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Research Article

Effect of ^{60}Co Irradiation on Biofilms Produced by *Salmonella Enterica*

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Abstract

Salmonella enterica constitutes a major contaminant in food matrices and its biofilm-forming capabilities are an important factor in their persistence in food, and the surrounding environments generating a public health concern. A number of irradiation sources have been used to control the growth of *Salmonella* spp. on various food and contact surfaces; however studies into the effectiveness of irradiation on biofilm-associated cells have been lacking, and the effect of ^{60}Co irradiation could destroy *S. enterica* biofilms. Radiation treatments above 250 krads resulted in complete elimination of *S. enterica* biofilms, while doses of 100 krads resulted in complete recovery of *S. enterica* biofilms following the Bacteriological Analytical Manual method used at the Food and Drug Administration. Pulse field yield electrophoresis analyses showed no changes in gene expression when biofilms were exposed to different irradiation doses, and transmission electron microscopy results showed no changes in exopolysaccharide production under different irradiation treatments. Moreover, all irradiated samples were positive in VIDAS™ indicating that *Salmonella* antigens were present, and although no viable biofilms were recover above 250 krads, these antigens were detected and not completely destroyed by any irradiation level tested in our study.

Keywords: *Salmonella enterica; ATCC 8324; Biofilms; Irradiation; Cobalt 60; Food safety*

Introduction

Salmonella are rod-shaped flagellated Gram-negative bacteria and common food-borne pathogens that are causative agents of human salmonellosis. They have widespread occurrence in animals and most are motile [1]. The ability of *Salmonella* to colonize fruits and vegetables makes these products frequent vehicles of salmonellosis. Compounding this situation is the perishable nature of these products, which generally prevents their analysis for human bacterial pathogens before marketing. In addition, microorganisms are naturally present in fresh produce, and the growing popularity of convenience foods, such as precut and prepackaged produce, introduces new public health concerns, because damaged plant tissues release nutrients and provide a favorable matrix for bacterial proliferation [3], which can lead to biofilm formation. Biofilms are microbial derived sessile communities characterized by cells attachment to a solid surface, a soluble interface, or by adherence to each other [4,10] The cells of a biofilm are embedded in an extracellular polymeric matrix (EPS), the hallmark of biofilm formation, and exhibit an altered phenotype with respect to growth rate and gene transcription [5]. It has previously been reported that *Salmonella* spp. form biofilms tightly attached to plastic, glass, cement and stainless steel [7]. Biofilm formation in food and surrounding environments has been a concern for food companies because of the resistance of biofilms to disinfectants and their enhanced survival mechanisms as compared to their planktonic counterparts [7,8,13].

The aim of irradiation of fresh fruit and vegetables is to inhibit postharvest contamination by reducing the microbial surface flora, thus minimizing storage problems at any temperature and consequently spoilage due to microorganisms [12]. Ionizing gamma irradiation (gamma ray) has been used to evaluate *Salmonella* radiation sensitivity on biofilms and planktonic cells [9] however; no studies

have investigated the phenotypic variations of changes in genetic patterns after gamma irradiation or re-culturing of cells from biofilms according to the Bacteriological Analytical Method [1].

The goals of our research were to determine the effectiveness of ^{60}Co irradiation in eradicating *Salmonella enterica* biofilms and to evaluate its effects on biofilm re-formation, structure, and gene expression.

Materials and Methods

Bacterial strains and biofilm culture conditions: Aliquots of *Salmonella enterica* ATCC 8324 strains previously diluted in phosphate buffer and were frozen in phosphate buffer and 50% glycerol at <80°C. Aliquots were plated and counted to determine ~30 CFUs for inoculation purposes. Approximately thirty colony forming units (CFU) of *S. enterica* ATCC 8324 were inoculated into each well of six-well multidish plates (Nunclon Delta SI, Thermo Scientific, Waltham, MA) containing 5 mL of nutrient broth (Difco, Co). Plates were incubated for 72 h at 35°C in a water bath (Precision, Thermo Scientific and Marietta, OH).

Irradiation protocol: Bacterial cultures were irradiated in the Gamma Cave Facility at the University of Massachusetts Lowell Radiation Laboratory. Prior to exposing the plates containing *S. enterica* cultures, a dosimetric analysis was performed by using Alanine Pellets to determine the positional dose rate and dose distribution to the plates. Alanine Pellet Dosimeters (Batch #T030901) and Bruker e-scan EPR reader #0162 were used to measure the radiation dose. The Bruker dosimetry system uses Electron Paramagnetic Resonance (EPR), a dosimetric method of radiation detection in which the quantity of unpaired electrons or free radicals is directly measured. Free radicals are formed when a material such as Alanine is exposed to an ionizing radiation field. The Bruker system measures the EPR signal in the Alanine dosimeter and correlates it to a dose by applying a fitting function. The fitting function is generated based on the system response to reference dosimeters irradiated and certified by the National Institute of Standards and Technology (NIST).

For efficient use of the allotted radiation time, the samples were bundled and irradiated simultaneously. The dosimetric analysis consisted of attaching dosimeters to the outside surface of each bundled group, as well as inserting them within the empty plate cavities. The samples were positioned on a rotating platform in a square configuration. The geometric center of the samples was located 9 inches from the source window. The measured dose distribution at the outside surface of each bundled sample was within +/-3%. The measured dose distribution through each bundled group was within +/-20%. The average dose rate was used to calculate the exposure time for each required dose level. For example, group 1 was removed after 30 minutes and received 100 krad while group 4 was the last to be removed after approximately 5 hours and received 1000 krad. Three six- well plates were used as controls; the remaining plates were irradiated with Co at 100, 250, 500 or 1,000 krad. Experiments were conducted two times in triplicate.

Microbiological analyses after irradiation: After irradiation, the cells on each plate were enumerated by withdrawing a 100 µl aliquot of the liquid culture and plating serial dilutions onto triplicates of Tryptic Soy Agar with yeast extract (TSAye) plates (Difco, Co.). Plates were incubated at 35°C for 24 h, at which time the colonies were counted. In addition, cells from each plate well were streaked with a sterile swab (Fisher Scientific, Houston, TX) onto Hektoen Enteric, Bismuth Sulfite and Xylose-Lysine-Deoxycholate agars (XLD) (Difco, Co.).

For resuscitation analyses, each plate was tested as a separate sample and one milliliter from each of five wells (5 ml total) was inoculated into 25 ml of Lactose broth and incubated for 24 h at 35°C as per the AOAC method [2]. After incubation, 0.1 ml from each composite was transferred to Rappaport Vasiliadis (RV) (Difco, Co.) medium and 1 ml into Tetrathionate broth (Difco, Co.) and incubated for 18-24h at 42°C. One ml from each culture was transferred to an individual tube of M broth (Difco, Co.) and incubated 6-8 h at 42°C. After 6h incubation, 1 ml from each M broth tube was transferred to a glass tube and heated for 15 min at 100°C.

Composites from RV and TT were streaked onto BS, HE and XLD agars and incubated for 24h at 35^{0}C. Five colonies were selected from BS medium and transferred to Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA) following the Bacteriological Analytical Manual method for Salmonella [1]. TSI cultures from colonies were used to inoculate individual API 20E strips (bioMérieux®, Marcy l'Etoile, France), and Trypticase soy-tryptose broth for serology, and TSA with sheep blood agar (Remel, Lenexa, KS) for PFGE analyses.

Enzyme-linked fluorescent assay (ELFA): Identification of *Salmonella* antigens was performed using an automated VIDAS™ system (BioMérieux® SA, Marcy l'Etoile, France) as described elsewhere (bioMérieux® SA, User's Manual 2005) [2]. An aliquot of the boiled test suspension is placed into the reagent strip and is cycled in and out of the SPR for a specific length of time. *Salmonella* antigens, if present, bind to the monoclonal antibodies coating the interior of the SPR. All other unbound material is washed away. The substrate, 4-methyl umbelliferyl phosphate, is converted by the enzyme on the SPR wall to the fluorescent product, 4-methyl umbelliferone. The intensity of fluorescence is measured by the optical scanner in VIDAS™. The fluorescence intensity is measured twice at 450 nm. The first result is related to the background, the second is the value after incubation of the substrate with enzyme. Based on that, the apparatus calculates the result of the test and interprets it as a positive or negative one. The RFV (Relative Fluorescence Value) is calculated as the difference between the sample and background fluorescence. The printed report contains the RFV of the sample, RFV of the standard, and test value (TV), which is a quotient of the sample value and standard value. A result was interpreted by the apparatus as positive, if TV ≥ 0.23, while as negative if TV ≤ 0.23. Results are interpreted after the test values and control are compared to thresholds stored in the computer.

Samples from RV and TT tubes were streaked onto BS, HE and XLD agars and incubated for 24h at 35°C. Colonies were selected from each medium and transferred to TSI and LIA following the Bacteriological Analytical Manual analyses for *Salmonella* [1]. Growth from TSI cultures was used to inoculate API strips for biochemical analyses, and Trypticase soy-tryptose broth for serology, and TSA with blood agar for PFGE analyses (as described in Microbiological Analyses after Irradiation section).

Transmission Electron Microscopy: After irradiation, 1 ml of each biofilm sample was used for Transmission Electron Microscopy (TEM) analysis and the non-irradiated (NI) planktonic sample was used as a control. Biofilms were asceptically pipette and placed in vials. TEM was performed using ruthenium red to stain the extracellular polymeric matrix produced by the cells. Previous to stain with ruthenium red, samples were fixed for at least 2h at room temperature in a 0.1 M sodium cacodylate buffer containing (pH 7.4) 2.5% glutaraldehyde 1.25% paraformaldehyde and 0.03% picric acid. Next 0.5% Ruthenium Red (RR) [12] (EMS catalog # 20600) was added to the same fixative and the cells were incubated for 3 more hours at room temperature. The pellets were then washed 3 xs with 0.1M cacodylate buffer, with 0.5% RR and postfixed with 1% Osmiumtetroxide (OsO4) and 0.5% RR for 2h. They were then washed in water 3x and incubated in 1% aqueous uranyl acetate for 1h followed by 2h washes in water and subsequent dehydration in increasing grades of alcohol (10 min each; 50%, 70%, 90%, 2 x 10 min 100%). The samples were then put in propyleneoxide for 1 hr and infiltrated overnight in a 1:1 mixture of propyleneoxide and Spurr's Low Viscosity Embedding media (EMS, Hatfield, PA). The following day, the samples were embedded in Spurr's Low Viscosity Embedding media and polymerized at 60°C for 48h.

Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids, stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG^2 Spirit BioTWIN. Images were recorded with an AMT 2k CCD camera. (Most Chemicals were purchased from EMS Hatfield, PA).

Pulse-field gel electrophoresis protocol: Pulsed-field gel electrophoresis (PFGE) was carried out according to the CDC PulseNet protocol [11]. PFGE plugs were prepared from growth on TSA with sheep blood agar plates. Plugs were digested with XbaI (Fisher Scientific, Fair Lawn, NJ). Electrophoresis was performed in a CHEF-Mapper apparatus (Bio-Rad Laboratories, Hercules, CA). The gel was stained with GelRed™ (Biotium, Hayward, CA) and digitally photographed with a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA). Patterns were analyzed by visual comparison of the digital gel images.

API20E: API20E results were performed following the manufacturer instructions (bioMérieux® SA, Marcy l'Etoile - France). Each well in the multi-test strip was inoculated with the culture suspension in sterile saline. Test strips were incubated for 24 h at 35^{0}C. Strip results were read and interpreted by browsing the APIweb™ (bioMérieux, apiweb.biomerieux.com).

Serology: Serological somatic (O) and (H) antigen tests for *S. enterica* after irradiation were conducted as recommended by the manufacturer (Beckton Dickinson and Co., Sparks, MD), added one drop of culture suspension and one drop of *Salmonella* polyvalent somatic (O) antiserum or (H) antiserum, mixing the suspension for one minute and then observing against a dark background for agglutination.

Results and Discussion

Enumeration and viability

^{60}Co is a common gamma source used to reduce bacterial growth, and it is approved for food matrices and packing materials [12]. In our study, microbial biofilms of *S. enterica* were effectively reduced when using different doses of ^{60}Co as compared to non-irradiated biofilms. Enumeration results were as follow: 0 CFUs at 1,000 krad, 0 CFUs for 250 krad, 5 x 10^4 CFUs for 1krad, and 18 x 10^4 CFUs for NI. Significant differences were also observed during visual inspection of the biofilms of irradiated samples when compared to the non-irradiated control sample (Figure 2). Non-irradiated biofilms consisted of large cream colored units (Figure 2a) mostly developed in the liquid interface, while the irradiated biofilms disrupted into very small sand-type cream color units (Figure 2b-e). This reduction was dose-dependent and it was significantly noticeable when samples were tested immediately after irradiation for viability and in three different agars (Figure 1). For all NI, 100krad and positive control, growth in BS agar (Figure 1a) showed a dark gray color, growth in XLD agar (Figure 1b) showed glossy black color, and growth in HEK (Figure 1c) showed a blue-green color. NI and 100 krad typical colonies on the BS agar were transferred to triple sugar iron agar (TSI) and lysine iron agar (LIA). TSI and LIA slants incubated at 35°C for 24 ± 2h showed in TSI an alkaline (red) slant and acid (yellow) butt, with production of H_2S (blackening of agar) (pictures not shown). NI and 100 krad, produced a positive reaction (purple) in LIA (pictures not shown).

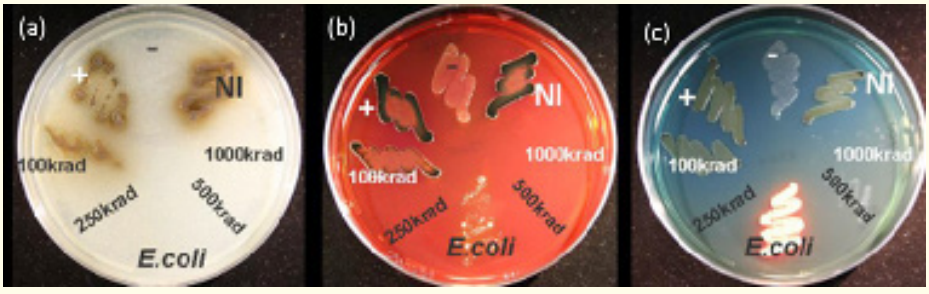


Figure 1: *Salmonella enterica after exposure to four irradiation doses (100, 250, 500, 1000 krad) of ^{60}Co and subsequent inoculation onto BS (a), XLD (b), and HE (c) agar plates. Positive control (12) ATCC 8324 (Salmonella enterica), Negative control (12) ATCC 9842 (atypical Salmonella), Non- irradiated sample (9) ATCC 8324 (Salmonella enterica), E. coli (Escherichia coli). For all NI, 100 krad and positive control, growth in BS agar (Figure 1a.) showed a dark gray color, growth in XLD agar (Figure 1b) showed glossy black color, and growth in HEK showed a blue-green color.*

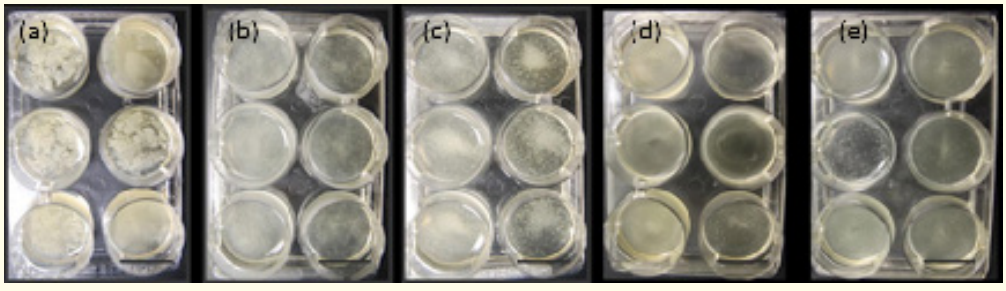


Figure 2: *Salmonella enterica control and irradiated samples at 72 h in nutrient broth.*
(a) non-irradiated (NI) sample showing large cream units, (b) 100 krad showing smaller cream units (c) 250 krad showing sand-type cream units (d) 500 krad and (e) 1,000 krad, both showing sand-type cream units. Scale bar: 25mm.

Microscopy

Transmission electron microscopy using ruthenium red stain revealed that planktonic cells did not have a surrounding EPS material (Figure 3a), in contrast, biofilms from *Salmonella* had a surrounding EPS matrix (Figure 3b-arrow). EPS is consider the hallmark of biofilm formation, and in this study, EPS material produced and surrounding the cell was not disrupted at any of the irradiation levels tested (Figure 4a-d), when stained with ruthenium red, and examined under TEM.

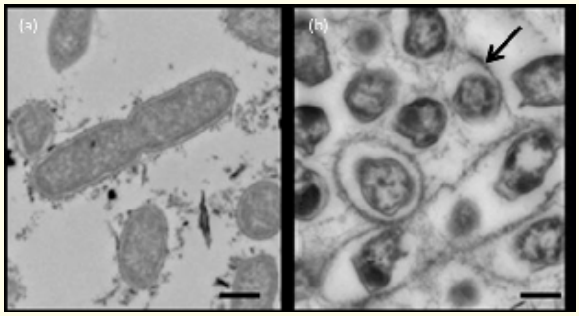


Figure 3: *TEM results of S. enterica planktonic and biofilm cells.*
(a) TEM of planktonic cells of S. enterica ATCC 8324
(b) TEM of biofilm of S. enterica ATCC 8324 produced and fixed after 72 h.
Planktonic cell of S. enterica ATCC 8324 lacks the surrounding material (Fig. 3a). Biofilm cells were surrounded by an extracellular polymeric substance (EPS), the hallmark of biofilm formation (Figure 3b-arrow).

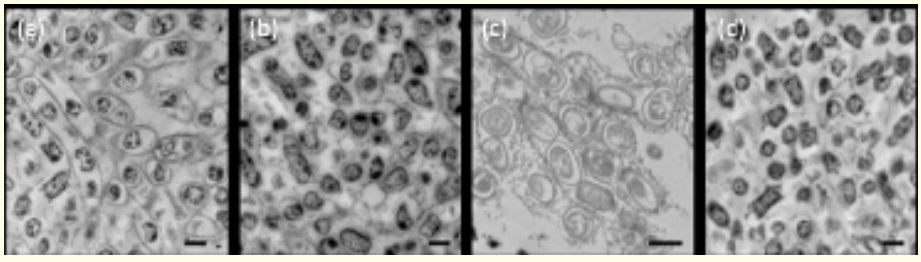


Figure 4: *TEM of Salmonella enterica biofilms when irradiated with ^{60}Co at (a) 100, (b) 250, (c) 500 and (d) 1,000 krad. Salmonella cells surrounded by EPS material was not destroyed with ^{60}Co irradiation, as shown in this study when stained with ruthenium red, and examined under TEM, at all irradiation level tested (Figure 4a-d).*

Bacteriological Analytical Manual

RV tubes were incubated at 42°C for 18-24h as per the AOAC method (AOAC Official Methods 2004.03). Following incubation, a color change was noted for the non-irradiated and 100 krad samples. No color change was noted for the 250, 500 and 1000 krad samples.

All irradiated samples were positive in VIDAS™ indicating that *Salmonella* antigens were present and bound to monoclonal antibodies provided in the VIDAS solid-phase receptacle. These antigens were not completely destroyed by any irradiation level tested in our study. Although the fluorescence levels measured are directly proportional to the quantity of antibody or antigen present in the sample (bioMérieux®, SA, Marcy l'Etoile, France, 2005 VIDAS™ Instrument user manual), test values were inversely proportional to the irradiation levels when increase from 100 to 1,000 krad.

Only the non-irradiated sample control and the 100 krad showed growth and typical colonies on BS, HE and XLD agars; no cells were recovered from 250, 500 and 1,000 krad. It is noticeable that antigen detection in VIDAS™ is not directly related to viability of the cells in agar plates. Levels of 250 krads are known to lower the concentration of total and reducing sugars in fruits and vegetables. Therefore, levels of 250 krad and above are not recommended for irradiation of cellulose material [6].

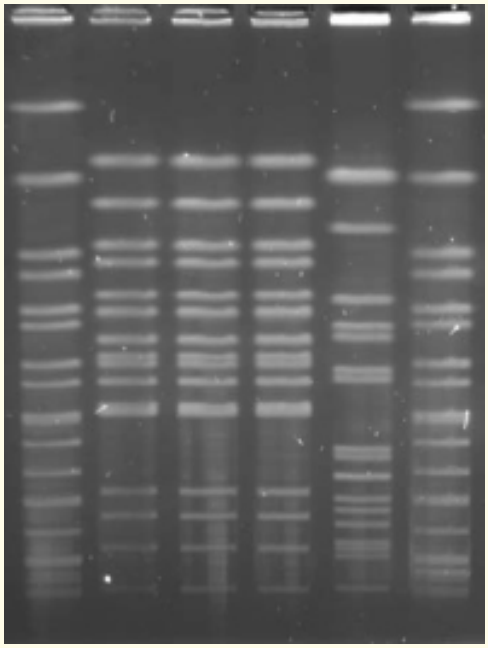
Following the BAM method, colonies from NI and 100 krad isolates from BS were inoculated into TSI and LIA slants incubated at 35°C for 24h. NI and 100 krad samples produced positive reaction (purple color) in LIA (picture not shown). Control and NI TSI tubes exhibited ammonium sulfate production following 24h incubation. However, 100 krad samples required a 48h incubation to show ammonium sulfate production. Irradiation appeared to delay this biochemical reaction (pictures not shown).

API Results and Serology

API demonstrated that non- irradiated sample of *S. enterica* had the same biochemical pattern as the 100 krad sample irradiated with ^{60}Co. Serology results revealed that both antigens were present in 100 krad sample when compared to the negative control (NI sample).

PFGE

The band pattern for 100 krad samples was identical to that of the NI sample and the control (Figure 5). Irradiation of 100 krad has been shown to be safe for food products [6]. Although, the biochemical characteristics of the cells may have been affected, genetic expression has not been compromised (Figure 5). The present studies show that ^{60}Co is useful in removing biofilms produced by a foodborne pathogen *Salmonella enterica*, and irradiation with ^{60}Co this dose does not affect the biochemical or genetic composition of this microbe ensuring microbial safety.



Lane 1 2 3 4 5 6

Figure 5: *Lane 1: PFGE standard; Lane 2: 100 krad; Lane 3: Non-irradiated sample; Lane 4: ATCC 8324; Lane 5: ATCC 9842, and Lane 6: PFGE standard.*

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