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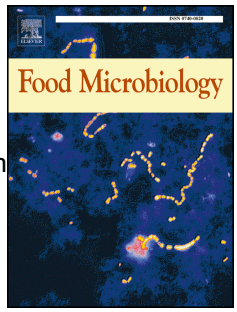

Crab-meat-isolated psychrophilic spore forming bacteria inactivation by electron beam ionizing radiation

S. Condón-Abanto, S. Pedrós-Garrido, G. Cebrián, J. Raso, S. Condón, J.G. Lyng, I. Álvarez

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Authors: Condón-Abanto, S.[1,2], Pedrós-Garrido, S.[1,3], Cebrián, G.[1], **Raso, J.** [1], Condón, S.[1], Lyng, J.G.[2] and Álvarez, I.[1]*

*** Corresponding author:** Ignacio Álvarez

[1]Grupo de Nuevas Tecnologías de Conservación de Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, C/ Miguel Servet 177, 50013, Zaragoza, Spain.

[2]UCD Institute of Food and Health, University College Dublin, Belfield Dublin 4 Ireland.

[3]School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

TEL.: 0034 876 55 54 52

FAX: 0034 976 76 15 90

E-mail: nacho.alvarez@unizar.es

Keywords: Ionizing radiation; psychrophilic spore inactivation; seafood; Brown crab.

Abstract

The present work was performed to evaluate the potential of electron beam ionizing radiation for the inactivation of three psychrophilic spore forming bacteria (*Bacillus mycoides, Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans*) isolated from ready-to-eat brown crab (*Cancer pagurus*). Inactivation curves for the three spores were performed in both types of crab meat, brown and white. Also the effect of pH and water activity (a_w) on the lethal efficacy of ionizing radiation, for the three different psychrophilic spore forming bacteria, was evaluated. The effects of pH, a_w and their possible interactions were assessed in citrate-phosphate buffers of different pH, ranging between 7 and 4, and a_w, ranging from <0.99 and 0.80. A reduction of a_w increased the spores resistance between >0.99 and 0.90, while an a_w reduction from 0.90 to 0.80 had a minor impact on their resistance. In contrast to a_w, the effect of pH showed a greater variability depending on the spore species. While pH did not affect the resistance of *B. weihenstephanensis* at any a_w, *B. mycoides* showed slightly higher resistance at pH 5.5 at a_w of 0.90 and 0.80. pH showed a significant effect on the resistance of *P. psychrodurans*. For the two types of crab meat, slightly differences were observed in 6*D* values. *B. weihenstephanensis* was the most resistant, requiring 7.3-7.6 kGy to inactivate 6 Log_{10}-cycles of this spore forming bacterium, while for *B. mycoides* and *P. psychrodurans* 6.1-6.3 and 5.4-5.3 kGy respectively were necessary to reach the same inactivation level in crab meat. An agreement between spore resistance in crab meats and lab media, with similar characteristics in pH and a_w, was also observed. The results obtained in this research demonstrated the potential for ionizing radiation to achieve an appropriate inactivation level of spores naturally present in brown crab with the application of doses lower than 10 kGy.

Keywords: Ionizing radiation; psychrophilic spore inactivation; seafood; Brown crab.

1. INTRODUCTION

The use of ionizing radiation for food decontamination was proposed in the 19th century, and since then a wide range of research has been performed to evaluate the potential of this technology for microbial inactivation (De Lara et al., 2002; Grant and Patterson, 1992; Jeong and Kang, 2017; Sarrías et al., 2003; Thayer and Boyd, 1993), and assess its influence on food properties (Byun et al., 2000 and 2008; Diehl, 1991; Graham and Stevenson, 1997; Lee et al., 2001). Currently, a number of organisations worldwide have accepted this technology as a safe alternative technology for food decontamination (WHO, FDA). The World Health Organization has established 10 kGy as the maximum dose for food processing without any adverse effect on food matrixes (WHO 1981). Though, a later study concluded that no limiting dose is required (WHO 1999). Either way, nowadays more than 60 countries worldwide have regulations regarding the use of ionizing radiation for food products (IAEA, 2017). In fact, the joint FAO/IAEA (International Atomic Energy Agency) Division of Nuclear Techniques in Food and Agriculture estimates that approximately 700,000 tonnes of food were irradiated in 2013 (IAEA, 2015). The main potential for the use of ionizing radiation in foods is its ability to extend the microbiological shelf-life with poultry, egg products, red meats, seafood products and spices proposed as good candidates for the use of radiation as decontamination technology, due to its potential to inactivate microorganisms at low temperatures (Farkas, 2006).

Fish and fishery products have a special interest due to their particular characteristics. Many of these products are commercially cooked as products in their own right or are cooked for use as ingredients in ready-to-eat products, where a thermal pasteurization to reduce 6 Log_{10}-cycles of non-proteolytic *Clostridium botulinum* type E is commonly applied to ensure food safety. However, the shelf-life of these products is directly dependent on the cold chain

during distribution, due to the presence of other more heat resistant psychrophilic. These microorganisms are able to survive conventional pasteurization treatments and germinate during chilled storage producing a noticeable reduction in the shelf-life of the product. A clear example of this issue is the preservation of ready-to-eat brown crab (*Cancer pagurus*).

Brown crab (*Cancer pagurus*) is one of the most consumed crustaceans in southern European countries where the market has been dominated by the fresh live products (Edwards & Early, 2001). However, the expansion of the market of this crustacean to the United States of America and Japan, where the consumption of ready-to-eat products is increasing (Edwrads & Early, 2001), makes it necessary to evaluate alternative technologies for the production of safe products with high quality attributes and prolonged shelf-life. One of the major problems in the production of ready-to-eat seafood products is the presence of psychrophilic bacterial spores (Faghri et al., 1984; Gram and Huss, 1996) which show high resistance against the thermal decontamination processes, requiring a severe heat treatment to reduce their population up to an acceptable level, though these treatments can affect the quality attributes of the final product. So, Electron Beam Ionizing radiation (EBI) could be an alternative in their production.

It is widely recognised that microbial inactivation induced by ionizing radiation is due to the DNA damage (Farkas, 2006). Despite the knowledge of its inactivation mechanism, a lack of data exists concerning the effects of treatment media characteristics on the lethal efficacy of EBI. It is also well known that physico-chemical characteristics of the treatment medium have an important effect on the microbial resistance against physical stress; however few studies in this respect related to EBI exists (Fan and Sommers, 2012; Huhtanen et al., 1989; Thayer and Boyd, 1993). To the best knowledge of the authors a systematic study to assess the effect of common variables, such as pH, water activity (a_w) and their interactions

on the lethal effect of EBI has not been previously described. This lack of knowledge is even larger in the case of psychrophilic bacterial spores.

The main objectives of the present study were to evaluate the potential application of ionizing radiation to reduce the spore population present in crab meats, to assess the influence of the pH and water activity of the treatment media on the lethal effect of EBI treatments on three different psychrophilic spores isolated from pasteurised crab (*Cancer pagurus*) and to analyse if the obtained inactivation results in lab media allows to predict the results obtained in the food matrix.

2. MATERIALS AND METHODS

2.1. Microorganisms, treatment media and sample preparation

The three spore forming bacteria used in this study were the three most isolated from Irish brown crab (*Cancer pagurus*): *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans*. During this investigation, the three spore suspensions were managed and prepared as described by Condon-Abanto et al. (2016). In brief, 1 mL from a pure culture in stationary phase was spread onto the surface of Tryptone Soya Agar with 0.6% (w/v) yeast extract (TSAYE) (Oxoid Ltd., Basingstoke, Hampshire, UK) agar plates containing 3 ppm (w/v) of manganese sulphate (Carlo Erba, Milan, Italy) and incubated at 25 °C for 10 days. Spores were then collected with sterile pH 7.0 McIlvaine citrate-phosphate buffer (Dawson et al., 1974) and washed and centrifuged three times. The final spore suspensions were then submerged in boiling water for 1 minute in order to inactivate the possible remaining population of vegetative cells and stored under refrigeration (4±1 ºC) until use. The presence of aggregates was evaluated by direct microscopic observation in a Thoma

chamber. The spore concentration was evaluated by pour plating in TSAYE (Oxoid). All suspensions contained a concentration of about 10^9 spores per mL.

To evaluate the effect of the different treatment media characteristics, such as pH and water activity (a_w), a series of McIlvaine citrate-phosphate buffers (Dawson et al., 1974) of different pH and a_w were prepared. pH was adjusted to 4.0, 5.5 and 7.0 using a pH meter BASIC 20 (Crison Instrument, Barcelona, Spain) and then the a_w was adjusted to 0.80, 0.90 and >0.99 by adding different proportions of glycerol with the a_w measured using a dew point instrument (Water Activity System mod. CX-1, Decagon Devices, Pullman, WA, USA). Once all treatment media were prepared, they were sterilized at 121 ºC for 20 min and stored under refrigeration (4±1 ºC) until required for use.

Immediately before treatments, the different media were distributed in 24-well plates. Each well was filled with 2 mL of buffer of a certain pH and a_w under aseptic conditions in a sterile laminar flow cabinet (Telestar mini-V/PCR, Telestar Technologies, S.L., Terrasa, Spain). Then, plates were inoculated by adding 0.1 mL of the corresponding dilution of each spore suspension, in order to reach an initial count of approximately 10^5 spores/mL in each well. The inoculated well plates were immediately treated. The pH and water activity of the treatment media did not differ before and after EBI treatments.

For crab meat samples, crabs were cooked at 95 ºC for 20 minutes. White meat from claws and brown meat from the body were then removed aseptically in a sterile laminar flow cabinet (Telestar mini-V/PCR, Telestar Technologies, S.L., Terrasa, Spain) to ensure the natural contamination was under the detection limit (data not shown). Then, meats were distributed by placing 1 g of each meat in sterile tubes of 10 mL, and 0.1 mL of the corresponding spore suspension dilution was added obtaining an initial concentration of 10^6 spores/g. The inoculated meat was manually mixed with a sterile spoon to uniformly

146 distribute the spores in the meat, and treated immediately. a_w of the crab meat, both white and
147 brown was 0.99 and the pH ranged from 7.5-8.0.
148
149 *2.2. Irradiation treatments*
150 Irradiation treatments were carried out in a 10-MeV circular electron accelerator
151 (Rhodotron) at the irradiation plant of Ionisos Ibérica (Tarancón, Cuenca, Spain). Well plates,
152 and inoculated meat samples were irradiated at programmed doses of 1, 2, 5, 10 and 15 kGy.
153 Irradiation dosimetry was carried out by using a band of cellulose triacetate located on the
154 surface of the samples (Nieto-Sandoval et al., 2000). The irradiation dosimetry indicated that
155 the actual doses applied were 1.13, 2.07, 5.38, 10.7 and 16.4 kGy, respectively. All
156 experiments were carried out in triplicate, by using different independently prepared spore
157 suspensions, applying irradiation doses in different runs during the same working day due to
158 limit accessibility to the circular electron accelerator.
159
160 *2.3. Recovery, incubation and survival counting of treated samples*
161 Immediately after treatments serial decimal dilutions in MRD of liquid samples were
162 pour-plated using TSAYE (Oxoid) as recovery media. Meat samples were diluted in 9 mL of
163 maximum recovery diluent (MRD) (Oxoid) and homogenized with an ultra-turrax® for 20
164 seconds. Then, proper dilutions in MRD were pour-plated in TSAYE (Oxoid). Plates were
165 incubated at 25 ºC for 24 hours for *B. mycoides* and *B. weihenstephanensis* and 48 hours for
166 *P. psychrodurans*. Longer incubation times did not change the obtained counts (data not
167 shown). Colony-forming units (CFU) were counted with an improved automatic colony-
168 counting image analyzer (Protos, Synoptics, Cambridge, UK), previously described by
169 Condón et al. (1987).
170

2.4. Modeling and Statistical analysis

Survival curves obtained from the electron beam irradiation treatments were obtained by plotting the Log_{10} fraction of survivors vs. the applied dose (kGy). Under most experimental conditions deviations from linearity were observed, determining survival curves with concave downwards profiles (shoulder). Because of this shape the Geeraerd et al. log-linear regression plus shoulder model was used (Geeraerd et al., 2000) to fit the survival curves, but swapping the parameter of time in the original model with the applied dose in kGy. Survival curves were fitted to the model by approach of least squares (i.e. by reducing the sum of square errors, between real and predicted values, to the minimum) using GraphPad PRISM® 5.0 software (GraphPad software, Inc., San Diego, CA, USA). The model includes two parameters to describe the survival curves (Equation 1): shoulder length (*Sl*) which defines the applied dose before the exponential inactivation begins, and the inactivation rate (k_{max}) that corresponds to the slope of the exponential portion of the survival curve.

$$N_t = N_0 e^{-k_{max} * dose} \left(\frac{e^{k_{max} Sl}}{1 + \left(e^{-k_{max} Sl} - 1\right) e^{k_{max} * dose}} \right) \qquad (1)$$

Based in k_{max} the traditional decimal reduction value (D_{10}) of each survival curve was calculated (Equation 2). In this case, the D_{10}value corresponds to the necessary dose (kGy) to produce a 90% reduction in the spore population.

$$D_{10} = 2.303/k_{max} \qquad (2)$$

To determine the treatment parameters and compare the resistance between the three spores under study, 6*D* values were calculated. In this case 6*D* is defined as the necessary dose to inactivate 6 Log_{10}-cycles of the initial spore population, and is calculated by Equation 3.

$$6D = Sl + 6 * D_{10} \qquad (3)$$

Where *Sl* is the shoulder length duration and D_{10} is the inactivation parameter calculated from Equation 2.

196 R^2 and *RMSE* values provided by the software were used to evaluate the goodness of
197 fits. Statistical analyses (*t*-test and one-way ANOVA) were performed with the GraphPad
198 PRISM® and differences were considered significant if $p \leq 0.05$. The standard deviations (*SD*)
199 are given in the figures as the error bars.
200
201 **3. RESULTS**

202 *3.1. Spore inactivation kinetics by electron beam irradiation: Effect of pH and water*
203 *activity (a_w).*

204 Figure 1(A-C) shows, as examples, the inactivation curves obtained in citrate-
205 phosphate buffer at pH 7.0 at three different a_w for *B. mycoides* (A), *B. weihenstephanensis*
206 (B) and *P. psychrodurans* (C) (inactivation curves at pH 5.5 and pH 4 are shown in
207 supplementary material as Figure S1A-C and S2A-C respectively) . As observed, inactivation
208 increased with increasing irradiation dose. For the three spores under study, concave
209 downwards profiles were generally observed at neutral pH in all water activities. The profile
210 of some inactivation curves at other pHs (i.e. 4.0 and 5.5) did not showed shoulders. As
211 indicated in the Materials and Methods section, Geeraerd et al. log-linear regression plus
212 shoulder model (Geeraerd et al., 2000) was used to fit the inactivation curves and to calculate
213 the resistance parameters shoulder length (*Sl*), and decimal reduction doses (D_{10}). Figure 1
214 also presents the line obtained from modelling (black line) to show the goodness of fit.
215 Model parameters are shown in Table 1 as well the root mean square error (*RMSE*). In all
216 cases the obtained R^2 values were >0.99.

217 For the three spore species, a_w affected their irradiation resistance influencing both,
218 the *Sl* and the D_{10} values. The maximum resistance was observed at the lowest investigated
219 a_w (0.80), whereas pH hardly affected the irradiation resistance. The *Sl* of *B. mycoides*,
220 ranged between 0 and 0.6 kGy and D_{10} values ranged from 0.8 to 2.1 kGy, showing the

highest D_{10} of the three bacterial species investigated. As in *Sl*, the pH hardly changed the D_{10} values, while the reduction of a_w showed an important influence. The reduction of a_w from >0.99 to 0.90 induced an increase in the D_{10} values, close to a 2-fold order of magnitude, while further reductions hardly changed this parameter.

In the case of *B. weihenstephanensis*, the *Sl* ranged from 1.1 and 2.5 kGy, being the species which showed the longest *Sl*. When a_w was reduced from >0.99 to 0.90 it induced increases of 72%, 90% and 66% in the *Sl* at pH 7.0, 5.5 and 4.0, respectively, while the reduction from 0.90 to 0.80 only increased the *Sl* by 26%, 19% and 17%, respectively, at the same pHs. On the other hand, D_{10} values ranged from 0.8 to 1.6 kGy, and were scarcely affected by pH at any a_w. And, as in *B. mycoides*, the reduction of a_w increased irradiation resistance. In this case, a a_w variation from >0.99 to 0.90 supposed increases of 77%, 67% and 100% at pH 7.0, 5.5 and 4.0, respectively, on the D_{10} values. However, further a_w reductions hardly change this parameter at any pH.

Finally, *P. psychrodurans* showed a similar behaviour in terms of the effect of a_w on *Sl* values to *B. weihenstephanensis* with a reduction in a_w leading to an increase in the *Sl*. However, the influence of pH was more noticeable. At pH 5.5 and 4.0, *Sl* values drastically increased when a_w of the treatment medium was reduced from >0.99 to 0.90, although further reductions scarcely produced any change in this parameter. Surprisingly, the same reductions in a_w at neutral pH slightly affected the *Sl* values, showing the lowest values compared to other pHs. On the other hand, D_{10} values ranged from 0.7 to 1.9 kGy varying with both a_w and pH. Similarly to the other investigated spores, D_{10} values of *P. psychrodurans* increased when a_w was reduced but its irradiation resistance was higher at neutral pH.

In summary, the inactivation curves obtained for the evaluated spore forming bacteria showed a great variability with regard to the shoulder length duration. Also pH and a_w effects varied notably respect to the studied species: in *B. weihenstephanensis* both factors seemed to

be independent of each other, but for *P. psychrodurans* an interaction between these two factors was observed. The differences detected in D_{10} values between species and also the effects of pH and a_w were smaller than those detected in the *Sl* parameter. The most noticeable difference was observed in case of *P. psychrodurans* where a reduction in resistance was detected at acid pHs.

To define the irradiation treatment intensity required to apply at industrial level, both *Sl* and D_{10} values should be taken into account. To evaluate more clearly the effect of pH and a_w on the irradiation resistance of the investigated spores, 6*D* values were compared. The advantage of using this value is that it comprises both inactivation model parameters, *Sl* and D_{10}. Therefore, it is possible to compare directly the resistance between spores at all investigated conditions. In addition, 6*D* is the inactivation level of the target microorganism to ensure the safety in processed ready-to-eat seafood products (FDA, 2011). Figure 2 shows the effect of a_w and pH on the 6*D* values for *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C). As observed, pH had a lower effect on the spore resistance than the effect observed for a_w. For *P. psychrodurans* (Figure 2C), the highest resistance was observed at neutral pH regardless the a_w, while at the other pHs no differences in resistance were detected at a_w of 0.90 and 0.80. Regarding *B. mycoides* the major difference between pHs was detected at a_w of 0.90 where a slightly higher resistance at pH 5.5 was observed (Fig. 2A).

In general, the maximum increase in the spore resistance was observed with a_w reduction from >0.99 to 0.90, while further reductions of a_w hardly affected the spore resistance. Only in the case of *P. psychrodurans* at pH 5.5, 6*D* value increased linearly from 5.9 kGy to 9.1 kGy with the a_w reductions. This species was also the most affected by the variation of a_w, increasing 6*D* values from 4.1 kGy to 8.5 kGy at pH 4 and from 5.8 to 11.7 at pH 7 when reducing a_w from >0.99 to 0.80.

From Figure 2, besides the influence of pH and a_w, differences in radiation resistances between species can be observed. *B. mycoides* showed the lowest resistance (5.5 kGy) in pH 7.0 and a_w of >0.99, and the highest (12.6 kGy) at pH 5.5 and 0.80 of a_w. *B. weihenstephanensis* showed the lowest resistance (6.2 kGy) at >0.99 of a_w and pH 4, and the highest (11.0-11.1 kGy) at all pH and both a_w 0.90 and 0.80. *P. psychrodurans* showed the lowest resistance (4.2 kGy) at pH 4.0 and a_w >0.99, while the highest resistance (11.7 kGy) was detected in media of pH 7.0 and a_w of 0.80. *B. weihenstephanensis* showed the greatest resistance at most pHs and water activities investigated. Only at the lowest a_w tested (0.80) did *B. mycoides* became the most resistant spore at pH 5.5. Therefore choice of irradiation reference organism is dependent upon a_w of the product.

3.2. Spores inactivation in crab meats

As occurred in lab media, the inactivation curves obtained in crab meat showed downwards profiles in all cases (Figures 3A and 3B). Therefore, the Geeraerd log-linear regression plus shoulder model (Geeraerd et al., 2000) was used to describe the curves. Table 2 shows the resistance parameters for the three spores in white and brown meat: D_{10}, *Sl* and 6*D* values. R^2 and *RMSE* have been included to show the goodness of fit of Equation 2 to the survival curves. A slight increase of the radiation resistance parameters *Sl*, D_{10} and 6*D* was observed when spores were treated in crab brown meat. *B. weihenstephanensis* was the most resistant requiring 7.3 and 7.6 kGy for white and brown meat, respectively, to reach 6 Log_{10}-reductions, while *P. psychrodurans* was the most sensitive requiring 5.4 and 5.3 kGy, respectively, to reach a similar inactivation level.

Figure 4 allows the comparison among the resistances of the three bacterial spores to different food preservation technologies: heat, ultrasonic waves under pressure at sublethal temperatures (manosonication; MS) (Álvarez et al., 2003) and at lethal temperatures

295 (manothermosonication; MTS) (Arroyo et al., 2011), and EBI. Data for heat, MS and MTS
296 were extracted from Condón-Abanto et al. (2016). As observed, the maximum differences in
297 resistance between the most and lowest resistant spores were 1.7-fold for MS, 4.4-fold for
298 MTS, 44.4-fold for heat and less than 1.2-fold for EBI.
299

300 **4. DISCUSSION**

301 Electron beam ionizing radiation appears to be one of the few non-thermal
302 technologies with the capability to inactivate spores in an effective way without requiring a
303 combination with other technologies such as heat, a phenomenon noted with other non-
304 thermal technologies (Bermúdez-Aguirre et al., 2012; Cléry-Barraud et al., 2004; Condón-
305 Abanto et al., 2016; Sevenich et al., 2015; Uemura and Isobe, 2003). Most published data
306 shows that spores are more resistant than vegetative cells with D_{10} values in the range of 1-4
307 kGy, (De Lara et al., 2002; Farkas 2006). The D_{10} values obtained in the present work at an
308 a_w of >0.99, independent of the pH, ranged from 0.8 to 1.1 kGy. These D_{10} values were lower
309 than those observed in other *Bacillus* species, which showed D_{10} values higher than 2 kGy
310 (De Lara et al., 2002; Sarrías et al., 2003; Valero et al., 2006), but when different a_w were
311 considered, D_{10} values ranged from 0.8 to 2.1 kGy.

312 According to our results, all the studied spores showed inactivation curves with no
313 exponential kinetics, and in most of the investigated conditions shoulders were observed.
314 Similar kinetics were described by other authors for *Bacillus* spores (Blatchley et al., 2005).
315 However, log linear inactivation kinetics have also been described for *B. cereus* and *B.*
316 *subtilis* spores (De Lara et al., 2002). The presence of shoulders has been explained by the
317 capacity of microorganisms to repair damage caused by low intensity treatments, the
318 activation of dormant spores and due to the presence of agglomerates (Mathys et al., 2007;
319 Sapru et al., 1993). The microscopic observation of our suspensions did not show the

presence of aggregates and the presence of tails was not detected in any of the survival curves obtained, which allows discarding that the shoulders observed are produced due to the presence of aggregates. On the other hand, the comparison of the microscopic counts with the plate counts allowed to conclude that the presence of superdormant spores would represent less than 10% of the population, which would indicate that the shoulders are not related to activation phenomena either. On the other side, the repair of damages inflicted by technological treatments has some special characteristics in bacterial spores since they can only occur once germination has begun (Setlow, 2006). A detailed study on the damage inflicted by radiation on the spores of *B. subtilis* were performed by Moeller et al. (2014).

Condón-Abanto et al. (2016) reported the presence of shoulders in the inactivation curves when applying heat, manosonication (MS) and manothermosonication (MTS) treatments, for *B. weihenstephanensis* and *P. pshychrodurans*, but not for *B. mycoides*. These results suggested that the capacity of damage repair would depend on both the bacterial species and the main target of the applied technology in terms of mechanism of action. Considering that the main mechanism involved in the microbial inactivation produced by EBI is the damage on the DNA, and the fact that the presence of shoulders is common in the inactivation curves obtained with other technologies which act on the same target, such as the case of UV-C light (Gayán et al., 2013), it is not surprising the detection of these shoulders in the inactivation curves obtained in our research.

However, it has been postulated that pH and a_w do not affect the antimicrobial effect of UV-C light (Gayán et al., 2014), while our results suggest that the pH and more significantly a_w of the treatment medium affects the irradiation resistance. This fact would be related to the mechanism of action by which each radiation technology, UV-C or e-beam, affects DNA. While UV-C radiation induces the formation of photoproducts due to the direct absorption of photons (Gayán et al., 2014, Lopez-malo & Palou 2005), EBI reacts through

two mechanisms affecting the DNA. The most simple would be comparable with the UV-C mechanisms where the damage in the DNA is produced when an energy photon or electron crash randomly with the genetic material (Dickson 2001; Goodhead 1994; Yokoya et al., 2008); while the second one involved more complex reactions based on the radiation chemistry of water. EBI, in presence of water, produces reactive species, from which hydroxyl radicals (OH•) and hydrogen peroxide (H_2O_2) are considered the main factors responsible for the reactions with nucleic acids (Sutherland et al., 2000; Lomax et al., 2002). The protective effect of a_w observed in this investigation shows the importance of this second mechanism for the inactivation efficacy of EBI. These series of reactions would also explain the results obtained by other authors, where the radiation resistance of different microorganisms increased when microorganism were treated in frozen media, where again a_w is reduced by the freezing process (Black and Jaczynski, 2006; Fan and Sommers, 2012; Thayer and Boyd, 1993 and 2001).

De Lara et al. (2000) suggested that the mechanism involved in bacterial spore inactivation by ionizing radiation would be very different from the mechanisms involved in heat destruction due to the different targets of each technology. However, since research about the effect of a_w on spore resistance against EBI has not been described yet, it would be convenient to compare the effect of this parameter between these two technologies. Thermobacteriology studies with different *Bacillus* species have reported a linear correlation between the Log of thermal D_{10} values and $(1-a_w)$ in different ranges of a_w (Guillard et al., 1998; Mazas et al., 1999), but in the present study no clear relations were detected between these two parameters. Mazas et al. (1999) reported that the effect of a_w on the heat resistance of several strains of *Bacillus cereus* spores begins to be noticed at a_w values lower than 0.85, while our results suggest that the main effect of a_w on the radiation lethal efficacy is produced between a_w values from >0.99 to 0.90. Additionally, they reported that a decrease in a_w from

370 0.96 to 0.71 increased D_{10} values to heat between 30 and 60-fold and Gillard et al. (1998)
371 observed an increase on D_{10} values to heat (of *B. cereus)* higher than ten-fold when the a_w
372 was reduced from >0.99 to 0.80. Contrarily, our results showed a much lower protective
373 effect of low a_w, since the resistance of spores hardly increased when a_w was reduced from
374 0.90 to 0.80. The protective effect of a_w reduction against EBI is related presumably with the
375 indirect inactivation mechanisms based on the formation of oxidative species (ROS) due to
376 the radiation chemistry of water but also due to a reduction of the intercellular water content
377 of the spore (Dickson, 2001; Moeller at al., 2014). The sorption isotherm of the most organic
378 materials indicates that, the reduction of a_w from >0.99 to 0.9 involves a great percentage
379 reduction of the water content, while a_w reduction from 0.90 to 0.80 requires a much smaller
380 reduction of the water content (Yanniotis and Blahovec, 2009). This would explain the great
381 protective effect of a_w between >0.99 and 0.90 and the low protective effect between 0.90 and
382 0.80 observed in this research.

383 To date, as in the case of a_w, no data about the effect of pH on EBI lethal efficacy, are
384 available in the literature in order to discuss with those obtained in the present work.
385 However, the effect of pH on the heat resistance of bacterial spores has been widely
386 described (Casadei et al., 2001; Palop et al., 1996 and 1999). While the pH hardly affected to
387 spore inactivation by EBI, it is reported that the heat resistance of *B. licheniformis* and *B.*
388 *cereus* changed 20 and 3-fold respectively when the pH was reduced from 7 to 4 (Palop et al.
389 1996 and 1999). Mazas et al. (1999) and Casadei et al. (2001) also reported reductions of 5
390 and 7-fold in the heat resistance of *B. cereus* for similar reduction of pH on the treatment
391 media. All of these discrepancies support the hypothesis that very different mechanisms are
392 involved in the bacterial spore inactivation by heat and EBI. Although, the few effects of pH
393 on spore resistances with EBI treatments is similar to those observed on UV-C light, which
394 produce the microbial inactivation through similar mechanisms.

Another important difference which showed the distinct inactivation mechanisms for each technology is the resistance variability between species. Figure 4 shows a comparison among the three investigated bacterial spores against heat, MS, MTS and EBI. The variability in resistance among spores was different depending on the inactivation technology. The maximum differences in resistance among the three spores were 1.7-fold for MS, 4.4-fold for MTS, 44.4-fold for heat and less than 1.2-fold in the case of EBI. These differences in resistance between species would be attributable to the different targets of each technology. As it has been already pointed out, while cell envelopes are the main target for ultrasound (Condón et al., 2011), the most sensitive targets in heat inactivation of bacterial spores seems to be DNA, core enzymes or spore membranes (Palop et al. 1998; Setlow, 1995). On the other hand, as was suggested previously, the most sensitive target to ionizing radiation is the DNA which would explain the small differences in resistance between species as it has been previously suggested for other technologies which act on the same targets such as UV-C (Gayán et al., 2013).

In general, the obtained results in this research could involve important practical implications. While changes in the contaminating flora, pH or a_w could increase the risk of microbial survival thousands of times in a sterilised product by heat, the same variables would hardly affect the safety and stability of a sterilised product by ionizing radiation.

It has been reported that a radiation dose $\leq$ 2 kGy produced a significant extension of the shelf-life of different crab products (Chen et al., 1996; ICGFI, 1998). However, to the best of our knowledge, no studies assessing the radiation resistance of naturally present bacterial spores in crab products have been reported in the form presented in the present work. The obtained results showed that, similar to observations in lab media, the inactivation kinetics of the three spore species showed a shoulder followed by an exponential decay, as it has been reported for other *Bacillus* species in different media (Blatchley et al., 2005). Our

results also proved that, despite the different composition and chemical characteristics of the two kinds of crab meat (Anacleto et al., 2011; Barrento et al., 2010), the specific resistance of each spore was scarcely affected by the type of meat. Moreover, the specific resistances of each species in meat were similar to those detected in lab media at similar pH and a_w levels. These results would indicate that unlike other technologies, the irradiation dose applied to lab media could be used as reference to calculate the necessary treatments for each specific foodstuff. Nevertheless, this important aspect would require further more exhaustive studies. Finally, the inactivation curves obtained in both types of meats suggested that a dose below 10 kGy, which is the maximum permitted and recommended legal dose by FAO/WHO for foods, would permit a reduction of 6 Log_{10}-cycles of any of the investigated bacterial spores present in crab and crab products. These results would indicate that EBI could be an adequate technology to preserve brown crab. However, further research would be necessary to determine the impact of those treatments in the crab meat quality and the maximum applicable dose to avoid possible undesirable changes on the sensory characteristics.

5. CONCLUSIONS

In summary, this work covers a knowledge gap in the field of bacterial spore inactivation by electron beam ionizing radiation. The obtained results showed that the pH of the treatment media could affect the spore resistance, although the effect would be dependent on the specific spore under study. On the other hand, an important protective effect of low a_w of the treatment medium was observed, but the impact of this parameter is present in a larger or smaller magnitude depending on the bacterial spore. The protective effect of the reduction on a_w has the major effect in the range from >0.99 to 0.90, regardless the investigated spore. The studied spores showed, in both lab media and crab meat, shoulders followed by an exponential decay profiles in their inactivation kinetics. Crab meat type and its composition

445 hardly affected the specific resistance of each spore. The observed radiation resistances in
446 meats were comparable with the resistances determined in lab media of similar pH and a_w.

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Table 1. Electron beam radiation resistance parameters obtained from the fitting of the Geeraerd log-linear plus shoulder model (Equation 1) to the survival curves of *B. mycoides*, *B. weihenstephanensis* and *P. psychrodurans* in citrate-phosphate buffers of different pH and a_w.

Microorganism	pH	a_w	*Sl* (kGy)	D_{10} (kGy)	RMSE
B. mycoides	7	>0.99	0.6 (0.032)^{a}	0.8 (0.002)^{a}	0.069
		0.90	0.5 (0.243)^{abcd}	1.5 (0.319)^{b}	0.111
		0.80	0.3 (0.063)^{bc}	1.9 (0.009)^{bc}	0.115
	5.5	>0.99	0.4 (0.005)^{c}	1.0 (0.043)^{c}	0.031
		0.90	-	1.9 (0.028)^{c}	0.052
		0.80	-	2.1 (0.068)^{c}	0.101
	4	>0.99	0.5 (0.040)^{b}	0.9 (0.025)^{a}	0.107
		0.90	0.3 (0.003)^{d}	1.8 (0.046)^{bc}	0.110
		0.80	0.3 (0.085)^{cd}	1.9 (0.016)^{bc}	0.122
B. weihenstephanensis	7	>0.99	1.1 (0.067)^{a}	0.9 (0.014)^{a}	0.042
		0.90	1.9 (0.045)^{b}	1.6 (0.027)^{b}	0.038
		0.80	2.4 (0.013)^{c}	1.4 (0.005)^{c}	0.083
	5.5	>0.99	1.1 (0.064)^{a}	0.9 (0.043)^{a}	0.010
		0.90	2.1 (0.060)^{d}	1.5 (0.008)^{d}	0.083
		0.80	2.5 (0.049)^{e}	1.4 (0.010)^{c}	0.085
	4	>0.99	1.2 (0.053)^{a}	0.8 (0.024)^{e}	0.039
		0.90	1.8 (0.012)^{b}	1.6 (0.001)^{b}	0.047
		0.80	2.1 (0.117)^{d}	1.5 (0.023)^{d}	0.049
P. psychrodurans	7	>0.99	0.2 (0.012)^{a}	0.9 (0.002)^{a}	0.076
		0.90	0.3 (0.179)^{abc}	1.6 (0.083)^{b}	0.008
		0.80	0.4 (0.003)^{b}	1.9 (0.003)^{c}	0.015
	5.5	>0.99	0.3 (0.028)^{c}	0.9 (0.001)^{a}	0.060
		0.90	1.0 (0.028)^{d}	1.0 (0.014)^{d}	0.078
		0.80	0.9 (0.012)^{e}	1.4 (0.061)^{e}	0.020
	4	>0.99	-	0.7 (0.003)^{f}	0.235
		0.90	0.8 (0.035)^{f}	1.0 (0.013)^{d}	0.080
		0.80	0.9 (0.029)^{e}	1.3 (0.024)^{e}	0.041

a_w, water activity; *Sl*, shoulder length; D_{10}, decimal reduction dose calculated from k_{max} with Equation 2 ; *RMSE*, root mean square error. Numbers in brackets represent standard deviation of three replicates. Letters show differences within columns for each spore specie ($p<0.05$).

Table 2. Electron beam radiation resistance parameters obtained from the fitting of the Geeraerd log-linear plus shoulder model (Equation 1) to the survival curves of *B. mycoides*, *B. weihenstephanensis* and *P. psychrodurans* in white and brown crab meats.

		D_{10} (kGy)	*Sl* (kGy)	6*D* (kGy)	R^2	*RMSE*
White meat	*B. mycoides*	0.8 (0.135)^{a,b}	1.3 (0.218)^{a}	6.1 (0.038)^{a}	0.99	0.026
	B. weihenstephanensis	1.0 (0.063)^{a}	1.0 (0.067)^{a,d}	7.3 (0.055)^{b}	0.99	0.021
	P. psychrodurans	0.8 (0.010)^{b}	0.6 (0.030)^{b}	5.4 (0.019)^{c}	0.99	0.039
Brown meat	*B. mycoides*	0.9 (0.052)^{a}	0.8 (0.026)^{c}	6.3 (0.118)^{d}	0.99	0.012
	B. weihenstephanensis	1.1 (0.060)^{a}	1.0 (0.018)^{a}	7.6 (0.109)^{e}	0.99	0.023
	P. psychrodurans	0.7 (0.005)^{c}	0.9 (0.012)^{d}	5.3 (0.007)^{f}	0.99	0.038

D_{10}, decimal reduction dose (kGy) calculated from k_{max} whit Equation 2; *Sl*, shoulder length (kGy); 6*D*, necessary doses (kGy) to reached 6 Log_{10}-reductions ; *RMSE*, root mean square error; R^2, determination coefficient. Numbers in brackets represent standard deviation of three replicates. Letters show differences within columns ($p<0.05$).

656 **Figure legend**

657 **Figure 1**. Survival curves to electron beam ionizing radiation at room temperature of *B.*
658 *mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate
659 buffer of pH 7 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars
660 represent standard deviation of three replicates.

661 **Figure 2.** Effect of the water activity (a_w) on the dose necessary to reduce 6-Log_{10} cycles of
662 *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) at pH 7.0 (●), 5.5 (○)
663 and 4.0 (●).Error bars represent standard deviation of three replicates.

664 **Figure 3.** Survival curves to electron beam ionizing radiation at room temperature of *B.*
665 *mycoides* (●), *B. weihenstephanensis* (■) and *P. psychrodurans* (▲) in crab's white meat
666 (A) and brown meat (B). Error bars represent standard deviation of three replicates.

667 **Figure 4.** Specific resistance of *B. mycoides* (black bars), *B. weihenstephanensis* (grey bars)
668 and *P. psychrodurans* (white bars) to different inactivation technologies in citrate-phosphate
669 buffer of pH 7.0 and a_w >0.99 (data for MS, MTS and Heat are adapted from Condon-Abanto
670 et al., 2016).

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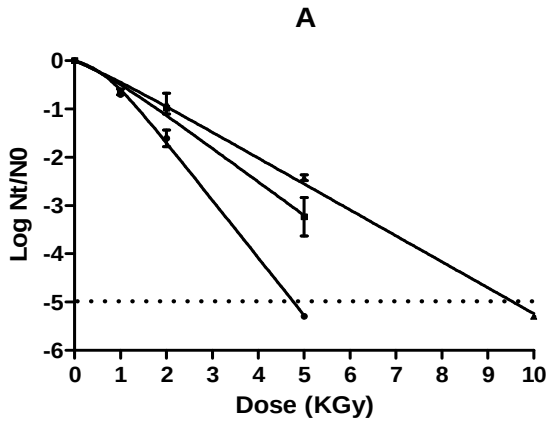
673

674 **Figure 1**



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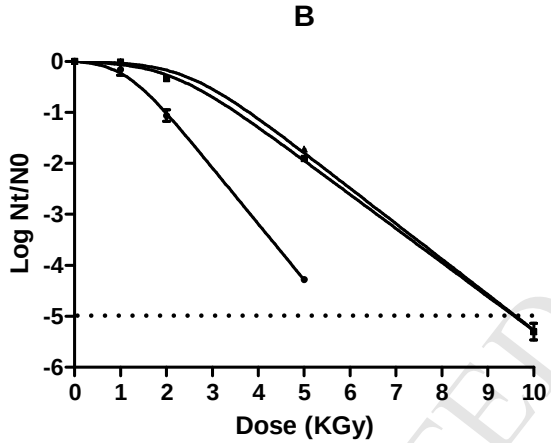

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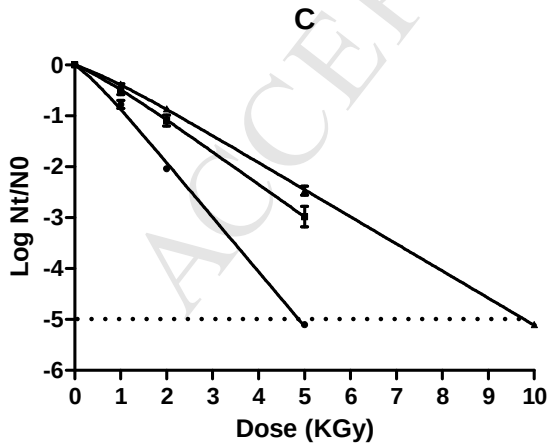

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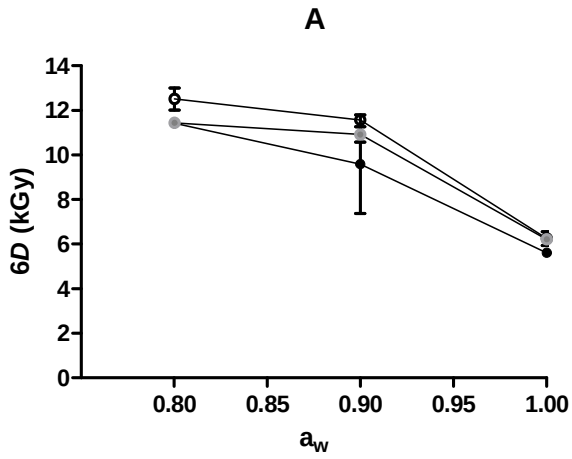
680 **Figure 2**



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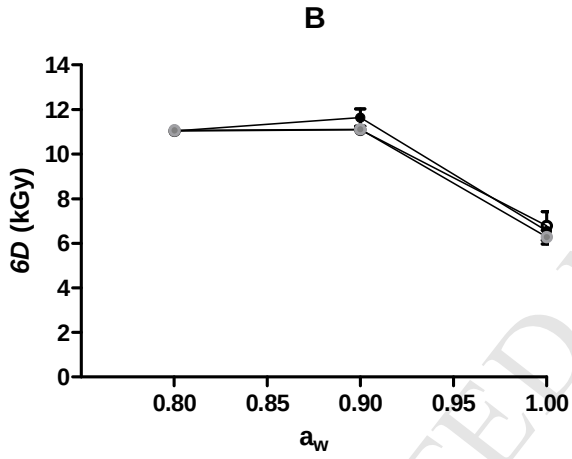

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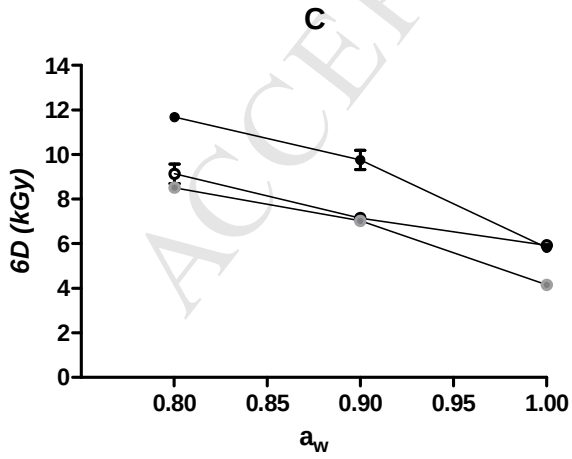

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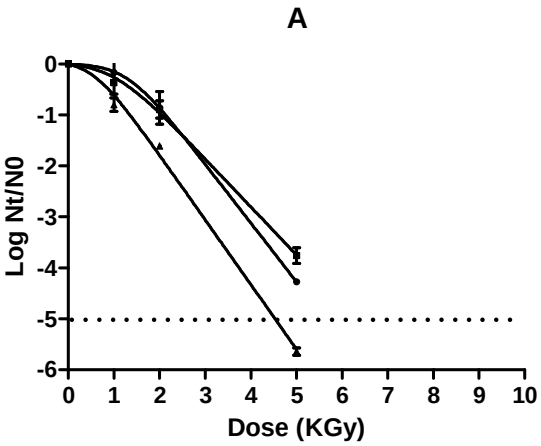
686 **Figure 3**



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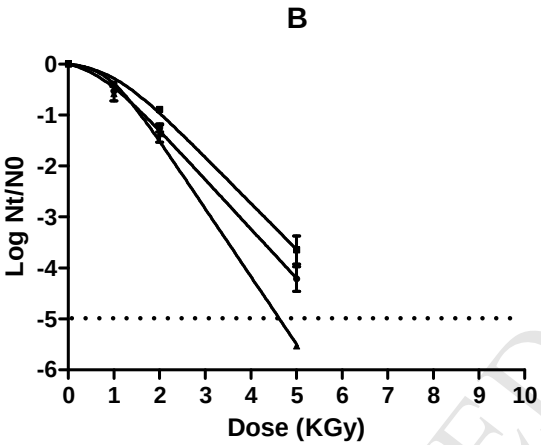

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699 **Figure 4**

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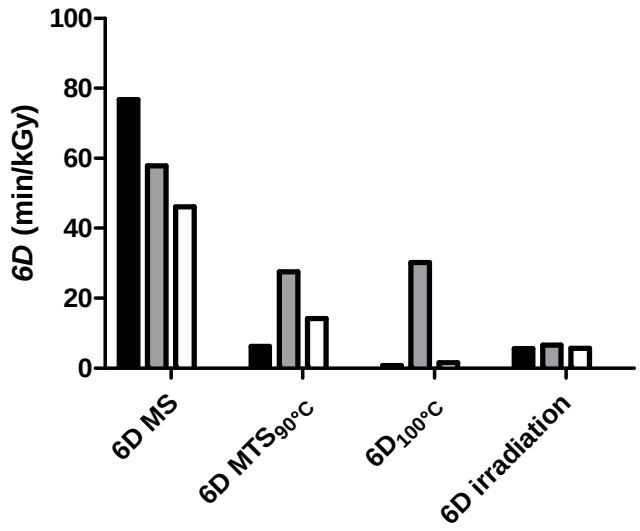

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718 **SUPPLEMENTARY MATERIAL**

719 **Figure S1.** Survival curves to electron beam ionizing radiation at room temperature of *B.*
720 *mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate
721 buffer of pH 5.5 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars
722 represent standard deviation of three replicates.

723 **Figure S2.** Survival curves to electron beam ionizing radiation at room temperature of *B.*
724 *mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate
725 buffer of pH 4 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars
726 represent standard deviation of three replicates.

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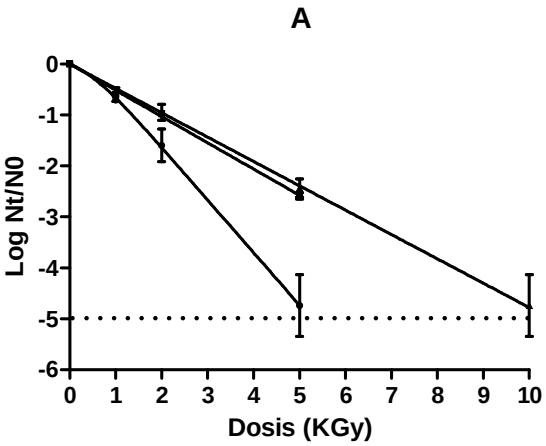
744 **Figure S1.**



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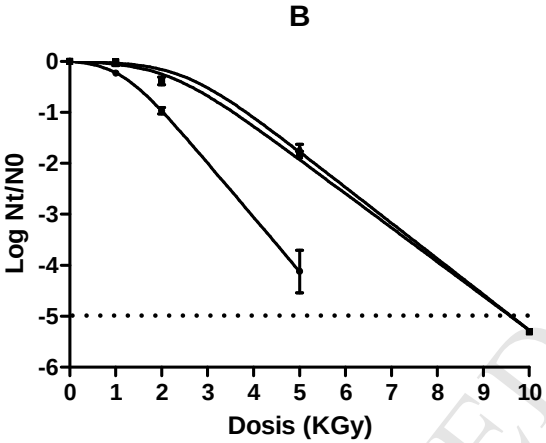

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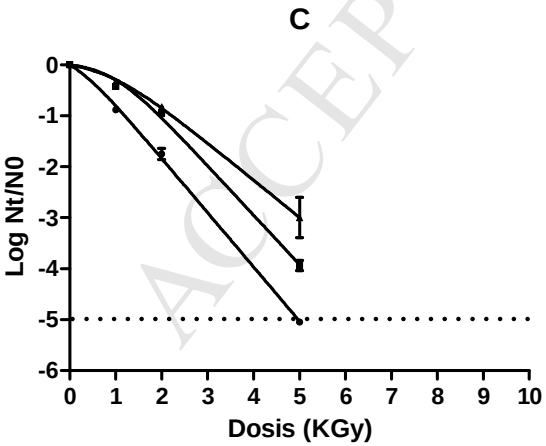

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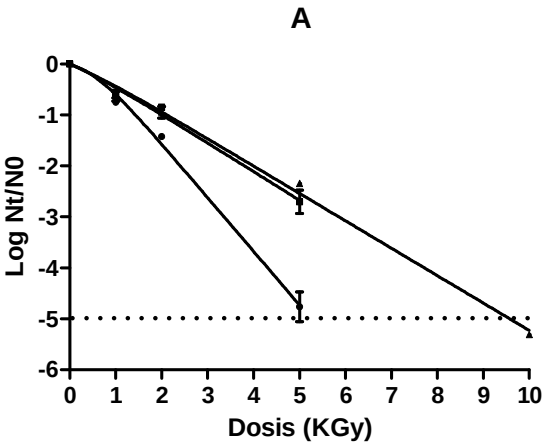
750 **Figure S2.**



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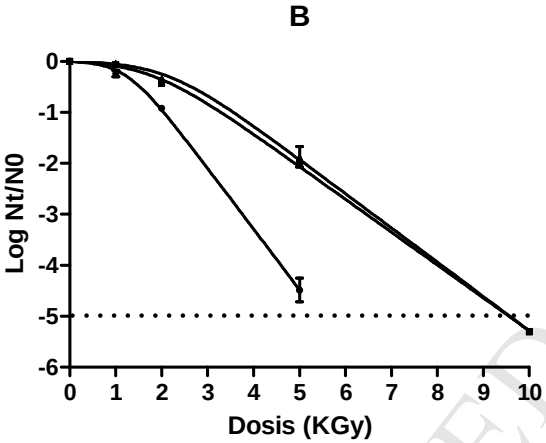

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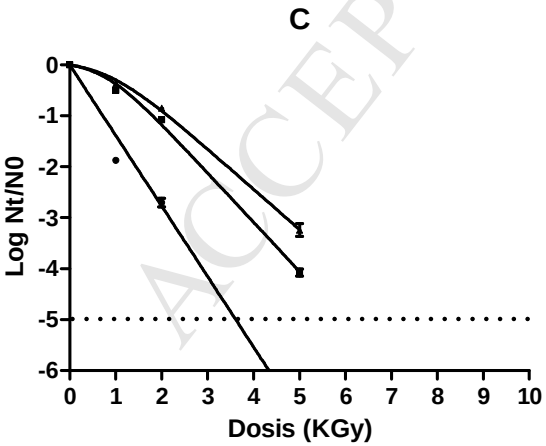

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Highlight

- The effect of the treatment media pH was different for the different spores forming bacteria
- A protective effect of low a_w of the treatment medium was observed
- The protective effect of the reduction on a_w has the major effect in the range from >0.99 to 0.90
- Ionizing radiation could be a suitable technology to reduce the naturally present bacterial spore populations present in crab meat products