Title: Application of ultrasound in combination with heat and pressure for the inactivation of spore forming bacteria isolated from edible crab (*Cancer pagurus*)

Authors: Condón-Abanto, S., Arroyo, C., Álvarez, I., Condón, S. and Lyng, J. G. *

* Corresponding author: Prof. James G. Lyng
School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4,
Ireland

Keywords: Manothermosonication, MTS, spore inactivation, seafood, Brown crab

Abstract

This research was performed to characterize the resistance of three different bacterial spores species isolated from pasteurized edible crab (*Cancer pagurus*) meat to heat treatments and to assess the potential of manosonication (MS) and manothermosonication (MTS) as an alternative for their inactivation. The spore-forming bacteria used in this study were *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans*. The thermal resistance of these three species was determined at different temperatures ranging from 80 to 110 °C and their resistance to ultrasound under pressure from 35 to 95 °C. GInaFit Excel tool was used to fit the Geeraerd's 'Log-linear + shoulder' and Bigelow & Easty's equations to the survival curves for heat and MS/MTS treatments. From the results obtained it can be concluded that the profile of the survival curves either for heat or for ultrasound treatments depended on the bacterial spore species. When shoulders were detected in the inactivation curves for heat they were also present in the curves for MS/MTS treatments, although the application of an ultrasonic field reduced the shoulder length. *B. weihenstephanensis* was found to be the most resistant species to heat, requiring 1.4 min to reduce 4 log₁₀ cycles at 107.5 °C ($z_T = 7.1$ °C) while *B. mycoides* was the most sensitive requiring 1.6 min at 95 °C ($z_T = 9.1$ °C). By contrast, *B. mycoides* was the most resistant to MS. The efficiency of the combination of ultrasonic waves under pressure with heat (MTS) for bacterial spore inactivation was directly correlated with the thermal resistance. Indeed, MTS showed a synergistic effect for the inactivation of the three spores. The highest percentage of synergism corresponded to the spore species with higher z_T value (*B. mycoides*), but the highest temperature at which this synergism was detected corresponded to the most heat tolerant spore species (*B. weihenstephanensis*). This study revealed that MTS treatment is capable of inactivating spore-forming bacteria and that the inactivation efficiency of the combined treatment is correlated with the thermal resistance of the spore species.

1. Introduction

It is globally accepted that the consumption of seafood is important in a healthy and balanced diet (WHO, 2003), since it is an essential source of valuable nutrients and is low in saturated fat (Barrento *et al.*, 2009). Crustaceans are widely appreciated and consumed mostly in the Mediterranean countries. In particular, *Cancer pagurus*, commonly known as brown crab or edible crab, is one of the most consumed crustaceans in south European countries such as France, Spain, Italy and Portugal (EUROSTAT, 2009). Over 84% of the total catch of this species comes from the English Channel and the Welsh, Scottish and Irish coasts (EUROSTAT, 2009). This type of seafood has been traditionally sold alive and is cooked at home with its price dictated by factors such as capture origin and animal sex. In more recent times, new ready-to-eat brown crab products and whole pasteurized brown crabs have been launched into the market but industrial practices have not evolved to keep pace with these developments. Therefore, there is a niche for process optimisation and/or for the incorporation of new technologies to enhance the processing of these type of products. One of the major problems encountered in the elaboration of this type of products is the presence of bacterial spores in the raw product (Faghri *et al.*, 1984; Gram and Huss, 1996; Huss, 1997; Ward *et al.*, 1977) which can survive the thermal treatments commonly applied in the manufacturing process. The high thermo-resistance of these spores is the reason for severe heat treatments required to inactivate them but the down side is the negative impact on the organoleptic and nutritional quality of the final product which is especially pronounced in seafood products.

One of the current challenges in food processing is the identification of new technologies that can be used as an alternative to conventional thermal treatments or technologies that can be used to supplement traditional heating to contribute to an increase in the heat lethal efficacy while maintaining the quality attributes of the final product at a higher

level than those encountered in conventional heating (Condón *et al.*, 2005). One of these supplement type technologies is ultrasound, which can be combined with other technologies for food processing and also for food preservation (Chemat *et al.*, 2011; Earnshaw, 1998; Hielscher, 2008; Knorr *et al.*, 2004). High-power (high-energy, high-intensity) ultrasound involves the use of intensities greater than 1 W/cm² at frequencies between 18 and 100 kHz and the main mechanism responsible for the microbial inactivation is the physical/mechanical forces generated by acoustic cavitation. However, on its own, the lethal effectiveness of high power ultrasound on vegetative cells is low and it is even lower when used for the inactivation of bacterial spores, which have a very complex structure with mechanically resistant envelopes. However, while the potential of using ultrasound as a stand-alone ‘non-thermal’ alternative preservation treatment to traditional heat processing has long since been disregarded (Berger and Marr, 1960), the combination of ultrasound with other techniques such as hypochlorite, mild heat or pressure has been demonstrated to enhance its lethal effectiveness (Arroyo *et al.*, 2011, 2012; Baumann *et al.*, 2005; Furuta *et al.*, 2004).

The majority of research conducted to assess the effects of ultrasound on microbial inactivation have been performed on vegetative cells (Allison *et al.*, 1996; Álvarez *et al.*, 2003; Baumann *et al.*, 2005; Bermúdez-Aguirre *et al.*, 2009; Gao *et al.*, 2014), with only a few research studies carried out on spores (Evelyn and Silva, 2015a,b; Joyce *et al.*, 2003; Raso *et al.*, 1998b). From these studies it can be concluded that if ultrasound were to be used in any practical application as a decontamination process, the combination with other physical or chemical decontamination methods is necessary in order to increase its lethality. Its combination with heat (thermosonication, TS) (Ordoñez *et al.*, 1987), with pressure (manosonication, MS) (Álvarez *et al.*, 2003), and with heat and pressure acting simultaneously (manothermosonication, MTS) (Arroyo *et al.*, 2011), are promising alternatives leading to increased microbial inactivation in a much more efficient way in terms

of treatment time and energy consumption compared to each treatment individually (Chemat *et al.*, 2011; Knorr *et al.*, 2004; Mason *et al.*, 1996). However, more data would be useful to evaluate the real potential of MTS treatments as an alternative to thermal sterilisation particularly in the context of microorganisms of commercial significance in products based on crab meat such as chowders, where the permanence of spores coming from crab meat would be high, and where the potential of this technology has not been explored yet.

The aim of this study was to characterize the thermal resistance of three different bacterial spores isolated from pasteurised crab meat. A second objective was to assess the potential of MTS as an alternative operation for their inactivation.

2. Material and Methods

2.1. Microorganisms and treatment media

The three spore forming microorganisms used in this study were the three most isolated spore forming bacteria from Irish brown crab (*Cancer pagurus*) meat after a cooking step at 75 °C for 50 min and further storage at 4 °C for 15 days under vacuum conditions. These three wild strains were confirmed to be *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* by 16S rDNA sequencing.

During this investigation, the cultures were maintained frozen at -80 °C in cryovials. Microorganisms were recovered from the cryovial by surface spreading on a Tryptone Soya Agar with 0.6% (w/v) yeast extract (TSAYE) (Oxoid Ltd., Basingstoke, Hampshire, UK) plate and incubated for 48 h at 25 °C. A broth subculture was prepared by inoculating a tube containing 10 mL of sterile Tryptone Soya Broth (Oxoid), supplemented with 0.6% yeast extract (w/v) (Oxoid) (TSBYE), with one of the colonies isolated as described above and incubated overnight. 250 mL Duran bottles containing 50 mL of sterile TSBYE were

inoculated with the overnight subculture and incubated at 25 °C. Once the stationary phase of growth was achieved (after 48 h for *B. mycoides* and *B. weihenstephanensis* and 72 h for *P. psychrodurans*), 1 mL of the suspension was spread onto the surface of TSAYE agar plates containing 3 ppm (w/v) of manganese sulphate (Carlo Erba, Milan, Italy) to trigger the sporulation. The plates were further incubated at 25 °C for 10 days. Is well known that higher sporulation temperatures induce higher thermal resistances, for this reason 25 °C was selected as sporulation temperature, since the natural sporulation temperature in the environment inhabited by these crabs is lower. Finally spores were collected by flooding the agar surface with sterile pH 6.8 McIlvaine citrate-phosphate buffer (Dawson *et al.*, 1974). This pH corresponds to the pH of brown crab meat where the three spore species were isolated. After harvesting, spores were washed 5 times by centrifugation at 2500 g for 20 min at 4 °C (Jouan centrifuge, CR 4.11, Saint-Herblain, France). Finally, spores were resuspended in the same buffer and introduced in a bath with boiling water for 1 min to inactivate the remaining vegetative bacteria. The spore suspensions were kept at 5 °C until further use.

2.2. Heat, MS and MTS treatments

Heat treatments were carried out in a thermoresistometer TR-SC (Condón *et al.*, 1989, 1993). This instrument consists of a 350 mL vessel provided with an electrical heater for maintaining a constant temperature, an agitation device to ensure the distribution and temperature homogeneity of the inoculum and ports for injecting the microbial suspension and sample extraction. Once the target temperature attained stability (± 0.1 °C), 0.2 mL of the spore suspension was inoculated into the treatment medium: pH 6.8 McIlvaine citrate-phosphate buffer. After inoculation, 0.1 mL samples were collected at different heating times and immediately pour plated. Survival curves were obtained at different temperatures ranging from 80 to 110 °C.

MS/MTS treatments were carried out in a resistometer previously described by Raso *et al.* (1998a) which was modified to ease its use. Figure 1 shows a diagram of the improved equipment. Modifications included:

1. Removal of the original agitation shaft and blade inside the treatment chamber (E) as these pieces were not essential when ultrasonic treatments were applied. In addition, not only this change facilitated the assembly and disassembly of the equipment but also its maintenance and cleaning.
2. The two one-way inverted sensor valves in the original design were also removed from the top of the treatment chamber and replaced by a pressure tube (#3) which connects the treatment chamber to the pressure or vacuum circuits through a two-way valve (#1). The main advantage of this new design was to avoid any fluid exchange between the main vessel and the treatment chamber and as well as to allow each vessel to be filled with different liquids.
3. The procedure for filling the treatment chamber was also modified. In this modification a new valve (#9), connected to the bottom of the treatment chamber throughout the sonication probe housing, allowed the treatment chamber to be filled ensuring the removal of the air inside the vessel. This modification avoided the presence of bubbles during ultrasonic treatments.
4. The cooling system was also improved. In the original design a coiled tube (containing a coolant) was wrapped around the treatment chamber. However, in the modified version the refrigeration was applied in a more direct fashion by removing the coiled tube and circulating the coolant directly through the main vessel (F). This more direct use of the refrigeration system allowed for a more efficient removal of heat generated by cavitation.

Preliminary experiments demonstrated that these improvements did not significantly change the survival curve profiles for MS/MTS (data not shown).

The ultrasonic generator used was a 2000 W Branson Sonifier (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) which operates at a constant frequency of 20 kHz. Survival curves to ultrasound treatments were obtained at different temperatures ranging from 35 to 95 °C at a constant pressure (100 kPa) and ultrasonic wave amplitude (90 µm). The temperature of the treatment medium was continuously monitored by a thermocouple (type K, NiCr-Ni sensor class 1, ALMEMO®, Ahlborn, Germany). The ultrasound generator was switched on and once the treatment temperature was stable (± 0.2 °C), 0.2 mL of the spore suspension was injected into the 23 mL treatment chamber containing pH 6.8 McIlvaine buffer. After injection 0.1 mL samples were collected and immediately pour-plated. All experiments were conducted at least in triplicate on independent working days.

2.3. Incubation of treated samples and survival counting

Survival curves to heat and MTS treatments were obtained by plotting the log₁₀ fraction of survivors vs. treatment time (min). Under some experimental conditions deviations from linearity were observed. Since the survival curves did not show tails but shoulders, Geeraerd et al. log-linear regression plus shoulder model was used (Geeraerd *et al.*, 2000). Other models such as Weibull, were consider but finally Geeraerd's log-linear plus shoulder model was chosen because it defines separately the shoulder duration which allowed the effect of the different process parameters on it to be evaluated. To fit the survival curves to the model the Geeraerd and Van Impe inactivation model fitting tool (GInaFiT) was used (Geeraerd *et al.*, 2005). The model describes the survival curves through two parameters (Equation 1): the shoulder length (*Sl*), defined as the time before the exponential inactivation begins, and the inactivation rate ($k_{\max}$) which corresponds to the slope of the exponential portion of the survival curve. For comparison purposes, the GInaFiT tool also provides the

parameter $4D$, defined as the treatment time required to inactivate 99.99% of the microbial population ($4 \log_{10}$ cycles).

$$N_t = N_0 e^{-k_{\max} t} \left(\frac{e^{k_{\max} S l}}{1 + (e^{-k_{\max} S l} - 1) e^{k_{\max} t}} \right) \quad (1)$$

Those survival curves which showed a log-linear profile were fitted to Bigelow & Easty's model (1920), which is also included in the GInaFiT software, providing the exponential inactivation rate ($k_{\max}$) as well as the $4D$ parameter. The traditional decimal reduction time value (D_T value) i.e. the time to inactivate 90 % of the bacterial population can be easily calculated as follows:

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

Consequently, the parameter $4D$ will only match with $4 \times D_T$ if the microbial inactivation follows a first order kinetic where $Sl = 0$.

Statistical analyses: t -test and one-way ANOVA were performed with the GraphPad PRISM® 5.0 software (GraphPad software, Inc., San Diego, CA, USA) 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

As an example, Figure 2 shows the survival curves to heat in pH 6.8 McIlvaine buffer (Fig. 2A and Fig. 2A') and to MTS treatments (Fig. 2B) of *B. mycoides*, *P. psychrodurans* and *B. weihenstephanensis* spores. For both treatments different inactivation kinetics were observed depending on the spore forming species. Whereas the survival curves of *B. mycoides* to heat and MTS followed a log-linear kinetic (Fig. 2A and 2B), the survival curves of *P. psychrodurans* and *B. weihenstephanensis* showed a concave downwards profile, which is known as 'shoulder'. The same profiles were observed at all temperatures tested. As indicated above the Geeraerd log-linear regression plus shoulder model (Geeraerd *et al.*,

2000) was used to calculate the resistance parameters. Table 1 and 2 show the mean value of the parameters calculated with the model including the shoulder length (Sl), the inactivation rate ($k_{\max}$) and the $4D$ values for heat ($4D_T$) and MS/MTS treatments ($4D_{MTS}$), respectively. The standard deviations (SD) for each parameter, the root mean square error value ($RMSE$) and the R-square value (R^2) have been also included to show the accuracy of the fitting.

3.1. Spore resistance to heat

As pointed out above, the survival curves of *B. weihenstephanensis* and *P. psychrodurans* to heat showed shoulders in all the conditions tested (Fig. 2A and Fig 2A', Table 1). Figure 3 shows the relationship between the logarithms of the shoulder length (min) and the treatment temperature ($^{\circ}\text{C}$) for *B. weihenstephanensis* (Fig. 3A) and *P. psychrodurans* (Fig. 3B). In both cases the Sl was an exponential function of the treatment temperature and a linear relationship between both parameters was found (Equations 3 and 4):

$$\text{For } B. \text{ weihenstephanensis: } \text{Log } Sl = -0.173 \times T + 18.07 \quad (3)$$

$$\text{For } P. \text{ psychrodurans: } \text{Log } Sl = -0.098 \times T + 9.335 \quad (4)$$

From each equations, z_{Sl} values (temperature increase required to reduce the shoulder length by 10-fold), conceptually equal to traditional z value (temperature increase required to reduce the D_T value by 10-fold) were calculated for *B. weihenstephanensis* ($z_{Sl} = 5.8^{\circ}\text{C}$) and *P. psychrodurans*, ($z_{Sl} = 10.2^{\circ}\text{C}$). A t-test revealed that these z values were significantly different ($P \leq 0.05$).

As indicated above, the thermal inactivation of *B. mycoides* spores followed a log-linear kinetics, therefore Sl values equalled 0 (Fig. 2A and Fig. 2B, Table 1).

As can be deduced from the definition of the thermal resistance parameters, the direct comparison of the D_T values could be misleading, since some microbial species show

shoulders and others do not. In order to compare the resistance of the three spores a 4D value (that incorporates the Sl and D_T values) was calculated for each spore species for each treatment (Table 1). Results showed that *B. mycoides* was the most heat sensitive whereas *B. weihenstephanensis* was the most heat resistant. For example, the time to reduce 4 $\log_{10}$ cycles were 1.6 min at 95 °C for *B. mycoides*, 1.4 min at 107.5 °C for *B. weihenstephanensis* and 1.1 min at 100 °C for *P. psychrodurans*. Also, thermal death time curves for 4 $\log_{10}$ cycles of inactivation (TDT-4D) were obtained by plotting $\log_{10}$ 4D values versus the corresponding treatment temperatures (Fig. 4A). As expected, an exponential relationship between both variables was found. From the equations of the regression lines, z_T values (i.e. the negative inverse of the slope of the TDT curve) of 9.1, 7.1 and 8.0 °C were calculated for *B. mycoides*, *B. weihenstephanensis* and *P. psychrodurans*, respectively. The statistical analysis of the z values revealed significant differences ($P \leq 0.05$) indicating that the impact of the treatment temperature on the 4D reduction time is different for each species. *B. weihenstephanensis*, with the lower z_T value, proved to be the most sensitive to changes in temperature whereas *B. mycoides* was the least sensitive.

3.2. Spore resistance to MS/MTS

Like for the thermal inactivation, *B. weihenstephanensis* and *P. psychrodurans* showed shoulders in the survival curves to MS/MTS treatments at all temperatures tested (Fig. 1B, Table 2). By contrast, shoulders were not observed at any temperature for *B. mycoides* (Fig. 1B, Table 2). Therefore, and similarly to the heat treatments, the comparison of the resistance of the three spores to MS/MTS can be done by means of the 4D values (Table 2). When the resistance to MS (35 °C) was compared, *B. mycoides* was the most resistant species with a 4D of 51.2 min, followed by *B. weihenstephanensis* with a 4D of 40.6 min while *P. psychrodurans* was the most sensitive with a 4D of 25.6 min.

When heat was included in the process (i.e. MTS), a change in tolerance was observed. At 80 °C no significantly different $4D_{\text{MTS}}$ values ($P>0.05$) were calculated for *B. weihenstephanensis* (23.4 min) and *P. psychrodurans* (21.5 min) but a lower $4D_{\text{MTS}}$ was calculated for *B. mycoides* (3.7 min) ($P\leq 0.05$). However, above 80 °C *B. weihenstephanensis* was the most resistant followed by *P. psychrodurans* while *B. mycoides* was the most sensitive to MTS. This shift in resistance to MTS with temperature seemed to indicate that the thermo-dependence of the inactivation by ultrasonic waves under pressure is different for each spore forming species. As *B. weihenstephanensis* and *P. psychrodurans* showed MTS survival curves with shoulders and *B. mycoides* did not, for comparison purposes it was necessary to obtain the MTS death time curves (MTSDT) by plotting the $\log_{10} 4D$ values versus the corresponding treatment temperature (Fig. 4B). As it is shown in Figure 5, and as opposed to the results for heat treatments, there was no exponential relationship between the $4D_{\text{MTS}}$ values and the temperature since the MTSDT curves showed concave downward profiles for the three bacterial spores. This result did not allowed for the calculation of z values. Overall, Fig. 4B demonstrates that when ultrasound was applied under pressure, the inactivation of *B. mycoides* was the most thermo-dependent and that of *B. weihenstephanensis* was the least thermo-dependent. Fig. 4B also demonstrated that the thermal dependence was not related with the resistance to MS treatments at 35 °C.

3.3. Synergistic effect for the combination of ultrasound and heat under pressure

As ultrasonic waves under pressure are lethal for microorganisms as heat treatments are, it is logical to assume that spore inactivation by ultrasound will increase with temperature. The lethality due to the combined process could be the result of adding the lethal effect of ultrasound under pressure to the lethal effect of heat (additive effect), higher than the addition (synergistic effect) or lower (antagonistic effect). Establishing whether the observed

effect was additive, synergistic or antagonistic can be evaluated by comparing the inactivation levels obtained experimentally with those calculated theoretically by assuming that the effect is additive. If both values match, the effect would be additive but if the theoretical value is less than the observed value the effect would be synergistic, or if greater, antagonistic.

This comparison can be done with the equation proposed by Raso *et al.* (1998a) by assuming that the inactivation by ultrasonic under pressure treatments and heat treatments are an exponential function of the treatment time and that both occurs simultaneously but independently. The equation proposed by Raso *et al.* (1998a) used as resistance parameter the decimal reduction time values (D_T) which could be misleading now because the survival curves here reported showed shoulders. Therefore, the equation modified by Arroyo *et al.* (2011) which used as resistance parameter the 4D value (Equation 5) was employed.

$$\text{Theoretical } 4D_{\text{MTS}} = \frac{(4D_T) \times (4D_{\text{MS}})}{(4D_T) + (4D_{\text{MS}})} \quad (5)$$

In order to quantify the magnitude of the synergistic effect the percentage of synergism was calculated with Equation 6.

$$\% \text{ Synergism} = \frac{\text{Theoretical value} - \text{Experimental value}}{\text{Theoretical value}} \times 100 \quad (6)$$

A synergistic effect was detected for the inactivation by MTS of all spore suspensions. Figure 6 shows the percentage of synergism calculated for *B. mycoides* (solid line), *B. weihenstephanensis* (dashed line) and *P. psychrodurans* (dotted line) in the range of temperatures tested. For each spore species there was a defined range of temperature for the synergistic effect. At temperatures above 90 °C for *B. mycoides* and above 95 °C for *P. psychrodurans* no advantages were observed in the combined treatment (synergy values below 1%), thus it seems correct to consider that at temperatures above 90 and 95 °C, respectively, the inactivating effect would be solely due to heat. By contrast, for *B.*

weihenstephanensis the synergistic effect was detected in all temperatures tested. Maximum synergistic effects of 69% at 75-80 °C for *B. mycoides*, 53% at 90 °C for *B. weihenstephanensis* and 63% at 80-85 °C for *P. psychrodurans* were calculated for the inactivation of 4 log₁₀ cycles (Fig. 4C).

4. Discussion

In this study the resistance to heat, ultrasound under pressure and the combined process (MTS) of three spores isolated from pasteurized Irish brown crab was studied in McIlvaine pH 6.8 buffer. Low lethality of ultrasound treatments applied alone (especially for the inactivation of spore forming bacteria) prompted the application of ultrasound in combination with other hurdles (e.g. heat and/or pressure) (Evelyn and Silva 2015a,b; Joyce *et al.*, 2003; Raso *et al.*, 1998b). Economic reasons also come into play since the energetic cost of ultrasonic treatments is high and combinations would significantly reduce the costs (Chemat *et al.*, 2011; Knorr *et al.*, 2004). When ultrasound treatments are applied in combination with heat at lower temperatures for shorter times than conventional heat processing it is possible to achieve the same inactivation levels with less impact on quality (Mason *et al.*, 1996; Piyasena *et al.*, 2003; Zenker *et al.*, 2003).

The survival curves to heat and MS/MTS of two of the three spores assessed in this study (*B. weihenstephanensis* and *P. psychrodurans*) did not follow an exponential inactivation rate but a concave downwards profile. By contrast, *B. mycoides* followed an exponential inactivation rate. Shoulders in the survival curves to heat of bacterial spores is usual (Baril *et al.*, 2011, 2012; Coton *et al.*, 2011). These shoulders have been associated to heat damage and repair phenomena (Condón *et al.*, 1996) and also to the activation of dormant spores (Sapru *et al.*, 1993). The shoulder phase can be also attributed to the multi-hit nature of thermal inactivation as heat damages several structures which compromise cell

viability. However, bacteria have developed different strategies to cope with heat damage and restore their functionality which can result in shoulders phases and when repair mechanisms are surpassed, additional heating would be lethal for microorganisms and the number of survivors will decline exponentially. Besides, it is also well known the presence of superdormant spores within a spore suspension that do not germinate in growth media but under some physical stresses such as heat they will activate and germinate. Therefore at the beginning of a heat treatment two phenomena can coexist: the microbial death which will decrease the count and the activation phenomenon which will increase it. The shoulder is then result of the balance of both phenomena. Once all the bacterial spores are activated the death follows an exponential rate.

Both technologies, heat and ultrasound, can damage and/or activate bacterial spores and be responsible for the shoulders observed in the survival curves.

Many of the studies on the effect of MTS on microbial inactivation have been carried out with vegetative cells where log-linear profiles (Condón *et al.*, 2011; Sala *et al.*, 1995) and deviations from linearity have been observed (Bermúdez-Aguirre *et al.*, 2009; Lee *et al.*, 2009). However, a lower number of studies have been carried out with spores though even in these cases survival curves with different profiles have been reported (Evelyn and Silva 2015a,b; Joyce *et al.*, 2003; Raso *et al.*, 1998b). Results reported here demonstrate that the shoulder length is shorter when ultrasound under pressure is applied in combination with heat (Table 1 and 2). These results are in agreement with data obtained with vegetative cells (Arroyo *et al.*, 2011) but not with the statement of Evelyn and Silva (2015b) who pointed out that the inactivation kinetics of *B. cereus* spores changed with the treatment temperature. Our results suggest that the kinetic of inactivation strongly depends on the spore species but not on the treatment temperature because when shoulders were observed, they were present at all temperatures tested. However, the duration of shoulder certainly changes with the treatment

temperature. A deeper analysis revealed an exponential relationship between the logarithm of the shoulder length and the treatment temperature (Fig. 3). This relationship has not been previously reported for bacterial spores but is similar to what Arroyo *et al.* previously observed with *Cronobacter sakazakii* (Arroyo *et al.*, 2011). Authors suggested that the study of this correlation may be of interest from a practical point of view since a simple correlation between both variables might simplify Geeraerd equation to build more robust secondary and tertiary models.

Since two different inactivation kinetics were observed in the survival curves to both heat and MS/MTS treatments, the comparison of resistance of the three bacterial spores was based on the $4D$ parameter which is also provided by the model. According to the results obtained in this research *B. weihenstephanensis* was the most resistant spore against heat and *B. mycoides* the most sensitive. The z_T values calculated in this study $z_T = 9.1, 8.0$ and 7.1 °C for *B. mycoides*, *P. psychrodurans* and *B. weihenstephanensis* (Fig. 4A) were in agreement with those calculated by Coton *et al.* (2011) which were in the range from 7.4 to 8.6 °C for different spore formers.

For ultrasound, MS resistance of *B. mycoides* was the highest and that of *P. psychrodurans* the lowest. However, the classification of spore resistance to MTS was highly dependent on the temperature applied. Up to 50 °C *B. mycoides* was the most resistant, between 50 °C and 80 °C it was *B. weihenstephanensis* whereas at 80 °C *B. weihenstephanensis* and *P. psychrodurans* showed similar resistance. Over 80 °C *B. weihenstephanensis* was the most resistant. This behaviour supports the hypothesis that the spores which have higher heat resistance will show higher resistance to MTS at high temperatures. As Álvarez *et al.* (2003) showed for *Salmonella enterica*, the percentage of synergistic effect was higher in the spore which had the higher z_T value.

As Raso *et al.* (1998b) reported for the inactivation of *B. subtilis* spores by MTS a synergistic effect was detected for the three spores studied (Fig. 4C). The results indicated that the maximum synergism effect and the temperature at which this was observed, was dependent on the bacterial spore.

The synergistic effect of the combination of heat with ultrasound could be due to the ultrasound sensitising bacterial spores to heat or *vice versa*. Since the main effect of ultrasound on the microbial inactivation is a mechanical effect (Condón *et al.*, 2011; Pagán *et al.*, 1999; Raso *et al.*, 1998b), the synergistic effect described for MTS treatments could be a consequence of ultrasound rendering cell envelopes weaker to heat but it could also be the opposite. Bacterial spores have a very complex structure and mechanically resistant envelopes, which isolate the protoplast from the environment. It has been reported that ultrasonic treatments are able to disrupt the spore exosporium (Berger and Marr, 1960) and that ultrasound provokes the release of dipicolinic acid and low molecular weight polypeptides from the cortex of some bacterial spores (Palacios *et al.*, 1991). Furthermore, Raso *et al.* (1998b) found that MS treatment sensitized spores of *B. subtilis* to lysozyme action. Some strains of spore forming bacteria are known to have naturally leaky coats and are therefore lysozyme sensitive, but, in most cases, lysozyme is only capable of hydrolysing the peptidoglycan of the spore cortex if the overlying coat is first made leaky. These results suggest that the mechanism of action of ultrasonic waves on bacterial spores is also based on the mechanical disruption of the most external envelopes. The external damage would lead to the rehydration of the protoplast, which would result in a loss of heat tolerance (Sala *et al.*, 1995). This would explain the observed synergistic effect of MTS on bacterial spores. Ultrasonic waves under pressure would act, in this case, by sensitizing bacterial spores to heat.

5. Conclusions

The main objective of this work was to evaluate the efficiency of the application of ultrasound under pressure in combination with heat for bacterial spore inactivation. From the results obtained it can be concluded that the profile of the survival curves either for heat or for ultrasound treatments depended on the bacterial spore species. When shoulders were detected in the inactivation curves for heat they were also present in the curves for MS/MTS treatments, although the application of an ultrasonic field reduced the shoulder length. The efficiency of the combined process (MTS) for bacterial spore inactivation was directly correlated with the thermal resistance since the bacterial spore which showed the highest resistance to MS also showed the lowest resistance to heat, and as result, it was the most sensitive to MTS. The combination of ultrasonic waves under pressure with heat showed a synergistic effect for the inactivation of the three spores. The highest percentage of synergism corresponded to the spore species with higher z_T value (*B. mycoides*), but the highest temperature at which this synergism was detected corresponded to the most heat tolerant spore species (*B. weihenstephanensis*).

Acknowledgements

The authors wish to acknowledge the financial support of Food Institutional Research Measure (FIRM) funded by the Irish Department of Agriculture, Food and the Marine (Project no. R13885). The authors would also like to sincerely thank the Novel Technologies for Food Preservation group at the University of Zaragoza, Spain.

References

Allison, D., D'emanuele, A., Eginton, P., Williams, A., 1996. The effect of ultrasound on *Escherichia coli* viability. Journal of basic microbiology 36, 1-11.

447 Álvarez, I., Mañas, P., Sala, F., Condón, S., 2003. Inactivation of *Salmonella enterica* serovar
 448 Enteritidis by ultrasonic waves under pressure at different water activities. *Applied and*
 449 *Environmental Microbiology* 69, 668-672.

450 Arroyo, C., Cebrián, G., Pagán, R., Condón, S., 2011. Inactivation of *Cronobacter sakazakii*
 451 by manothermosonication in buffer and milk. *International Journal of Food*
 452 *Microbiology* 151, 21-28.

453 Arroyo, C., Cebrián, G., Pagán, R., Condón, S., 2012. Synergistic combination of heat and
 454 ultrasonic waves under pressure for *Cronobacter sakazakii* inactivation in apple juice.
 455 *Food Control* 25, 342-348.

456 Baril, E., Coroller, L., Couvert, O., Leguérinel, I., Postollec, F., Boulais, C., Carlin, F.,
 457 Mafart, P., 2012. Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus*
 458 *licheniformis* spores as function of sporulation temperature and pH. *Food Microbiology*
 459 30, 29-36.

460 Baril, E., Coroller, L., Postollec, F., Leguerinel, I., Boulais, C., Carlin, F., Mafart, P., 2011.
 461 The wet-heat resistance of *Bacillus weihenstephanensis* KBAB4 spores produced in a
 462 two-step sporulation process depends on sporulation temperature but not on previous cell
 463 history. *International Journal of Food Microbiology* 146, 57-62.

464 Barrento, S., Marques, A., Teixeira, B., Carvalho, M.L., Vaz-Pires, P., Nunes, M.L., 2009.
 465 Accumulation of elements (S, As, Br, Sr, Cd, Hg, Pb) in two populations of *Cancer*
 466 *pagurus*: Ecological implications to human consumption. *Food and Chemical*
 467 *Toxicology* 47, 150-156.

468 Baumann, A.R., Martin, S.E., Feng, H., 2005. Power ultrasound treatment of *Listeria*
 469 *monocytogenes* in apple cider. *Journal of Food Protection* 68, 2333-2340.

470 Berger, J.A., Marr, A.G., 1960. Sonic disruption of spores of *Bacillus cereus*. *Journal of*
 471 *General Microbiology* 22, 147-157.

472 Bermúdez-Aguirre, D., Corradini, M.G., Mawson, R., Barbosa-Cánovas, G.V., 2009.
 473 Modeling the inactivation of *Listeria innocua* in raw whole milk treated under thermo-
 474 sonication. Innovative Food Science & Emerging Technologies 10, 172-178.

475 Chemat, F., Zill e, H., Khan, M.K., 2011. Applications of ultrasound in food technology:
 476 Processing, preservation and extraction. Ultrasonics Sonochemistry 18, 813-835.

477 Condón, S., Arrizubieta, M.J., Sala, F.J., 1993. Microbial heat resistance determinations by
 478 the multipoint system with the thermoresistometer TR-SC - Improvement of this
 479 methodology. Journal of Microbiological Methods 18, 357-366.

480 Condón, S., Lopez, P., Oria, R., Sala, F.J., 1989. Thermal Death Determination: Design and
 481 Evaluation of a Thermoresistometer. Journal of Food Science 54, 451-457.

482 Condón, S., Mañas, P., Cebrián, G., 2011. Manothermosonication for microbial inactivation.
 483 In: Feng, H., Babosa-Cánovas, G. V. and Weiss, J.; (Eds), Ultrasound Technologies for
 484 Food and Bioprocessing. Springer: New York, pp. 287–319.

485 Condón, S., Palop, A., Raso, J., Sala, F.J., 1996. Influence of the incubation temperature after
 486 heat treatment upon the estimated heat resistance values of spores of *Bacillus subtilis*.
 487 Letters in Applied Microbiology 22, 149-152.

488 Condón, S., Raso, J., Pagán, R., 2005. Microbial inactivation by ultrasound. In: Barbosa-
 489 Canovas, G.V., Tapia, M.S., Cano, M.P. (Eds), Novel Food Processing Techniques. CRC
 490 Press LLC, Boca Raton, FL, pp. 423–442.

491 Coton, M., Denis, C., Cadot, P., Coton, E., 2011. Biodiversity and characterization of aerobic
 492 spore-forming bacteria in surimi seafood products. Food Microbiology 28, 252-260.

493 Dawson, R.M.C., D. C. Elliot, W. H. Elliot, and K. M. Jones, 1974. pH, buffers and
 494 physiological media Dawson, R. M. C., D. C. Elliot, W. H. Elliot, and K. M. Jones (Eds.),
 495 data for biochemical research 2nd Ed. Clarendon Press, Oxford, pp. 484-485.

496 Earnshaw, R., 1998. Ultrasound: a new opportunity for food preservation. *Ultrasound in food*
 497 *processing*, 183.

498 EUROSTAT, 2009. <http://epp.eurostat.ec.europa.eu/portal/page/portal/fisheries/data/database>.

499 Evelyn, E., Silva, F.V.M., 2015a. Use of power ultrasound to enhance the thermal
 500 inactivation of *Clostridium perfringens* spores in beef slurry. *International Journal of*
 501 *Food Microbiology* 206, 17-23.

502 Evelyn, E., Silva, F.V.M., 2015b. Thermosonication versus thermal processing of skim milk
 503 and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus*
 504 spores. *Food Research International* 67, 67-74.

505 Faghri, M.A., Pennington, C.L., Cronholm, L.S., Atlas, R.M., 1984. Bacteria associated with
 506 crabs from cold waters with emphasis on the occurrence of potential human pathogens.
 507 *Applied and Environmental Microbiology* 47, 1054-1061.

508 Furuta, M., Yamaguchi, M., Tsukamoto, T., Yim, B., Stavarache, C., Hasiba, K., Maeda, Y.,
 509 2004. Inactivation of *Escherichia coli* by ultrasonic irradiation. *Ultrasonics*
 510 *Sonochemistry* 11, 57-60.

511 Gao, S., Hemar, Y., Ashokkumar, M., Paturel, S., Lewis, G.D., 2014. Inactivation of bacteria
 512 and yeast using high-frequency ultrasound treatment. *Water Research* 60, 93-104.

513 Geeraerd, A.H., Herremans, C.H., Van Impe, J.F., 2000. Structural model requirements to
 514 describe microbial inactivation during a mild heat treatment. *International Journal of*
 515 *Food Microbiology* 59, 185-209.

516 Geeraerd, A.H., Valdramidis, V.P., Van Impe, J.F., 2005. GInaFiT, a freeware tool to assess
 517 non-log-linear microbial survivor curves. *International Journal of Food Microbiology*
 518 102, 95-105.

519 Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. *International*
 520 *Journal of Food Microbiology* 33, 121-137.

521 Hielscher, H.F.W.Y.T., 2008. Power ultrasound. Food Science Technology International 14,
522 433-436.

523 Huss, H.H., 1997. Control of indigenous pathogenic bacteria in seafood. Food Control 8, 91-
524 98.

525 Joyce, E., Phull, S.S., Lorimer, J.P., Mason, T.J., 2003. The development and evaluation of
526 ultrasound for the treatment of bacterial suspensions. A study of frequency, power and
527 sonication time on cultured *Bacillus* species. Ultrasonics Sonochemistry 10, 315-318.

528 Knorr, D., Zenker, M., Heinz, V., Lee, D.-U., 2004. Applications and potential of ultrasonics
529 in food processing. Trends in Food Science & Technology 15, 261-266.

530 Lee, H., Zhou, B., Liang, W., Feng, H., Martin, S.E., 2009. Inactivation of *Escherichia coli*
531 cells with sonication, manosonication, thermosonication, and manothermosonication:
532 Microbial responses and kinetics modeling. Journal of Food Engineering 93, 354-364.

533 Mason, T.J., Paniwnyk, L., Lorimer, J.P., 1996. The uses of ultrasound in food technology.
534 Ultrasonics Sonochemistry 3, S253-S260.

535 Ordoñez, J.A., Aguilera, M., Garcia, M.L., Sanz, B., 1987. Effect of combined ultrasonic and
536 heat treatment (thermoultrasonication) on the survival of a strain of *Staphylococcus*
537 *aureus*. Journal of Dairy Research 54, 61-67.

538 Pagán, R., Manas, P., Raso, J., Condón, S., 1999. Bacterial resistance to ultrasonic waves
539 under pressure at nonlethal (manosonication) and lethal (manothermosonication)
540 temperatures. Applied and Environmental Microbiology 65, 297-300.

541 Palacios, P.B., J.; Hoz, L.; Sanz, B.; Ordoñez, J.A., 1991. Study of substances released to
542 ultrasonic treatment from *Bacillus stearothermophilus* spores. Journal of Applied
543 Bacteriology 71, 445-451.

544 Piyasena, P., Mohareb, E., McKellar, R.C., 2003. Inactivation of microbes using ultrasound:
545 a review. International Journal of Food Microbiology 87, 207-216.

546 Raso, J., Pagán, R., Condón, S., Sala, F., 1998a. Influence of temperature and pressure on the
547 lethality of ultrasound. *Applied and Environmental Microbiology* 64, 465-471.

548 Raso, J., Palop, A., Pagán, R., Condón, S., 1998b. Inactivation of *Bacillus subtilis* spores by
549 combining ultrasonic waves under pressure and mild heat treatment. *Journal of Applied*
550 *Microbiology* 85, 849-854.

551 Sala, F., Burgos, J., Condon, S., Lopez, P., Raso, J., 1995. Effect of heat and ultrasound on
552 microorganisms and enzymes. In: G.W Gloud (Ed), *New methods of food preservation*.
553 Springer, pp. 176-204.

554 Sapru, V., Smerage, G.H., Teixeira, A.A., Lindsay, J.A., 1993. Comparison of predictive
555 models for bacterial spore population resources to sterilization temperatures. *Journal of*
556 *Food Science* 58, 223-228.

557 Ward, D., Pierson, M., Tassell, K., 1977. The microflora of unpasteurized and pasteurized
558 crabmeat. *Journal of Food Science* 42, 597-600.

559 WHO, 2003. Food based dietary guidelines in the world health organization European region.
560 Report EUR/03/5045414.

561 Zenker, M., Heinz, V., Knorr, D., 2003. Application of ultrasound-assisted thermal
562 processing for preservation and quality retention of liquid foods. *Journal of Food*
563 *Protection* 66, 1642-1649.

564

Table 1. Heat resistance parameters from the fitting of the Geeraerd log-linear plus shoulder model to the survival curves of *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* spores treated in pH 6.8 citrate-phosphate buffer.

Microorganism	T	<i>Sl</i> mean (<i>SD</i>)	$k_{\max}$ mean (<i>SD</i>)	<i>4D</i> mean (<i>SD</i>)	<i>RMSE</i>	R^2
<i>B. mycoides</i>	80	0	0.13 (0.02)	70.4 (7.80)	0.1067	0.93
	85	0	0.54 (0.05)	17.7 (1.70)	0.16	0.96
	90	0	1.93 (0.22)	4.8 (0.53)	0.22	0.92
	95	0	5.81 (0.48)	1.6 (0.12)	0.3	0.93
<i>B. weihenstephanensis</i>	102.5	1.79 (0.17)	1.26 (0.17)	9.2 (0.95)	0.3484	0.93
	105	0.62 (0.05)	2.55 (0.28)	4.28 (0.48)	0.3667	0.93
	107.5	0.48 (0.04)	9.94 (0.52)	1.41 (0.02)	0.3373	0.95
	110	0.07 (0.001)	11.11 (0.52)	0.9 (0.04)	0.2787	0.95
<i>P. psychrodurans</i>	90	3.67 (0.11)	0.57 (0.01)	19.83 (0.09)	0.0872	0.99
	93	1.62 (0.04)	1.28 (0.00)	8.8 (0.02)	0.076	0.99
	96	0.83 (0.046)	3.45 (0.01)	3.5 (0.04)	0.1085	0.99
	100	0.38 (0.007)	12.09 (0.01)	1.14 (0.01)	0.1718	0.99

T, temperature (°C); *Sl*, shoulder length (min); $k_{\max}$, inactivation rate; *4D*, time for a 4-log reduction (min); *SD*, standard deviation; *RMSE*, root mean square error; R^2 , determination coefficient.

Table 2. MS/MTS resistance parameters from the fitting of the Geeraerd log-linear plus shoulder model to the survival curves of *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* spores treated in pH 6.8 citrate-phosphate buffer.

Microorganism	T	<i>Sl</i> mean (SD)	$k_{\max}$ mean (SD)	<i>4D</i> mean (SD)	RMSE	R^2
<i>B. mycoides</i>	35	0	0.18 (0.02)	51.18 (0.34)	0.0702	0.94
	50	0	0.25 (0.03)	36.85 (0.52)	0.1132	0.91
	65	0	0.44 (0.03)	21.04 (1.33)	0.1842	0.92
	80	0	0.68 (0.06)	13.67 (1.21)	0.339	0.89
	90	0	2.23 (0.08)	4.14 (0.16)	0.1785	0.96
<i>B. weihenstephanensis</i>	35	4.71 (0.88)	0.26 (0.00)	40.62 (1.01)	0.0686	0.95
	80	3.09 (0.65)	0.44 (0.03)	23.58 (0.52)	0.1212	0.97
	85	2.97 (0.52)	0.48 (0.02)	22.3 (1.09)	0.1174	0.98
	90	3.66 (0.56)	0.58 (0.03)	19.26 (0.74)	0.1671	0.98
	95	4.55 (0.42)	0.8 (0.04)	16.27 (1.30)	0.1919	0.98
<i>P. psychrodurans</i>	35	7.7 (0.6)	0.36 (0.07)	25.59 (1.23)	0.0427	0.95
	80	2.66 (0.06)	0.5 (0.05)	21.5 (1.9)	0.1324	0.95
	85	2.38 (0.03)	0.65 (0.00)	16.48 (0.07)	0.0585	0.99
	90	2.27 (0.04)	1.16 (0.02)	10.22 (0.18)	0.1026	0.99
	95	1.05 (0.09)	2.51 (0.00)	4.72 (0.08)	0.2265	0.98

MS, manosonication; MTS, manothermosonication; T, temperature (°C); *Sl*, shoulder length (min); $k_{\max}$, inactivation rate; *4D*, time for a 4-log spore reduction (min); *SD*, standard deviation; *RMSE*, root mean square error; R^2 , determination coefficient.

FIGURE LEGEND

Figure 1. Diagram of the MTS resistometer. A, MTS resistometer main unit; B, ultrasound generator; C, heating/cooling water bath; D, agitation motor; E, Main vessel; F, treatment chamber; G, temperature data logger; 1, two way valve; 2, mano-reducer; 3, pressure/vacuum valve; 4, treatment chamber thermocouple; 5, cooling inlet (only MS); 6, main vessel filling empty tube; 7, ultrasound probe housing; 8, ultrasound probe; 9, treatment chamber filling valve; 10, solenoid sampling valve; 11, automatic injection syringe; 12, agitation shaft; 13, heating element; 14, cooling outlet (only MS); 15, main vessel pressure inlet; 16, main vessel thermocouple; 17 and 18 bottom and top caps.

Figure 2. Survival curves to heat at 95 °C (A) or 105 °C (A') and to MTS at 90 °C (B) in pH 6.8 citrate-phosphate buffer of *B. mycoides* (●), *P. psychrodurans* (■) and *B. weihenstephanensis* (▲).

Figure 3. Influence of the temperature on the shoulder length calculated with the Geeraerd log-linear regression plus shoulder model for *B. weihenstephanensis* (A) and *P. psychrodurans* (B) by heat (open symbols) and MTS (solid symbols) in pH 6.8 citrate phosphate buffer. Dotted line represents the theoretical DRT curves to MTS calculated with Equation 6.

Figure 4. (A) Influence of temperature on the 4D values calculated with the Geeraerd log-linear regression plus shoulder model for *B. mycoides* (●), *P. psychrodurans* (■) and *B. weihenstephanensis* (▲) inactivation by heat in pH 6.8 citrate-phosphate buffer. **(B)** Influence of temperature on the 4D values calculated with the Geeraerd log-linear regression

plus shoulder model for *B. mycoides* (●), *P. psychrodurans* (■) and *B. weihenstephanensis* (▲) inactivation by MS/MTS in pH 6.8 citrate-phosphate buffer. (C) Percentage of synergism calculated with Equation 6 for the inactivation by MTS of 4 log₁₀ cycles (4D values calculated with Geeraerd log-linear regression plus shoulder model) of *B. mycoides* (block line), *P. psychrodurans* (dotted line) and *B. weihenstephanensis* (dashed line).

Figure 1

Figure 2

Figure 3

Figure 4