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The Effect of Cryogenic Freezing Followed by Gamma Radiation on the Survival of *Salmonella* spp. on Frozen Shrimp

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Abstract

Unfortunately, contraction of food borne illness due to consumption of contaminated seafood, including shrimp, is an occasional occurrence. Cryogenic freezing and gamma irradiation are safe and effective technologies that can be used to control and inactivate pathogenic bacteria in foods. In this study, the effect of cryogenic freezing and gamma irradiation for inactivation of *Salmonella* spp. on shrimp was investigated. We found that cryogenic freezing of raw shrimp (-82°C, 3 min), using a pilot scale industrial liquid nitrogen freezer, resulted in a 1.27 log reduction of *Salmonella* spp. on whole shrimp, which was maintained during 12 weeks of frozen storage (-20°C). During our evaluation of selective microbiological media for recovery and enumeration of *Salmonella* spp. we found that Brilliant Green Sulfur Agar produced results indistinguishable from that of non-selective Tryptic Soy Agar when determining the effect of cryogenic freezing on *Salmonella* spp. survival. Radiation D_{10} values for *Salmonella* spp. on frozen shrimp were approximately 0.56 kGy. Cryogenic freezing (-82°C), followed by gamma irradiation (2.25 kGy) produced a >5 log reduction of *Salmonella* spp., and that reduction was maintained during 12 weeks frozen storage (-20°C). These results indicate that both cryogenic freezing and gamma irradiation contribute to inactivation of *Salmonella* spp. on frozen shrimp.

Keywords: Gamma radiation; Cryogenic freezing; Shrimp; *Salmonella*

Introduction

The United States imported approximately 1.2 billion pounds of shrimp in 2008, at a value of $4.1 billion, with the majority of non-canned shrimp being imported in the frozen form, and the per capita consumption of shrimp (all forms) estimated to be approximately 4.1 lbs [1]. While consumption of shrimp and other seafood is significantly less than meat and poultry, food borne illness associated with consumption of seafood can occur on occasion [2,3]. Consumption of shrimp contaminated with *Salmonella* spp. has been associated with many food borne illness outbreaks, and that 0.5-34.4% of shrimp products, sold in multiple nations, at the retail level, and from processors and exporters, tested positive for *Salmonella* [4]. Shrimp accounted for approximately 58% of imported seafood quarantined for *Salmonella* (considered to be an adulterant) contamination by the U.S. Food and Drug Administration (FDA), while lobster accounted for 5%, tilapia 4%, and squid 3% [5].

A recent study conducted by the FDA concluded that antibiotic resistant *Salmonella* were readily isolated from imported foods, primarily of seafood origin, and that continued surveillance of food-borne zoonotic bacterial pathogens from imported foods entering the United States is required [4]. Noda et al. [6] found that *Salmonella* populations on shrimp decreased during long-term frozen storage, but were still recoverable, and that storage temperature affected the recovery of the pathogen. It is relatively easy to isolate *Salmonella* spp. from many types of tropical and non-tropical seafood including retail frozen shrimp, lobsters, cuttlefish, catfish, and seer fish [7-9].

Ionizing (gamma) irradiation is a safe and effective process that has been approved in many countries to improve the microbiological safety and shelf-life of foods [10]. In the U.S, a petition to allow irradiation of crustaceans is currently being evaluated by the FDA [11]. While there is a great deal of data available regarding irradiation of seafood, the majority of the published studies target refrigerated, as opposed to frozen seafood [12]. The majority of raw seafood sold in the U.S. is either frozen or previously frozen. Additional research is needed to determine radiation D_{10} values of food borne pathogens on raw frozen seafood such as shrimp. While treatment of foods with gamma irradiation is an effective intervention technology, the effect of freezing on pathogen survival in frozen meat, poultry, and seafood is less clear as most published studies did not use pilot scale commercial freezing equipment, but laboratory scale freezers [13-16]. Additional research is needed to determine the effect of quick freezing on food borne pathogen survival.

The purpose of this study was to: 1) Determine the effect of cryogenic freezing (-82°C) on the survival of *Salmonella* spp. inoculated onto raw whole shrimp using pilot scale commercial equipment; 2) To determine the radiation D_{10} for *Salmonella* spp. isolated from seafood in frozen shrimp; 3) To determine the effect of freezing and irradiation for *Salmonella* inoculated onto shrimp during long-term frozen storage (-20°C); and 4) Evaluate selective and non-selective microbiological media for use in cryogenic freezing studies.

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Materials and Methods

Bacterial strains

The *Salmonella* isolates used in this study, were isolated from seafood, including shrimp, were obtained from the U.S. Food and Drug Administration and included *Salmonella Schwarzengrund, S. Bahrenfeld, S. Weltevreden, and S. Panama*. Identity of the isolates was confirmed by Gram stain, followed by analysis with Gram positive or negative identification cards using the Vitek Automicrobic System (bioMerieux Vitek, Inc, Hazelwood MO). The bacterial strains were cultured on Tryptic Soy Agar (TSA, BBL/Difco, Inc., Sparks, MD) at 37°C and maintained at 0-4°C until use.

Shrimp

The shrimp was purchased from an internationally-recognized retailer with a strong quality assurance program. The shrimp were kept frozen (-20°C) and irradiated to a radiation dose of 10 kGy for inactivation of background microflora as described below [17]. Irradiation of the shrimp to 10 kGy decreased the background microflora to less than 0.1 CFU/g. The shrimp were thawed overnight in a refrigerator (4°C) prior to inoculation.

Bacterial growth and inoculation

The procedure for inoculation, irradiation, and enumeration of bacteria was followed as published previously [17,18]. Each bacterial strain was cultured independently in 25-mL of Tryptic Soy Broth (TSB, BBL/Difco Laboratories, Sparks, MD) in baffled 500-mL sterile flasks at 37°C (150 rpm) for 18 h. The bacterial cells were then diluted into four liters of sterile distilled water in a sterile polypropylene pan. The thawed shrimp were then dip-inoculated in the same species mixture for approximately 30 min. The excess fluid was then allowed to drip off; the shrimp placed in a separate sterile polypropylene pan, and kept on ice until cryogenic freezing. This procedure resulted in approximately 7 $\log_{10}$ CFU/g of the *Salmonella spp.* multi-isolate cocktail being inoculated onto the shrimp. Samples (six shrimp) were then removed to assess *Salmonella* levels, and served as the untreated controls for determination of $\log_{10}$ reduction as described below.

Freezing

For the freezing study, the inoculated whole shrimp were cryogenically frozen (– 82°C) for 3 min using a Cryo-Test Chamber (Air Products and Chemicals, Allentown, PA USA), which exposed the shrimp to liquid nitrogen vapor in a controlled manner. The frozen shrimp samples (six shrimp, approximately 21 g each) were then placed in individual polynylon bags (Uline, Inc., Philadelphia, PA) which were sealed using a Multivac A300 packager (Kansas City, MO) and kept frozen at -20° C with sufficient bags of frozen shrimp to complete an experimental replicate lasting three months. This entire procedure was then repeated five times (n=5).

Gamma irradiation

A Lockheed Georgia Company (Marietta, GA) self-contained ^{137}Cs radiation source was used for all exposures. The radiation source consisted of 23 individually sealed source pencils placed in an annular array. The 22.9 cm × 63.5 cm cylindrical sample chamber was located central to the array when placed in the operating position. The dose rate was 0.082kGy/min, which was determined using ISO/ASTM Standard 51900-019 in cooperation with the National Institute of Standards and Tests (NIST). The temperature during irradiation was maintained at -20 °C by the gas phase of a liquid nitrogen source that was introduced directly into the top of the sample chamber [5]. To ensure that uniform radiation dose was delivered, sample bags were placed centrally and vertically within the cylindrical chamber. Because of the irradiator design, dose uniformity-ratio's (DUR's) were less than 1.1: 1.0 for the samples types used in this study. The temperature was monitored using two thermocouples placed on the side of the sample bags. The radiation absorbed dose was then verified using radio chromic film dosimeters (Far West, Inc., Goleta, CA).

Enumeration of bacteria

Following freezing and irradiation, the samples were assayed for surviving bacteria by standard microbiological procedures. The shrimp samples were allowed to thaw at room temperature, approximately 25°C. Ninety-mL of sterile BPB was then added to sample bags that contained 10g of inoculated sample, and the sample mixed by stomaching for 90 s (Seward, UK). The samples were then serially diluted in BPB, using tenfold dilution, and 0.1-mL of diluted sample was surface plated onto TSA, Brilliant Green Sulfur Agar (BGSA), XLT-4, or Hektoen Agar (BD-Difco, Sparks, MD). Three 0.1-mL aliquots were plated per dilution. The plates were then incubated for 24-48 h at 37°C prior to enumeration.

D_{10} values

The average CFU/g of an irradiated sample (N) was divided by the average CFU/g of the untreated control (N_o) to produce a survivor ratio (N/N_o). The untreated controls were the *Salmonella* spp. Inoculated onto the shrimp and then frozen for one week. Radiation D_{10} value is defined as the radiation dose required to achieve a 90% reduction in viable microorganism. Radiation D_{10} values were determined by calculating the reciprocal of the slope of the $\log_{10}$ (N/N_o) ratios versus irradiation dose [10].

Statistical analysis

Each experiment was conducted independently five times (n=5). Determination of D_{10}, descriptive statistics, and analysis of variance (ANOVA) were completed using Microsoft Excel Office 2000 (Microsoft Corp, Redmond, WA).

Results and Discussion

The majority of seafood and aquaculture products purchased in the U.S. are purchased as either frozen or previously frozen, and cryogenic freezing of shrimp is an effective means for maintaining product quality, even under repeated freezing and thawing [19]. Some studies indicate that cryogenic freezing may be used to inactivate food-borne pathogens and spoilage microorganisms [13-16]. However, very few, if any, of these studies utilized commercial quality cryogenic freezing equipment. There is very little literature available that has investigated the use of selective media on recovery of *Salmonella* spp. from frozen foods. Therefore we investigated the effect of cryogenic freezing, and the effect of the microbiological media, including tryptic soy agar (TSA) as a non-selective recovery medium and Brilliant Green Sulfur Agar (BGSA), Hektoen Agar (HA), and XLT-4 as the selective media to recover and enumerate *Salmonella* spp. from non-irradiated and irradiated frozen shrimp.

When the shrimp were cryogenically frozen (-82°C), stored for one week (-20°C), and the *Salmonella* spp. recovered and enumerated using TSA, BGSA, HA, and XLT-4 media, the $\log_{10}$ reductions were 1.27 (+0.09), 1.26 (+0.10), 1.82 (+0.07), and 1.96 (+0.08), respectively.

The log reductions obtained using HA and XL-4 and were statistically greater than those obtained using TSA and BGSA (ANOVA, n=5, $p<0.05$). In contrast, the log reductions obtained using TSA and BGSA were statistically similar. The difference in log reductions and recovery (approximately 0.6 $\log_{10}$) were maintained during frozen storage regardless of whether the shrimp were non-irradiated, irradiated to a dose of 1.5 kGy, or irradiated to a dose of 3.0 kGy (Figure 1). Therefore, we recommend the use of BGSA as a selective medium for the enumeration of *Salmonella* spp. after cryogenic freezing as opposed to HA and XLT-4.

The effect of selective microbiological agars on gamma radiation D_{10} values has been determined in our laboratory using a number of food borne pathogens (Sommers and Boyd (2006); Sommers, unpublished data). In this study, the D_{10} values for *Salmonella* spp. on frozen shrimp were 0.56 (+0.02), 0.56 (+0.02), 0.55 (+0.03) and 0.46 (+0.03) using TSA, BGSA, HA, and XLT-4, respectively. This is in agreement with results previously obtained in our laboratory that XLT-4 agar can sometimes provide statistically lower D_{10} values than other selective media.

The National Advisory Committee on Microbiological Safety of Foods (NACMCF) recommends a five log reduction of food borne pathogens for a food to be called pasteurized when using non thermal process interventions. Frequently, the minimum radiation dose needed to produce a five log reduction is calculated using a D_{10} value [10]. An alternative is to calculate the $\log_{10}$ reduction directly. In either case, care must be taken to account for and separate the effect of individual intervention technologies (e.g. freezing vs. gamma radiation), and the microbiological media used that may affect the calculation of either D_{10} or $\log_{10}$ reductions. For inactivation of *Salmonella* spp. on frozen shrimp using gamma irradiation, the use of BGSA as the selective agar provided the best results when compared to non-selective TSA medium. When calculating only D_{10}, independent of freezing, we have found both BGSA and HA to be acceptable as *Salmonella* selective media.

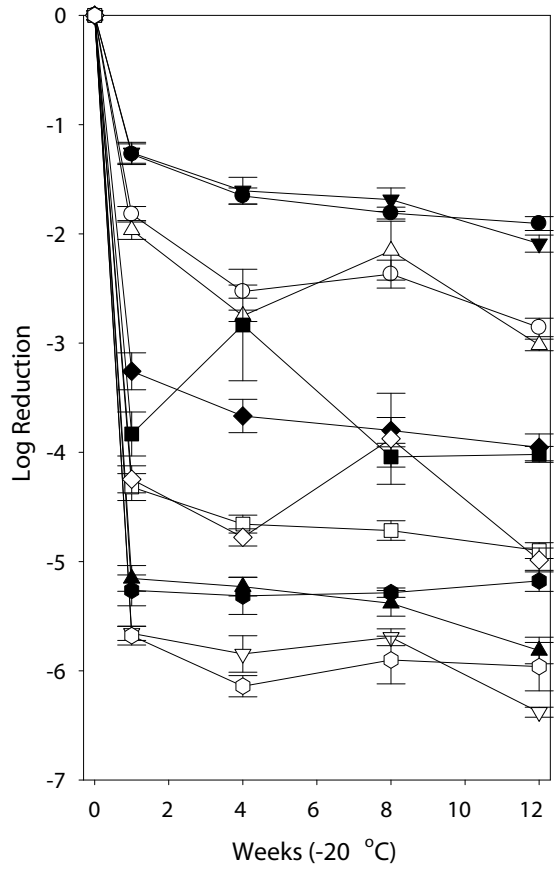

Figure 1: The Effect of Cryogenic Freezing and Gamma Radiation on the Survival of *Salmonella* spp. using Different Recovery Media. Each experiment was conducted independently 5 times (n=5). Standard error of the mean is shown as error bars. Time 0 is when samples were irradiated. TSA-0 kGy (closed circle); BGSA-0 kGy (inverted closed triangle); HA-0 kGy (open circle); XLT-4-0 kGy (open triangle). TSA-1.5 kGy (closed square); BGSA-1.5 kGy (closed diamond); HA-1.5 kGy (open diamond); XLT-4-1.5 kGy (open triangle). TSA-3 kGy (closed triangle); BGSA-3 kGy (closed pentagon); HA-3 kGy (inverted open triangle); XLT-4-3 kGy (open pentagon).

Gamma irradiation of frozen shrimp for inactivation of *Salmonella* spp. yielded interesting results. The radiation D_{10} values obtained for *Salmonella* spp. inoculated onto shrimp (0.46-0.56 kGy) are in agreement with radiation D_{10} values (0.47-0.70 kGy) for *Salmonella* spp. on a variety of frozen seafood products [18]. When the effect of cryogenic freezing on the survival of *Salmonella* spp. is included, a radiation dose of 3.0 kGy would be needed to produce a five $\log_{10}$ reduction of *Salmonella* on whole shrimp and other frozen crustaceans and fish. At the gamma radiation dose of 3.0 kGy used in the storage study, the numbers of *Salmonella* spp. recovered were at the lower limit of detection for the methodology used (Figure 1).

A radiation dose of 4 kGy produced a >6 $\log_{10}$ reduction of *Salmonella* Enteriditis, inoculated onto shrimp [20], while another report recommended a radiation dose of 3.5 kGy for a 5 $\log_{10}$ reduction of *Salmonella* spp. [21], and a third study recommended a radiation dose of 3.5 kGy for complete inactivation of *Salmonella* spp. on frozen shrimp [22]. Those studies did not account for the effect of cryogenic freezing on *Salmonella* spp. survival. The radiation doses needed to produce a five log reductions of *Salmonella* spp. on frozen shrimp typically have no, or negligible, effects on the chemistry or quality of the product [19-23].

Conclusions

In this study, we determined that cryogenic freezing (-82°C) of shrimp inactivated approximately 1.27 $\log_{10}$ of *Salmonella* spp. using a industrial pilot scale equipment, and that $\log_{10}$ reduction was maintained during 12 weeks of frozen storage (-20°C). Additionally, when the effect of freezing on *Salmonella* spp. was accounted for, the radiation D_{10} for *Salmonella* spp. on shrimp was approximately 0.56 kGy, which is consistent with D_{10} values obtained in previous studies in our laboratory [18]. Therefore, cryogenic freezing, in combination with a gamma radiation dose of 2.25 kGy, was sufficient to inactivate five $\log_{10}$ of *Salmonella* spp. on whole raw shrimp in this study. This information may be of value to seafood processors and radiation service providers during preparation of their Hazard Analysis and Critical Control Point Plans (HACCP), and regulatory agencies in the evaluation of pending petitions to allow irradiation of seafood products.

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