

The evaluation of gamma irradiation and cold storage for the reduction of *Campylobacter jejuni* in chicken livers[☆]

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ABSTRACT

Recent outbreaks of *Campylobacter* mediated disease attributed to undercooked chicken livers have highlighted a continuing need for methods to reduce *Campylobacter* numbers in these types of food products. In this study, gamma irradiation is evaluated for its effectiveness in reducing *Campylobacter jejuni* numbers in experimentally contaminated chicken livers. A wide range of radiation doses were evaluated in conjunction with cold storage parameters, before and after irradiation. Storage of chicken livers at -20°C prior to radiation treatment, as expected, increased *C. jejuni* radiation resistance. Livers previously stored at -20°C exhibited D_{10} values of 0.748 kiloGray (kGy) compared to livers without previous storage that had a significantly lower D_{10} value of 0.361 kGy. Cold storage conditions post-irradiation at both 4°C and -20°C further reduced the *C. jejuni* numbers over those reduced by the initial irradiation. The largest reduction (3.8 logs) of *C. jejuni* numbers in livers produced by combining irradiation and cold storage was achieved using 0.8 kGy of radiation followed by 1 week storage at -20°C . This reduction of 3.8 logs was not determined to be significantly different from the 3.5 log reduction achieved with the same radiation dose (0.8 kGy) after only 48 h of subsequent storage at -20°C .

1. Introduction

Campylobacter species are responsible for the largest number of bacterial mediated food-borne gastrointestinal infections in the developed world (CDC, 2006; Humphrey et al., 2007). Among the different *Campylobacter* species, *Campylobacter jejuni* is responsible for the majority of cases of human disease (Moore et al., 2005). Poultry are a common reservoir for *C. jejuni*, and provide a convenient pathway for transmission to humans by the consumption of undercooked poultry and the contamination of additional food products by raw poultry (Bryan and Doyle, 1995). Poultry products have been found to be contaminated with *Campylobacter* at a high frequency, with reports in the United States of 30% and in the European Union of 75% of products contaminated (European Food Safety Authority, 2010; USDA Food Safety Inspection Service (FSIS), 2012).

In recent years, a series of human disease outbreaks have occurred in the United States that have been attributed to *C. jejuni* contamination

of chicken livers (Glashower et al., 2017; Scott et al., 2015; Tompkins et al., 2013). Like many other poultry products, chicken livers have been found to be frequently contaminated with *Campylobacter* (Barot et al., 1983; Baumgartner et al., 1995; Strachan et al., 2012). For the most part, the outbreaks appear to be the result of undercooking the chicken livers that are subsequently used to manufacture a chicken liver pâté dish. Notably, these sort of outbreaks do not appear limited to the United States and have occurred in other locations in the developed world (Little et al., 2010; O'Leary et al., 2009). Given the reoccurring nature of the outbreaks, methods to greatly lower the levels of *Campylobacter* contamination in chicken livers should be prioritized.

Previous research efforts have investigated the effectiveness of gamma-irradiation for reducing the numbers of *Campylobacter* in poultry, with most cases producing promising results (Kudra et al., 2012; Lambert and Maxcy, 1984; Patterson, 1995; Raut et al., 2012; Xavier et al., 2017). Radiation damage to genetic material occurs via two mechanisms: direct photonic damage to the DNA backbone or

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indirect damage via generation of reactive oxygen species such as hydroxyl radicals, with indirect damage accounting for > 75% of bacterial inactivation (Sommers et al., 2002a; Sommers et al., 2002b). Irradiation of microorganisms at -20°C has been shown to increase their radiation resistance due to limited mobility of hydroxyl radicals in a frozen environment compared with those at temperatures of 4°C (Bruns and Maxcy, 1979; Thayer and Boyd, 1992, 2001). Lambert and Maxcy (1984) obtained D_{10} values of 0.32 kGy (-30°C), and 0.16 kGy ($0-5^{\circ}\text{C}$) for *Campylobacter* in beef. Patterson (1995) obtained D_{10} of 0.12–0.25 for *Campylobacter* spp. in raw poultry meat. Kudra et al. (2012) obtained D_{10} of 0.29 and 0.31 kGy for *C. jejuni* in refrigerated chicken meat, with or without modified atmospheric packaging. Raut et al. (2012) obtained D_{10} of 0.11–0.19 kGy for *C. jejuni* in refrigerated chicken meat. However, there exists little information specifically on the effectiveness of reducing *C. jejuni* numbers in chicken livers with gamma-irradiation, (Kawasaki et al., 2018); which has prompted the undertaking of this potentially useful investigation. Additionally, since chicken livers are often subjected to cold storage before being used by consumers, this investigation included common cold storage temperatures and times in conjunction with irradiation to determine the effects on reducing *C. jejuni* numbers.

2. Materials and methods

Bacterial Strains: Seven *Campylobacter* strains isolated from chicken livers implicated in outbreaks of human disease were kindly supplied to our lab by the Food Safety & Inspection Service (FSIS). The strains designated: FSIS11705135, FSIS11705136, FSIS11705137, FSIS11705138, FSIS11705139, FSIS11705140, FSIS11705141 were confirmed as *C. jejuni* by *gyrase B* sequencing (Gunther et al., 2011). The strains were stored frozen at -80°C in 15% glycerol until needed for experiments.

Chicken Livers: Whole chicken livers were purchased from a retail source. Each whole liver was divided into roughly three equal pieces for individual use in subsequent experiments. Livers were stored separately and frozen at -20°C . Next, the frozen livers were irradiated to sterility (20 kGy) using a self-contained ^{137}Cs gamma irradiator (Lockheed Georgia Company, Marietta, GA) at a dose of 0.07 kGy/min (NIST calibrated). The sample temperature (-20°C) was maintained during irradiation by the injection of liquid nitrogen into the sample chamber. After irradiation, samples were stored at -20°C until needed. Routine dosimetry was conducted using alanine pellets and a Bruker eScan (Bruker, Billerica, MA) with a dose uniformity ratio of less than 1.1: 1.0.

Gamma Irradiation Treatments: The seven *C. jejuni* strains were plated directly from freezer stocks onto Brucella agar plates (Becton Dickinson, Sparks, MD) and incubated for 24 h at 42°C in a microaerobic (5% O_2 , 10% CO_2 , 85% N_2) Concept-M growth chamber (Baker-Ruskin, Bridgend, UK). The colonies that grew on the agar plates from the seven *C. jejuni* strains were used to individually inoculate 3 ml Brucella broth cultures that were incubated ~ 16 h microaerobically at 42°C . A spectrometer was used to measure the OD_{600} of the seven resulting cultures, and based on these results, the cultures were all diluted to an OD_{600} of 0.3. The seven cultures were then combined to form a single *C. jejuni* cocktail. Sterile livers were defrosted and 0.5 ml of the *C. jejuni* cocktail was injected into the center of each chicken liver as three separate injections (150, 150, and 200 μl s) using fine point disposal pipette tips (Rainin, Columbus, OH). The livers were placed in stomacher bags (Nasco, Fort Atkinson, WI) and vacuum sealed before being weighed. At this point, half the number of experimentally contaminated livers were placed at -20°C for one week while the other half of the total livers were immediately irradiated with the appropriate dose. Irradiation was again accomplished using the self-contained ^{137}Cs gamma irradiator at a dose of 0.07 kGy/min, but this time in a chamber cooled to 4°C or -20°C . Irradiation was conducted at 0.2 kGy increments. Routine dosimetry was conducted using an eScan Alanine System (Bruker, Billerica, MA) according to ASTM standards. Control

samples that received no irradiation were also included as part of these experiments. After irradiation the livers were each transferred to a homogenizer filter bag (Nasco, Fort Atkinson, WI) and 1 ml of Brucella broth was added to each liver, the livers were briefly smashed by hand and were then placed in a stomacher type homogenizer (Triple mix paddle blender, Boekel Scientific, Feasterville, PA) for 1 min at 300 rpm. Three hundred microliters were removed from each homogenized liver, serially diluted five times (30 μl cell solution into 270 μl diluent) and each of the resulting six cell concentrations in the dilution series were plated six times onto Brucella agar in 10 μl drops following the 6×6 plating method (Chen et al., 2003). Agar plates were incubated for ~ 16 h and the resulting *C. jejuni* colonies present at the various dilutions were counted and recorded. The resulting colony forming units (CFU) per ml for each individual liver sample was determined and used in conjunction with the corresponding weight (previously measured) for each liver in order to quantify the number of surviving *C. jejuni* per liver. The samples previously stored for 1 week at -20°C were processed exactly as the samples described above that were not previously stored at -20°C . These experiments were performed as three separate experimental replicates.

2.1. Gamma irradiation plus subsequent cold storage

Irradiation treatment plus subsequent cold storage experiments were performed similarly to the experiments described above, except they included post-irradiation cold storage and did not include any storage at -20°C prior to irradiation. Cold storage was performed at two different temperatures (4°C and -20°C) and for two different time periods (48 h and 1 week). After the samples were irradiated, they were transferred directly to a -20°C freezer or a 4°C refrigerator. As was done previously, control strains that received no irradiation were included in the experiments as well as irradiated strains that received no subsequent cold storage. Samples were removed from the two cold storage conditions at the appropriate times and processed as previously described to determine the number of surviving *C. jejuni*. Again, these experiments were performed as three separate experimental replicates.

2.2. Statistical analysis

Colony counts resulting from the 6×6 plating method have a limit of detection of 100 cells per ml. For the experiments observing *C. jejuni* survival in chicken livers, this limit increased slightly to 1000 cells per ml as a result of the homogenized livers producing small particles that could not be completely filtered out of the samples, obscuring the *C. jejuni* colonies that grew in the undiluted sample plating making them impossible to properly count. Surviving *C. jejuni* cell counts were derived from multiple technical replicates and three experimental replicates for each mean survival number presented in this manuscript. From the same data, for each mean survival value, a standard deviation was calculated and included in the subsequent figures. The D_{10} value (the amount of irradiation energy need to decrease the bacterial numbers in the liver by one log) was calculated from the two sets of values presented in Fig. 1. The mean *C. jejuni* cell numbers were log transformed and the values plotted versus the corresponding irradiation amounts. A best fit line was applied to the resulting data plots and the slope of the lines were calculated. The D_{10} value was then derived from the formula: $-1/\text{slope}$.

3. Results

A range of gamma irradiation doses were applied to chicken livers experimentally contaminated with a seven strain cocktail of *C. jejuni*. The resulting surviving *C. jejuni* numbers were plotted in relationship to the applied irradiation dose (Fig. 1). Previous to irradiation, one group of samples were stored for 1 week at -20°C , while the other group was immediately irradiated without any additional treatments or storage.

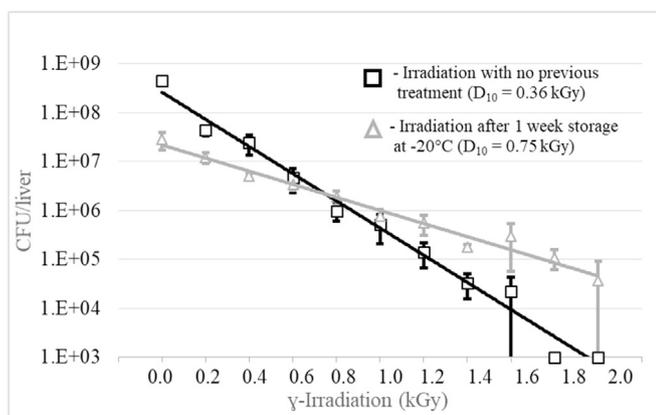


Fig. 1. Survival of a seven strain *C. jejuni* cocktail challenged with a range of different irradiation doses; both with (Δ) and without (\square) previous 1 week storage at -20°C . Best fit lines were applied to each data set and a regression value calculated for each line ($R^2 = 0.99$ for samples without previous -20°C storage and $R^2 = 0.98$ for samples previously stored at -20°C).

The average bacterial numbers in the livers without previous -20°C storage decreased steadily as the irradiation dose was increased reaching the limit of detection for the experiments at between a 1.6 and 1.8 kGy dose. When compared to the average *C. jejuni* numbers in livers that did not receive any irradiation (4.4×10^8 cell/liver), a radiation dose between 1.6 and 1.8 kGy was able to produce a > 5 log reduction on average in contaminating *C. jejuni* numbers ($\leq 1.0 \times 10^3$ cells/liver).

A fitted line was produced for the data set of irradiation doses to surviving bacterial numbers for the samples without previous -20°C storage. The resulting trend line adhered closely to the measured data producing a R^2 value of 0.99. The resulting slope of this fitted line was used to calculate a D_{10} ($-1/\text{slope}$) value for the data set, which produced a value of 0.36 kGy per one log reduction in *C. jejuni* numbers at 4°C .

The data set resulting from the contaminated chicken liver samples previously stored at -20°C for one week prior to irradiation produced results significantly different from the previous samples irradiated without prior -20°C storage (Fig. 1). The untreated samples with previous storage at -20°C had on average 2.8×10^7 cells/liver, which is just over one log less than the untreated livers without previous -20°C storage (4.4×10^8 cells/liver). Despite starting the experiments with significantly fewer *C. jejuni* cells, the samples stored at -20°C previous to irradiation experienced smaller reductions in bacterial cell numbers when exposed to irradiation compared with samples not previously stored at -20°C . The livers previously stored at -20°C never reached undetectable levels of *C. jejuni* contamination even at 2.0 kGy, the highest radiation dose used in this investigation. The average, 2.0 kGy dose *C. jejuni* survival numbers (3.7×10^4 cells/liver) did not even produce a full three logs of bacterial reduction compared to the corresponding untreated samples (2.8×10^7 cells/liver).

Similarly to the fitted line for the previous data, the data for the samples stored at -20°C prior to irradiation formed a line that adhered closely to the data points, producing a R^2 value of 0.98. The resulting slope of this fitted line produced a D_{10} value of 0.748 kGy. The irradiation dose of 0.748 kGy is on average, more than twice the dose of 0.36 kGy needed to produce the same one log reductions in *C. jejuni* when there was no previous -20°C storage.

In addition to -20°C storage prior to irradiation, different cold storage scenarios post-irradiation were investigated, with the goal of further decreasing *C. jejuni* numbers in chicken livers without increasing radiation doses. Two different irradiation doses followed by storage at two different temperatures (4°C or -20°C) for two different time periods (48 h and 1 week) were investigated to determine the

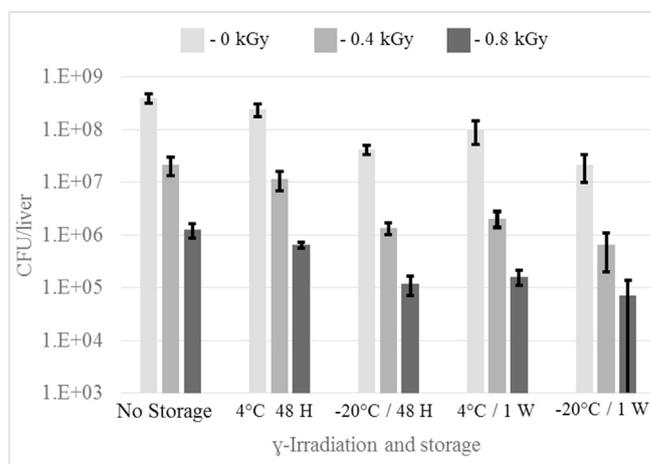


Fig. 2. Survival of a seven strain *C. jejuni* cocktail challenged with three different radiation doses (0.0 kGy, 0.4 kGy and 0.8 kGy), with two different cold storage temperatures (4°C and -20°C) and for two different time periods (48 h and 1 week).

effects on *C. jejuni* survival in chicken livers. The results are presented in Fig. 2. Without any subsequent storage, 0.4 and 0.8 kGy on average, reduced *C. jejuni* numbers in livers by ~ 1.3 and 2.5 logs, respectively. Storage at 4°C alone, without any irradiation, on average produced a 0.2 log reduction after 48 h and 0.6 log reduction after 1 week. Additionally, storage at -20°C alone, on average, produced a 1 log reduction after 48 h and 1.3 log reduction after 1 week. When storage was combined with either of the two different irradiation doses, the resulting *C. jejuni* reductions appeared to be produced by a simple additive effect of the reductions previously seen from irradiation or cold storage alone. As an example, the average reduction from 0.8 kGy irradiation followed by 48 h storage at -20°C compared with no treatments was determined to be 3.5 logs. This average 3.5 log reduction matches the combined reductions achieved by 0.8 kGy irradiation (2.5 logs) and 48 h storage at -20°C , (1.0 logs) achieved separately. The largest average reduction in *C. jejuni* numbers, 3.8 logs, observed in the combinational experiments resulted from 0.8 kGy of irradiation combined with storage at -20°C for one week. However, this reduction was not significantly different from the second largest reduction of 3.5 logs produced by the same dose but with only 48 h of storage at -20°C .

4. Discussion

Outbreaks of human disease caused by the presence of *Campylobacter* in improperly cooked food products is a significant public health problem. Studies looking at the presence of *Campylobacter* in chicken livers have reported prevalence percentages of 31, 77, 81 and 100%, respectively (Baumgartner and Felleisen, 2011; Baumgartner et al., 1995; Noormohamed and Fakhr, 2012; Strachan et al., 2012). Repeated outbreaks attributable to *C. jejuni* contamination of chicken livers underscores the need for interventions to reduce the numbers of *Campylobacter* present in the livers to compensate for undercooking this food product. This need appears to be particularly important given the observations that chicken livers are often intentionally undercooked to achieve a specific color characteristic in pâté, a dish commonly made from chicken livers and one often implicated in outbreaks. (Little et al., 2010; O'Leary et al., 2009; Whyte et al., 2006).

The research presented in this manuscript demonstrates that gamma irradiation offers a viable option for the significant reduction of *C. jejuni* numbers in chicken livers. On average, as little as 1.4 kGy was able to reduce *C. jejuni* numbers in livers by over 4 logs and 1.8 kGy was able to reduce the contaminating *C. jejuni* to levels undetectable by direct plating methods (> 5 log reduction). Previous irradiation studies of

Campylobacter in food have almost entirely focused on treating chicken muscle meat products (Kudra et al., 2012; Patterson, 1995; Raut et al., 2012). Only one article investigated applying radiation to an organ meat, chicken hearts, could be found in the literature (Xavier et al., 2017). Several of the studies irradiating *Campylobacter* in chicken muscle meat produced D₁₀ values lower than the lowest one we achieved in livers, 0.1–0.19 kGy compared to 0.36 kGy (Patterson, 1995; Raut et al., 2012). This difference is likely attributable to those studies using a minced meat product while this research examined whole liver pieces, which had the *C. jejuni* inoculated into the internal tissue that may have offered some protection against irradiation. It is important to look at the internal effects of irradiation on *C. jejuni* within the livers since *Campylobacter* are not just found on the surface of the livers (Baumgartner et al., 1995; Whyte et al., 2006). Previous research has presented mixed results in regards to the presence of detrimental effects to poultry products resulting from the use of radiation. One study reported no detrimental effects to the sensory properties of chicken products after irradiation at doses of 1.66–2.86 kGy (Hashim et al., 1995). However, another study described changes to the structure of muscle fibers within chicken meat following irradiation (Yoon, 2003). No color changes in the irradiated livers, at least that were obvious to visual inspection, were observed at any of the radiation doses assessed.

In addition to irradiation, cold storage was applied to the contaminated livers in an effort to further reduce *C. jejuni* numbers. Freezing livers ($\leq -20^\circ\text{C}$) had been previously shown to significantly reduce *C. jejuni* numbers in chicken livers. Another study described a sensory assessment that suggested a preference for pâtés made from previously frozen chicken livers (Harrison et al., 2013; Hutchison et al., 2015). Part of the current research presented in this article, details how cold temperature storage conditions (4°C and -20°C) applied post-irradiation were able to further decrease *C. jejuni* numbers to a sufficient degree so that 0.8 kGy combined with storage at -20°C for 1 week provided an almost four log reduction that previously required 1.4 kGy when applied alone. According to the research, storage at -20°C alone was only able to achieve a ~ 1 log reduction in *C. jejuni* numbers in livers, which was considerably less than the ~ 2.5 log reduction achieved by previous research through storage of livers at -25°C for 24 h (Harrison et al., 2013). This difference may be attributable to the previous research using a naturally contaminated livers with starting bacteria concentrations less than those used in the experiments presented in this manuscript. Additionally, the current research used a cocktail of seven different *C. jejuni* strains that may have supplied a level of genetic diversity not seen in the previous experiments.

Finally, it appears that the application of irradiation is most efficient when administered to fresh livers since -20°C storage before irradiation reduces the efficacy of the irradiation. In our experiments, the D₁₀ value more than doubled (from 0.36 kGy to 0.75 kGy) for livers stored at -20°C for one week compared to those without storage. Consistent with the results of this research, the application of 1.8 kGy irradiation to fresh chicken livers followed by storage at -20°C for between 48 h and 1 week should produce livers that are or almost devoid of *Campylobacter* contamination. An irradiation and cold storage treatment regimen capable of producing this sort of reduction in *Campylobacter* numbers is essential to maintaining food safety for this product.

5. Conclusions

The repeated multi-year outbreaks attributable to this product, the high percentage of *C. jejuni* prevalence in chicken livers, and the persistent preference for undercooking the livers leaves few options other than the complete eradication of bacterial contaminants. Current US Food and Drug Administration regulations permit the irradiation of poultry and red meat to a dose of 4.5 kGy for refrigerated (e.g. 4°C)

versus 7.0 kGy (-20°C) (US FDA, 2018). In this study, 4–5 log reductions of *C. jejuni* were easily obtained at radiation doses far below those limits. Irradiation of chicken livers has the potential to eliminate the public health issues associated with *C. jejuni* contamination.

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