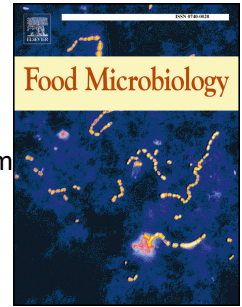


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Crab-meat-isolated psychrophilic spore forming bacteria inactivation by electron beam ionizing radiation

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1 **Title:** Crab-meat-isolated psychrophilic spore forming bacteria inactivation by electron beam
2 ionizing radiation.

3

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

25 **Abstract**

26 The present work was performed to evaluate the potential of electron beam ionizing
27 radiation for the inactivation of three psychrophilic spore forming bacteria (*Bacillus*
28 *mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans*) isolated from
29 ready-to-eat brown crab (*Cancer pagurus*). Inactivation curves for the three spores were
30 performed in both types of crab meat, brown and white. Also the effect of pH and water
31 activity (a_w) on the lethal efficacy of ionizing radiation, for the three different psychrophilic
32 spore forming bacteria, was evaluated. The effects of pH, a_w and their possible interactions
33 were assessed in citrate-phosphate buffers of different pH, ranging between 7 and 4, and a_w ,
34 ranging from <0.99 and 0.80. A reduction of a_w increased the spores resistance between
35 >0.99 and 0.90, while an a_w reduction from 0.90 to 0.80 had a minor impact on their
36 resistance. In contrast to a_w , the effect of pH showed a greater variability depending on the
37 spore species. While pH did not affect the resistance of *B. weihenstephanensis* at any a_w , *B.*
38 *mycoides* showed slightly higher resistance at pH 5.5 at a_w of 0.90 and 0.80. pH showed a
39 significant effect on the resistance of *P. psychrodurans*. For the two types of crab meat,
40 slightly differences were observed in 6D values. *B. weihenstephanensis* was the most
41 resistant, requiring 7.3-7.6 kGy to inactivate 6 Log₁₀-cycles of this spore forming bacterium,
42 while for *B. mycoides* and *P. psychrodurans* 6.1-6.3 and 5.4-5.3 kGy respectively were
43 necessary to reach the same inactivation level in crab meat. An agreement between spore
44 resistance in crab meats and lab media, with similar characteristics in pH and a_w , was also
45 observed. The results obtained in this research demonstrated the potential for ionizing
46 radiation to achieve an appropriate inactivation level of spores naturally present in brown
47 crab with the application of doses lower than 10 kGy.

48

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

50

51 **1. INTRODUCTION**

52 The use of ionizing radiation for food decontamination was proposed in the 19th
53 century, and since then a wide range of research has been performed to evaluate the potential
54 of this technology for microbial inactivation (De Lara et al., 2002; Grant and Patterson, 1992;
55 Jeong and Kang, 2017; Sarrías et al., 2003; Thayer and Boyd, 1993), and assess its influence
56 on food properties (Byun et al., 2000 and 2008; Diehl, 1991; Graham and Stevenson, 1997;
57 Lee et al., 2001). Currently, a number of organisations worldwide have accepted this
58 technology as a safe alternative technology for food decontamination (WHO, FDA). The
59 World Health Organization has established 10 kGy as the maximum dose for food processing
60 without any adverse effect on food matrixes (WHO 1981). Though, a later study concluded
61 that no limiting dose is required (WHO 1999). Either way, nowadays more than 60 countries
62 worldwide have regulations regarding the use of ionizing radiation for food products (IAEA,
63 2017). In fact, the joint FAO/IAEA (International Atomic Energy Agency) Division of
64 Nuclear Techniques in Food and Agriculture estimates that approximately 700,000 tonnes of
65 food were irradiated in 2013 (IAEA, 2015). The main potential for the use of ionizing
66 radiation in foods is its ability to extend the microbiological shelf-life with poultry, egg
67 products, red meats, seafood products and spices proposed as good candidates for the use of
68 radiation as decontamination technology, due to its potential to inactivate microorganisms at
69 low temperatures (Farkas, 2006).

70 Fish and fishery products have a special interest due to their particular characteristics.
71 Many of these products are commercially cooked as products in their own right or are cooked
72 for use as ingredients in ready-to-eat products, where a thermal pasteurization to reduce 6
73 Log₁₀-cycles of non-proteolytic *Clostridium botulinum* type E is commonly applied to ensure
74 food safety. However, the shelf-life of these products is directly dependent on the cold chain

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

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

91 It is widely recognised that microbial inactivation induced by ionizing radiation is due
92 to the DNA damage (Farkas, 2006). Despite the knowledge of its inactivation mechanism, a
93 lack of data exists concerning the effects of treatment media characteristics on the lethal
94 efficacy of EBI. It is also well known that physico-chemical characteristics of the treatment
95 medium have an important effect on the microbial resistance against physical stress; however
96 few studies in this respect related to EBI exists (Fan and Sommers, 2012; Huhtanen et al.,
97 1989; Thayer and Boyd, 1993). To the best knowledge of the authors a systematic study to
98 assess the effect of common variables, such as pH, water activity (a_w) and their interactions

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

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

107

108 **2. MATERIALS AND METHODS**

109 *2.1. Microorganisms, treatment media and sample preparation*

110 The three spore forming bacteria used in this study were the three most isolated from
111 Irish brown crab (*Cancer pagurus*): *Bacillus mycoides*, *Bacillus weihenstephanensis* and
112 *Psychrobacillus psychrodurans*. During this investigation, the three spore suspensions were
113 managed and prepared as described by Condon-Abanto et al. (2016). In brief, 1 mL from a
114 pure culture in stationary phase was spread onto the surface of Tryptone Soya Agar with
115 0.6% (w/v) yeast extract (TSAYE) (Oxoid Ltd., Basingstoke, Hampshire, UK) agar plates
116 containing 3 ppm (w/v) of manganese sulphate (Carlo Erba, Milan, Italy) and incubated at 25
117 °C for 10 days. Spores were then collected with sterile pH 7.0 McIlvaine citrate-phosphate
118 buffer (Dawson et al., 1974) and washed and centrifuged three times. The final spore
119 suspensions were then submerged in boiling water for 1 minute in order to inactivate the
120 possible remaining population of vegetative cells and stored under refrigeration (4±1 °C) until
121 use. The presence of aggregates was evaluated by direct microscopic observation in a Thoma

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

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

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

139 For crab meat samples, crabs were cooked at $95\text{ }^\circ\text{C}$ for 20 minutes. White meat from
140 claws and brown meat from the body were then removed aseptically in a sterile laminar flow
141 cabinet (Telestar mini-V/PCR, Telestar Technologies, S.L., Terrasa, Spain) to ensure the
142 natural contamination was under the detection limit (data not shown). Then, meats were
143 distributed by placing 1 g of each meat in sterile tubes of 10 mL, and 0.1 mL of the
144 corresponding spore suspension dilution was added obtaining an initial concentration of 10^6
145 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 °C 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

171 2.4. Modeling and Statistical analysis

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

$$184 \quad N_t = N_0 e^{-k_{\max} * \text{dose}} \left(\frac{e^{k_{\max} Sl}}{1 + (e^{-k_{\max} Sl} - 1) e^{k_{\max} * \text{dose}}} \right) \quad (1)$$

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

$$188 \quad D_{10} = 2.303/k_{\max} \quad (2)$$

189 To determine the treatment parameters and compare the resistance between the three
 190 spores under study, $\underline{6D}$ values were calculated. In this case $\underline{6D}$ is defined as the necessary
 191 dose to inactivate 6 Log_{10} -cycles of the initial spore population, and is calculated by Equation
 192 3.

$$193 \quad 6D = Sl + 6 * D_{10} \quad (3)$$

194 Where Sl is the shoulder length duration and \underline{D}_{10} is the inactivation parameter
 195 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. mycooides* (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. mycooides*,
220 ranged between 0 and 0.6 kGy and D_{10} values ranged from 0.8 to 2.1 kGy, showing the

221 highest D_{10} of the three bacterial species investigated. As in *Sl*, the pH hardly changed the D_{10}
222 values, while the reduction of a_w showed an important influence. The reduction of a_w from
223 >0.99 to 0.90 induced an increase in the D_{10} values, close to a 2-fold order of magnitude,
224 while further reductions hardly changed this parameter.

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

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

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

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

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

264 In general, the maximum increase in the spore resistance was observed with a_w
265 reduction from >0.99 to 0.90, while further reductions of a_w hardly affected the spore
266 resistance. Only in the case of *P. psychrodurans* at pH 5.5, $6D$ value increased linearly from
267 5.9 kGy to 9.1 kGy with the a_w reductions. This species was also the most affected by the
268 variation of a_w , increasing $6D$ values from 4.1 kGy to 8.5 kGy at pH 4 and from 5.8 to 11.7 at
269 pH 7 when reducing a_w from >0.99 to 0.80.

270 From Figure 2, besides the influence of pH and a_w , differences in radiation resistances
271 between species can be observed. *B. mycooides* showed the lowest resistance (5.5 kGy) in pH
272 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.*
273 *weihenstephanensis* showed the lowest resistance (6.2 kGy) at >0.99 of a_w and pH 4, and the
274 highest (11.0-11.1 kGy) at all pH and both a_w 0.90 and 0.80. *P. psychrodurans* showed the
275 lowest resistance (4.2 kGy) at pH 4.0 and a_w >0.99, while the highest resistance (11.7 kGy)
276 was detected in media of pH 7.0 and a_w of 0.80. *B. weihenstephanensis* showed the greatest
277 resistance at most pHs and water activities investigated. Only at the lowest a_w tested (0.80)
278 did *B. mycooides* became the most resistant spore at pH 5.5. Therefore choice of irradiation
279 reference organism is dependent upon a_w of the product.

280

281 3.2. Spores inactivation in crab meats

282 As occurred in lab media, the inactivation curves obtained in crab meat showed
283 downwards profiles in all cases (Figures 3A and 3B). Therefore, the Geeraerd log-linear
284 regression plus shoulder model (Geeraerd et al., 2000) was used to describe the curves. Table
285 2 shows the resistance parameters for the three spores in white and brown meat: D_{10} , Sl and
286 $6D$ values. R^2 and $RMSE$ have been included to show the goodness of fit of Equation 2 to the
287 survival curves. A slight increase of the radiation resistance parameters Sl , D_{10} and $6D$ was
288 observed when spores were treated in crab brown meat. *B. weihenstephanensis* was the most
289 resistant requiring 7.3 and 7.6 kGy for white and brown meat, respectively, to reach 6 Log_{10} -
290 reductions, while *P. psychrodurans* was the most sensitive requiring 5.4 and 5.3 kGy,
291 respectively, to reach a similar inactivation level.

292 Figure 4 allows the comparison among the resistances of the three bacterial spores to
293 different food preservation technologies: heat, ultrasonic waves under pressure at sublethal
294 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

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

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

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

345 two mechanisms affecting the DNA. The most simple would be comparable with the UV-C
346 mechanisms where the damage in the DNA is produced when an energy photon or electron
347 crash randomly with the genetic material (Dickson 2001; Goodhead 1994; Yokoya et al.,
348 2008); while the second one involved more complex reactions based on the radiation
349 chemistry of water. EBI, in presence of water, produces reactive species, from which
350 hydroxyl radicals (OH•) and hydrogen peroxide (H_2O_2) are considered the main factors
351 responsible for the reactions with nucleic acids (Sutherland et al., 2000; Lomax et al., 2002).
352 The protective effect of a_w observed in this investigation shows the importance of this second
353 mechanism for the inactivation efficacy of EBI. These series of reactions would also explain
354 the results obtained by other authors, where the radiation resistance of different
355 microorganisms increased when microorganism were treated in frozen media, where again a_w
356 is reduced by the freezing process (Black and Jaczynski, 2006; Fan and Sommers, 2012;
357 Thayer and Boyd, 1993 and 2001).

358 De Lara et al. (2000) suggested that the mechanism involved in bacterial spore
359 inactivation by ionizing radiation would be very different from the mechanisms involved in
360 heat destruction due to the different targets of each technology. However, since research
361 about the effect of a_w on spore resistance against EBI has not been described yet, it would be
362 convenient to compare the effect of this parameter between these two technologies.
363 Thermobacteriology studies with different *Bacillus* species have reported a linear correlation
364 between the Log of thermal D_{10} values and $(1-a_w)$ in different ranges of a_w (Guillard et al.,
365 1998; Mazas et al., 1999), but in the present study no clear relations were detected between
366 these two parameters. Mazas et al. (1999) reported that the effect of a_w on the heat resistance
367 of several strains of *Bacillus cereus* spores begins to be noticed at a_w values lower than 0.85,
368 while our results suggest that the main effect of a_w on the radiation lethal efficacy is produced
369 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 et 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.

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

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

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

420 results also proved that, despite the different composition and chemical characteristics of the
421 two kinds of crab meat (Anacleto et al., 2011; Barrento et al., 2010), the specific resistance of
422 each spore was scarcely affected by the type of meat. Moreover, the specific resistances of
423 each species in meat were similar to those detected in lab media at similar pH and a_w levels.
424 These results would indicate that unlike other technologies, the irradiation dose applied to lab
425 media could be used as reference to calculate the necessary treatments for each specific
426 foodstuff. Nevertheless, this important aspect would require further more exhaustive studies.
427 Finally, the inactivation curves obtained in both types of meats suggested that a dose below
428 10 kGy, which is the maximum permitted and recommended legal dose by FAO/WHO for
429 foods, would permit a reduction of 6 Log_{10} -cycles of any of the investigated bacterial spores
430 present in crab and crab products. These results would indicate that EBI could be an adequate
431 technology to preserve brown crab. However, further research would be necessary to
432 determine the impact of those treatments in the crab meat quality and the maximum
433 applicable dose to avoid possible undesirable changes on the sensory characteristics.

434

435 5. CONCLUSIONS

436 In summary, this work covers a knowledge gap in the field of bacterial spore
437 inactivation by electron beam ionizing radiation. The obtained results showed that the pH of
438 the treatment media could affect the spore resistance, although the effect would be dependent
439 on the specific spore under study. On the other hand, an important protective effect of low a_w
440 of the treatment medium was observed, but the impact of this parameter is present in a larger
441 or smaller magnitude depending on the bacterial spore. The protective effect of the reduction
442 on a_w has the major effect in the range from >0.99 to 0.90 , regardless the investigated spore.
443 The studied spores showed, in both lab media and crab meat, shoulders followed by an
444 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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632 **Table 1.** Electron beam radiation resistance parameters obtained from the fitting of the
 633 Geeraerd log-linear plus shoulder model (Equation 1) to the survival curves of *B. mycooides*,
 634 *B. weihenstephanensis* and *P. psychrodurans* in citrate-phosphate buffers of different pH and
 635 a_w .

Microorganism	pH	a_w	<i>Sl</i> (kGy)	D_{10} (kGy)	RMSE
<i>B. mycooides</i>	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
<i>B. weihenstephanensis</i>	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
<i>P. psychrodurans</i>	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

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

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

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

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 644 D_{10} , decimal reduction dose (kGy) calculated from k_{max} whit Equation 2; Sl , shoulder length
 645 (kGy); $6D$, necessary doses (kGy) to reached 6 Log₁₀-reductions ; $RMSE$, root mean square
 646 error; R^2 , determination coefficient. Numbers in brackets represent standard deviation of
 647 three replicates. Letters show differences within columns (p<0.05).

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656 **Figure legend**

657 **Figure 1.** Survival curves to electron beam ionizing radiation at room temperature of *B.*
658 *mycooides* (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-Log₁₀ cycles of
662 *B. mycooides* (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.

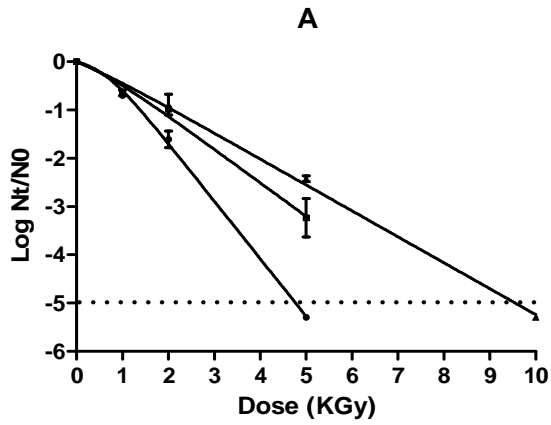
664 **Figure 3.** Survival curves to electron beam ionizing radiation at room temperature of *B.*
665 *mycooides* (●), *B. weihenstephanensis* (■) and *P. psychrodurans* (▲) in crab's white meat
666 (A) and brown meat (B). Error bars represent standard deviation of three replicates.

667 **Figure 4.** Specific resistance of *B. mycooides* (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).

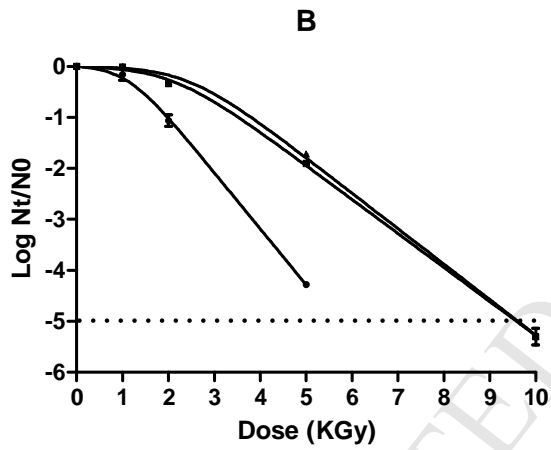
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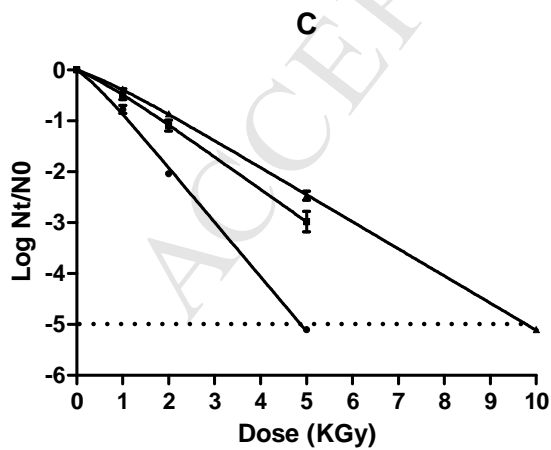
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674 **Figure 1**

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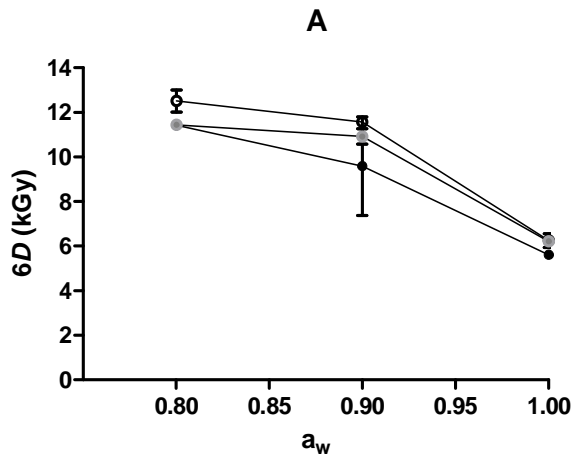
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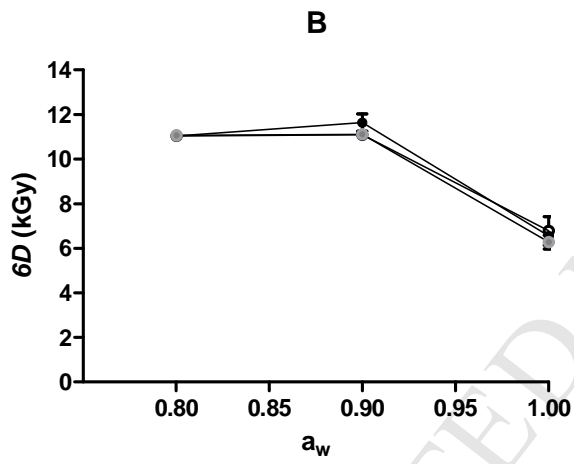
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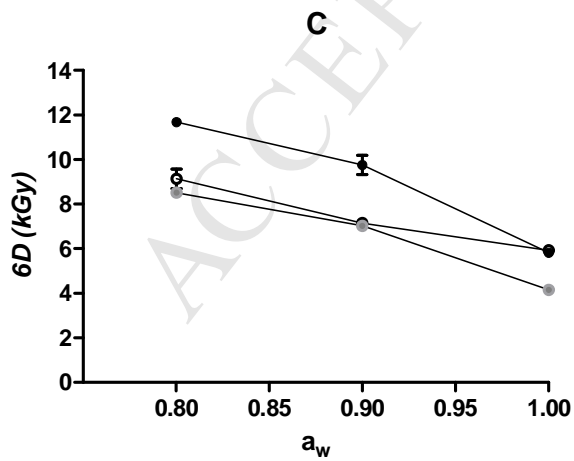
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680 **Figure 2**

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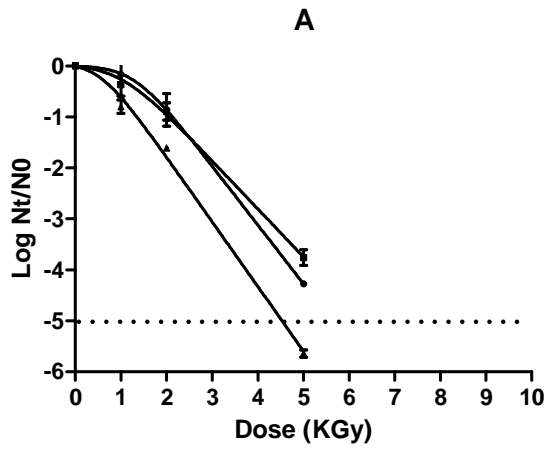
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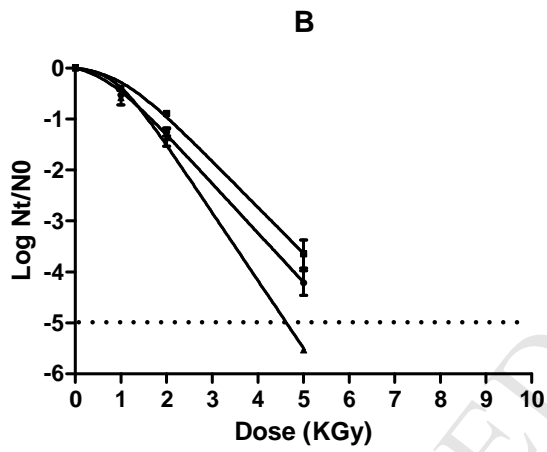
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686 **Figure 3**

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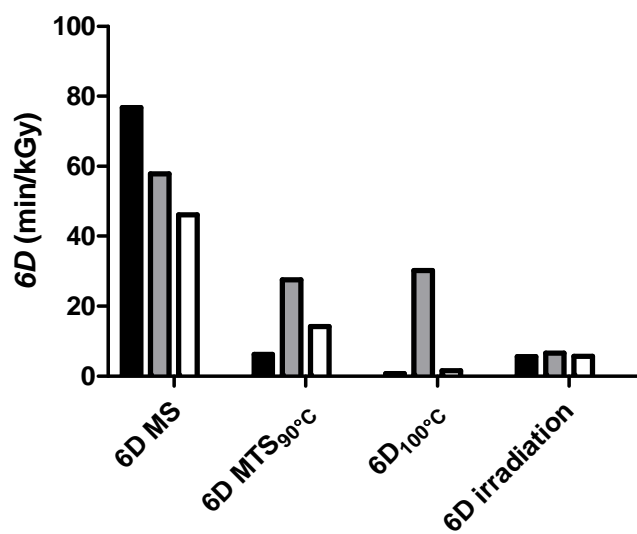
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699 **Figure 4**

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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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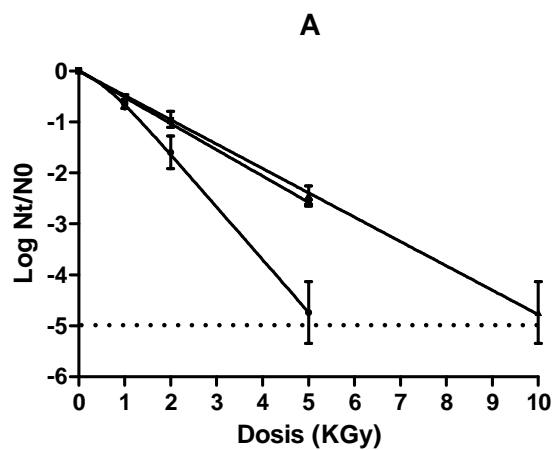
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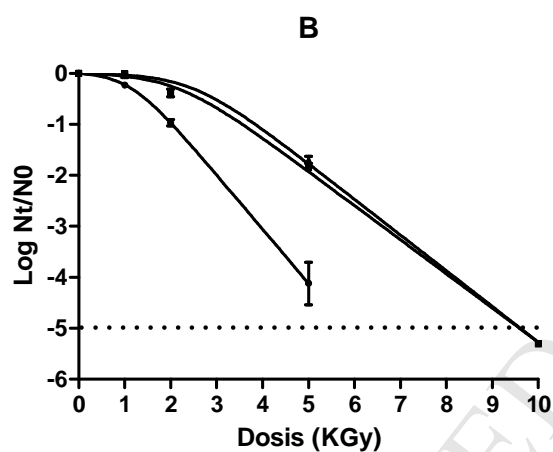
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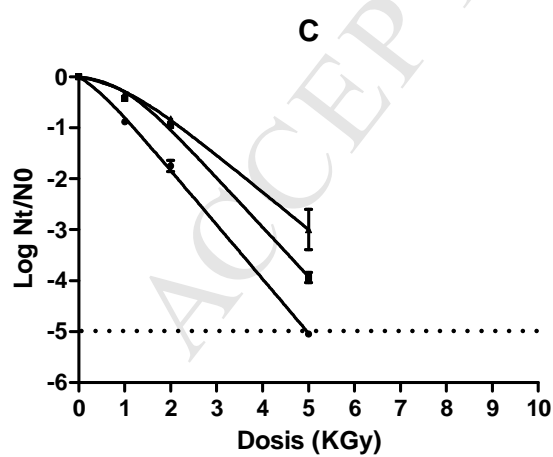
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744 **Figure S1.**

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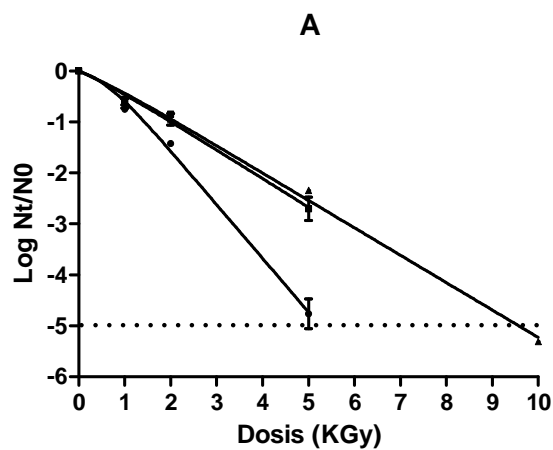
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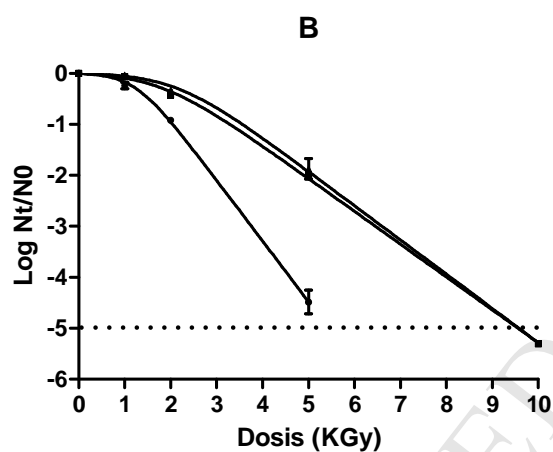
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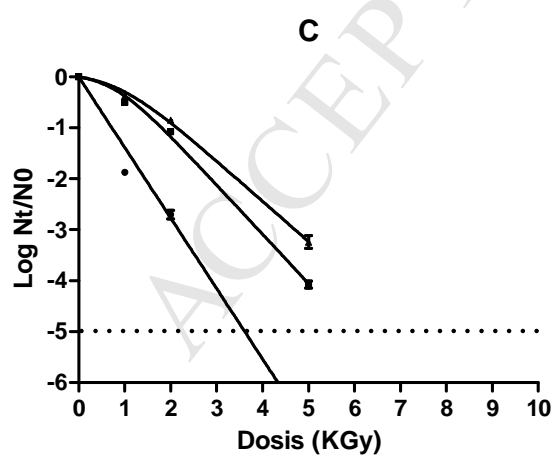
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750 **Figure S2.**

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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