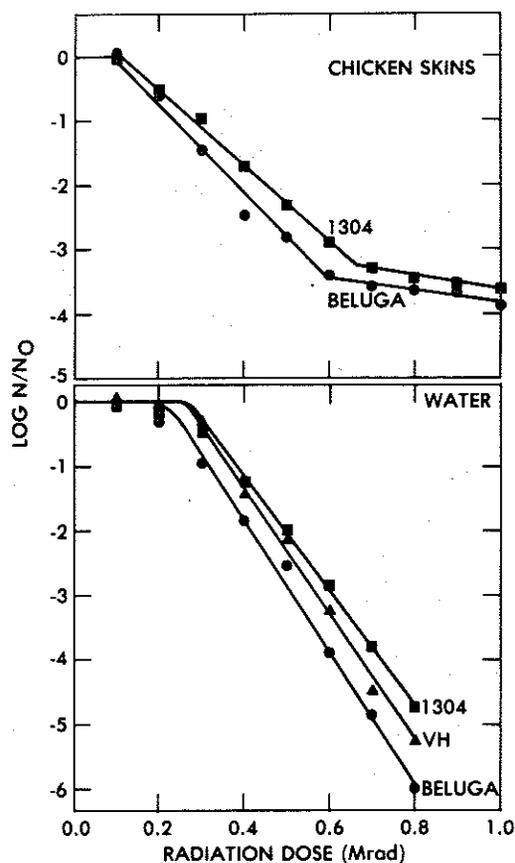


Fig. 3—Radiation resistance of *C. botulinum* type E spores on radiation sterilized chicken skins (ca.  $5 \times 10^6/7 \text{ cm}^2$ ) and in water (ca.  $5 \times 10^7/\text{ml}$ ). All containers were vacuum-sealed and irradiated at 5°C. Symbols: ●, Beluga; ▲, VH; ■, 1304.

#### Growth of *C. botulinum* in competition with the natural microbial flora of chicken skins

Nonsterilized chicken skins were inoculated with *C. botulinum* type E spores (ca.  $5 \times 10^4/7 \text{ cm}^2$ ), vacuum sealed in a pouch, irradiated with 0.3 Mrad at 5°C, and incubated at 30°C. The samples were checked daily for off-odors and toxin and total plate count (Table 2). The surviving natural flora multiplied (above  $10^8$ ) and produced an off-odor by the time that the first toxic sample was detected. When chicken skins were irradiated with 0.5 Mrad, fewer than 10 cells/7 cm<sup>2</sup> survived (data not shown). At least 8 days were needed at 10°C to get countable colonies. Some of the skins irradiated with 0.5 Mrad contained fewer than 10 cells/7 cm<sup>2</sup> even after 21 days at 10°C. At 30°C, the count of these irradiation survivors reached 10 colonies/7 cm<sup>2</sup> after 4 days and the skin had an off-odor only after 6 days.

#### DISCUSSION

UNSTRESSED SPORES of *C. botulinum* type E germinated, multiplied and produced toxin on chicken skins in vacuum sealed pouches at 10°C, within 3 days. On most surfaces of vacuum sealed fish (e.g. herring, cod, haddock) inoculated with *C. botulinum* type E (ca  $10^3$  spores/pack) toxin was detected after 6–12 days of incubation at 10°C (Cann and Taylor, 1979; Cann et al., 1966).

The growth of *C. botulinum* type E on chicken skins incubated under aerobic conditions at 30°C was not unex-

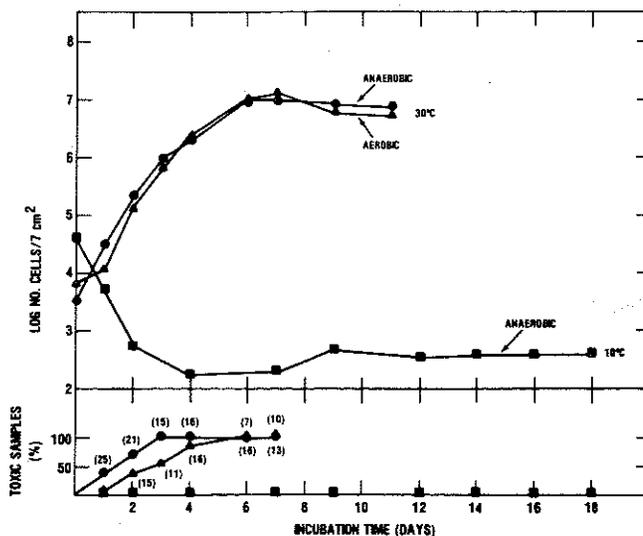


Fig. 4—Growth and toxin production of *C. botulinum* type E (Beluga) spores, inoculated and irradiated on radiation sterilized chicken skins. Symbols: ●, 30°C in vacuum-sealed flexible pouches; ▲, 30°C grown under aerobic conditions; ■, 10°C in vacuum sealed flexible pouches.

Table 1—Toxin titer produced by *Clostridium botulinum* type E (Beluga) on chicken skins incubated in vacuum sealed pouches

Incubation temp (°C)	Radiation <sup>a</sup> dose (Mrad)	Incubation time (days)	Avg cell number per 7 cm <sup>2</sup>	No. of samples tested	Mouse LD <sub>50</sub>	
					Range	Average
30	0.0	7	$2.0 \times 10^8$	4	340–520	430
30	0.3	9	$8.9 \times 10^7$	3	1200–1400	1300
10	0.0	10	$9.0 \times 10^7$	7	<1.0–128	54
10	0.3	18	$4.1 \times 10^2$	6	<1.0	<1.0

<sup>a</sup> Irradiated at 5°C

## C. BOTULINUM ON IRRADIATED CHICKEN SKINS...

pected. Similar results were shown by others. Abrahamsson et al. (1965) found toxin on inoculated surfaces of herring fillets incubated aerobically at 20°C. Kautter (1964) showed that inoculated smoked fish stored at 30°C became toxic during the same period whether the fish was exposed to air or incubated under anaerobic conditions. In all these experiments the inoculum level was high (above 10<sup>5</sup> spores/pack). The oxidation-reduction potential required for growth of type E spores on chicken skins was achieved on these tissues even under aerobic conditions. The mechanism by which anaerobic conditions were achieved may be similar to that found with fastidious anaerobes grown on the rumen epithelium (Cheng and Costerton 1979). The growth of anaerobes was due to the availability of an ecological niche in which facultative anaerobes generated enormous reducing capacity and thereby created local anaerobic conditions. However, our results showed that if broiler skins were kept under refrigeration (10°C or lower) or if the initial level of type E spores was low (<10<sup>2</sup>/7 cm<sup>2</sup>) no toxin was produced within 18 days under aerobic conditions.

The irradiation survival curve of *C. botulinum* type E spores in aqueous suspension was characterized by a shoulder (0.22–0.29 Mrad) followed by a logarithmic decline (D = 0.099–0.109 Mrad), similar to data found in the literature (Schmidt et al., 1962; Roberts et al., 1965; Segner and Schmidt, 1966). When the spores were suspended on chicken skins, there was a tail in the irradiation survival curve. This tail was not due to contamination or counting of low numbers of colonies. It is possible that some spores penetrated into crevices or channels of capillary size on the chicken skins, (Thomas and McMeekin, 1981), offering more protection against irradiation.

*C. botulinum* spores, surviving irradiation, grew and produced toxin under both aerobic and anaerobic conditions at 30°C. However, those irradiation survivors appeared to be injured, since unlike unirradiated spores they did not produce toxin at 10°C within 18 days. In fact, even under anaerobic conditions, there was a decrease in survivors at 10°C. Results of studies in progress support our hypothesis that type E *C. botulinum* spores surviving an irradiation dose of 0.3 Mrad were unable to produce toxin on chicken skin at 10°C because of radiation injury. These studies, to be published later, show that spores were indeed injured by radiation as evidenced by various criteria. The report on outgrowth and toxin production of *C. botulinum* type E at 3.3°C (Schmidt et al., 1961) did not consider the effect of processing conditions on injury of the spores and the possibility that such injured cells might be less able than uninjured cells to grow and produce toxin at low temperatures. It was found that injured spores have a much more restricted temperature range for growth (Adams, 1978). Therefore, stress factors should be considered when one recommends storage temperatures for specific foods.

A dose of 0.25–0.30 Mrad effectively reduces *Salmonellae* (Mulder et al., 1977). The presented data show that an irradiation dose of 0.3 Mrad (but not 0.5 Mrad) left enough of the natural flora to compete with *C. botulinum* type E cells, if present, on severely abused (30°C) chicken skins. Under anaerobic conditions at 30°C an off-odor was produced by the surviving natural flora as a warning against improper storage. The results indicate that there may be no need to specify a storage temperature of ≤3.0°C for low-dose irradiated chicken as was done for irradiated fish.

Table 2—Anaerobic growth of the surviving natural flora and production of *Clostridium botulinum* type E toxin at 30°C after 0.3 Mrad<sup>a</sup>

Incubation time (days)	No. of samples examined	Avg plate count <sup>b</sup> of normal flora/7 cm <sup>2</sup>	Toxic samples (%)	Odor of all the samples
0	9	1.1 × 10 <sup>2</sup>	0.0	Normal
1	9	8.6 × 10 <sup>5</sup>	0.0	Slight off odor
2	9	5.5 × 10 <sup>8</sup>	11.1	Off odor
3	8	1.2 × 10 <sup>9</sup>	50.0	Off odor
4	8	1.8 × 10 <sup>9</sup>	50.0	Strong off odor
7	9	4.0 × 10 <sup>8</sup>	55.5	Strong off odor
9	9	—	88.9	Strong off odor
14	9	—	88.9	Strong off odor
16	9	—	100.0	Strong off odor

<sup>a</sup> Prior to irradiation, the chicken skins were inoculated with ca 5 × 10<sup>4</sup> spores/7 cm<sup>2</sup>.

<sup>b</sup> Counts on Plate Count agar (BBL), the plates were stratified with the same medium and incubated at 25°C.

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