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

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**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, slight differences were observed in 6D 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; Sarrias 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 °C) 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
distribute the spores in the meat, and treated immediately. $a_w$ of the crab meat, both white and brown was 0.99 and the pH ranged from 7.5-8.0.

2.2. Irradiation treatments

Irradiation treatments were carried out in a 10-MeV circular electron accelerator (Rhodotron) at the irradiation plant of Ionisos Ibérica (Tarancón, Cuenca, Spain). Well plates, and inoculated meat samples were irradiated at programmed doses of 1, 2, 5, 10 and 15 kGy. Irradiation dosimetry was carried out by using a band of cellulose triacetate located on the surface of the samples (Nieto-Sandoval et al., 2000). The irradiation dosimetry indicated that the actual doses applied were 1.13, 2.07, 5.38, 10.7 and 16.4 kGy, respectively. All experiments were carried out in triplicate, by using different independently prepared spore suspensions, applying irradiation doses in different runs during the same working day due to limit accessibility to the circular electron accelerator.

2.3. Recovery, incubation and survival counting of treated samples

Immediately after treatments serial decimal dilutions in MRD of liquid samples were pour-plated using TSAYE (Oxoid) as recovery media. Meat samples were diluted in 9 mL of maximum recovery diluent (MRD) (Oxoid) and homogenized with an ultra-turrax® for 20 seconds. Then, proper dilutions in MRD were pour-plated in TSAYE (Oxoid). Plates were incubated at 25 °C for 24 hours for $B.\ mycoides$ and $B.\ weihenstephanensis$ and 48 hours for $P.\ psychrodurans$. Longer incubation times did not change the obtained counts (data not shown). Colony-forming units (CFU) were counted with an improved automatic colony-counting image analyzer (Protos, Synoptics, Cambridge, UK), previously described by Condón et al. (1987).
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.

$$N_l = N_0 e^{-k_{max} \cdot dose} \frac{e^{k_{max}Sl}}{1+(e^{-k_{max}Sl-1})e^{k_{max} \cdot dose}} \quad (1)$$

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.

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

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

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

Where $Sl$ is the shoulder length duration and $D_{10}$ is the inactivation parameter calculated from Equation 2.
$R^2$ and RMSE values provided by the software were used to evaluate the goodness of fits. Statistical analyses ($t$-test and one-way ANOVA) were performed with the GraphPad PRISM® and differences were considered significant if $p \leq 0.05$. The standard deviations (SD) are given in the figures as the error bars.

3. RESULTS

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

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

For the three spore species, $a_w$ affected their irradiation resistance influencing both, the $S_l$ and the $D_{10}$ values. The maximum resistance was observed at the lowest investigated $a_w$ (0.80), whereas pH hardly affected the irradiation resistance. The $S_l$ of B. mycoides, 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. psychrotrum* 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 $SI$ parameter. The most noticeable difference was observed in case of *P. psychrotrum* where a reduction in resistance was detected at acid pHs.

To define the irradiation treatment intensity required to apply at industrial level, both $SI$ 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, $6D$ values were compared. The advantage of using this value is that it comprises both inactivation model parameters, $SI$ and $D_{10}$. Therefore, it is possible to compare directly the resistance between spores at all investigated conditions. In addition, $6D$ 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 $6D$ values for *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrotrum* (C). As observed, pH had a lower effect on the spore resistance than the effect observed for $a_w$. For *P. psychrotrum* (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. psychrotrum* at pH 5.5, $6D$ 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 $6D$ 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
(manothermosonication; MTS) (Arroyo et al., 2011), and EBI. Data for heat, MS and MTS were extracted from Condón-Abanto et al. (2016). As observed, the maximum differences in resistance between the most and lowest resistant spores were 1.7-fold for MS, 4.4-fold for MTS, 44.4-fold for heat and less than 1.2-fold for EBI.

4. DISCUSSION

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

According to our results, all the studied spores showed inactivation curves with no exponential kinetics, and in most of the investigated conditions shoulders were observed. Similar kinetics were described by other authors for Bacillus spores (Blatchley et al., 2005). However, log linear inactivation kinetics have also been described for B. cereus and B. subtilis spores (De Lara et al., 2002). The presence of shoulders has been explained by the capacity of microorganisms to repair damage caused by low intensity treatments, the activation of dormant spores and due to the presence of agglomerates (Mathys et al., 2007; 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. psychrodurans*, 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*<sub>w</sub> 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*<sub>w</sub> 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 (H₂O₂) 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
0.96 to 0.71 increased $D_{10}$ values to heat between 30 and 60-fold and Gillard et al. (1998) observed an increase on $D_{10}$ values to heat (of *B. cereus*) higher than ten-fold when the $a_w$ was reduced from >0.99 to 0.80. Contrarily, our results showed a much lower protective effect of low $a_w$, since the resistance of spores hardly increased when $a_w$ was reduced from 0.90 to 0.80. The protective effect of $a_w$ reduction against EBI is related presumably with the indirect inactivation mechanisms based on the formation of oxidative species (ROS) due to the radiation chemistry of water but also due to a reduction of the intercellular water content of the spore (Dickson, 2001; Moeller et al., 2014). The sorption isotherm of the most organic materials indicates that, the reduction of $a_w$ from >0.99 to 0.9 involves a great percentage reduction of the water content, while $a_w$ reduction from 0.90 to 0.80 requires a much smaller reduction of the water content (Yanniotis and Blahovec, 2009). This would explain the great protective effect of $a_w$ between >0.99 and 0.90 and the low protective effect between 0.90 and 0.80 observed in this research.

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

Acknowledgements

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References


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.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>pH</th>
<th>a_w</th>
<th>Sl (kGy)</th>
<th>D_{10} (kGy)</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. mycoides</strong></td>
<td>7</td>
<td>&gt;0.99</td>
<td>0.6 (0.032)^a</td>
<td>0.8 (0.002)^a</td>
<td>0.069</td>
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<tr>
<td></td>
<td></td>
<td>0.90</td>
<td>0.5 (0.243)^abcd</td>
<td>1.5 (0.319)^b</td>
<td>0.111</td>
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<td></td>
<td></td>
<td>0.80</td>
<td>0.3 (0.063)^bc</td>
<td>1.9 (0.009)^bc</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>&gt;0.99</td>
<td>0.4 (0.005)^c</td>
<td>1.0 (0.043)^c</td>
<td>0.031</td>
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<tr>
<td></td>
<td></td>
<td>0.90</td>
<td>-</td>
<td>1.9 (0.028)^d</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.80</td>
<td>-</td>
<td>2.1 (0.068)^c</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;0.99</td>
<td>0.5 (0.040)^d</td>
<td>0.9 (0.025)^a</td>
<td>0.107</td>
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<tr>
<td></td>
<td></td>
<td>0.90</td>
<td>0.3 (0.003)^d</td>
<td>1.8 (0.046)^bc</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.80</td>
<td>0.3 (0.085)^cd</td>
<td>1.9 (0.016)^bc</td>
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<tr>
<td><strong>B. weihenstephanensis</strong></td>
<td>7</td>
<td>&gt;0.99</td>
<td>1.1 (0.067)^d</td>
<td>0.9 (0.014)^a</td>
<td>0.042</td>
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<td></td>
<td></td>
<td>0.90</td>
<td>1.9 (0.045)^d</td>
<td>1.6 (0.027)^b</td>
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<td></td>
<td></td>
<td>0.80</td>
<td>2.4 (0.013)^d</td>
<td>1.4 (0.005)^c</td>
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<tr>
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<td>5.5</td>
<td>&gt;0.99</td>
<td>1.1 (0.064)^d</td>
<td>0.9 (0.043)^b</td>
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<td>0.8 (0.024)^f</td>
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<tr>
<td><strong>P. psychrodurans</strong></td>
<td>7</td>
<td>&gt;0.99</td>
<td>0.2 (0.012)^d</td>
<td>0.9 (0.002)^a</td>
<td>0.076</td>
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<td>0.90</td>
<td>0.3 (0.179)^abc</td>
<td>1.6 (0.083)^b</td>
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<td></td>
<td>0.80</td>
<td>0.4 (0.003)^b</td>
<td>0.9 (0.001)^b</td>
<td>0.015</td>
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<td>5.5</td>
<td>&gt;0.99</td>
<td>0.3 (0.028)^d</td>
<td>0.9 (0.003)^c</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>1.0 (0.028)^d</td>
<td>1.0 (0.014)^d</td>
<td>0.078</td>
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<tr>
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<td></td>
<td>0.80</td>
<td>0.9 (0.012)^d</td>
<td>1.4 (0.061)^f</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;0.99</td>
<td>-</td>
<td>0.7 (0.003)^f</td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.90</td>
<td>0.8 (0.035)^d</td>
<td>1.0 (0.013)^d</td>
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<tr>
<td></td>
<td></td>
<td>0.80</td>
<td>0.9 (0.029)^d</td>
<td>1.3 (0.024)^f</td>
<td>0.041</td>
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</tbody>
</table>

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.

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

$D_{10}$, decimal reduction dose (kGy) calculated from $k_{max}$ with Equation 2; $SI$, shoulder length (kGy); $6D$, 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).
**Figure legend**

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

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

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

**Figure 4.** Specific resistance of *B. mycoides* (black bars), *B. weihenstephanensis* (grey bars) and *P. psychrodurans* (white bars) to different inactivation technologies in citrate-phosphate buffer of pH 7.0 and a\textsubscript{w} >0.99 (data for MS, MTS and Heat are adapted from Condon-Abanto et al., 2016).
Figure 1

A

B

C
Figure 2

A

B

C
Figure 3

A

B

Dose (KGy) vs. Log Nt/N0 for different treatments.
Figure 4

![Graph showing 6D (min/kGy) for different conditions]
SUPPLEMENTARY MATERIAL

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

**Figure S2.** Survival curves to electron beam ionizing radiation at room temperature of *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate buffer of pH 4 and water activity (*a_w*) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars represent standard deviation of three replicates.
Figure S1.
Figure S2.
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