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Santiago Condón Abanto

Adding Value to Ready-to-Crustacean Products: Process optimization for "entire" crustaceans using novel technologies

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Autor

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Producción Animal y Ciencia de los Alimentos

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Adding Value to Ready-to-eat Crustacean Products:

Process optimization for "entire" and "particulate" crustaceans using novel technologies

by

Santiago Condón-Abanto, Dipl. Ing (Agr.), B.Sc., M.Sc.

UNIVERSITY COLLEGE DUBLIN **UNIVERSIDAD DE ZARAGOZA**

AN *COLÁISTE* OLLSCOILE, BAILE ÁTHA CLIATH

A thesis submitted to University College Dublin and Universidad de Zaragoza for the degree of **Doctor of Philosophy (PhD**) in Food Science

at

UCD Dublin, School of Agriculture & Food science, Belfield, Dublin 4, Ireland
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Prof. Ignacio Álvarez
Prof. Javier Raso

Declaration

I hereby declare that the work herein submitted for the degree of Doctor of Philosophy of the National University of Ireland, Dublin is the result of my own investigation, except where the reference is made to the publish literature. I also declare that the material submitted in this thesis has not been accepted for any other degree and currently is only submitted to the University of Zaragoza as agreeded in the joint-PhD contract signed between both instittutions (Annex I).

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Que la Tesis Doctoral titulada "Adding Value to Ready-to-eat Crustacean Products: Process optimization for "entire" and "particulate" crustaceans using novel technologies" de la que es autor Santiago Condón Abanto, ha sido realizada bajo su dirección y en cotutela con el University College Dublin, donde el profesor James G. Lyng y la Dr. Cristina Arroyo han actuado como supervisores, y que su contenido corresponde con el Proyecto de Tesis aprobado en su momento y cumple con las condiciones requeridas para optar al grado de Doctor por la Universidad de Zaragoza.

Fdo.: Dr. Ignacio Álvarez Lanzarote

Fdo.: Dr. Javier Raso Pueyo

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ABBREVIATIONS

$6D$	Required treatment to reduce 6 Log_{10} reductions
A_f	Accuracy factor
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
a_w	Water activity
B	Brown meat
B_f	Bias factor
BI*	Browning index
BLAST	Basic Local Alignment Search Toll
BP	Baird-Parker agar
C*	Chroma
CAC	Codix Alimentarius Commission
CCFRA	Campden and Chorleywood Food Research Association
Cd	Cadmiun
CFA	Chartered Financial Analyst
CFC	Cetrimide Fusidin Cephaloridine
CFU	Colony Forming Unit
Co	Cobalt
Cs	Caesium
D_{10}	Decimal reduction time value
DNA	Deoxyribonucleic acid
DSS	Decision Support System
D_T	Decimal reduction time value
Dw	Dry weight
EBI	Electron Beam Ionizing radiation
EC	European Commission
ECFF	European Chilled Food Federation
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
FSAI	Food Safety Authority of Ireland
Gy	Gray
H*	Hue angle
HPP	High Pressure Processing
HTST	High temperature-short time
IAEA	International Atomic Energy Agency
ICGFI	International Consultative Group on Food Irradiation
ICMSF	International Commission on Microbial Specifications for Foods
ICP-MS	Inductively coupled plasma-mass spectroscopy
IE	Ireland
IEA	International Atomic Energy Agencia
IFT	Institute of Food Technologists
L.H	Long and Hammer's Agar
LAB	Lactic acid bacteria
LTLT	Low temperature-long time
MRD	Maximun Recovery Diluent
MRSA	Man Rogosa Sharpe agar
MS	Mano-sonication
MT	Metallothionein
MTS	Mano-thermo-sonication
MTSDT	Mano-thermo-sonication death time curves
NCBI	National Center for Biotechnology Information

ND	Not detectable
PCA	Plate count agar
PCR	Polymerase chain reaction
PEF	Pulsed Electric Field
PEMBA	Bacillus cereus agar base supplemented with polymyxin B
R^2	Coefficient of determination
RMSE	Root mean square error
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RTE	Ready to eat
SD	Standard deviation
SE	Standard error
SEP	Standard error of prediction
SPS	Sulfite Polymyin Sulfadizine
SSO	Specific spoilage organism
TBC	Total bacterial counts
TDT	Thermal Death Time curve
TS	Thermo-sonication
TSA	Tryptic Soy Agar
TSAYE	Tryptone Soya Agar Yeast Extract
TSB	Tryptic Soy Broth
TSBYE	Tryptone Soya Broth Yeast Extract
TVC_m	Total Viable Mesophilic Counts
TVC_p	Total Viable Psychrophilic Counts
TWI	Tolerable weekly intake
UHT	Ultra-High Temperature
UK	United Kingdom
US	Ultrasound
USDA	United Sates Department of Agriculture
VRBGA	Violet Red Vile Glucose Agar
W	White meat
w/v	weight/volume
WHO	World Health Organization
WI*	Whiteness index
ww	Wet weight
YE	Yeast Extract
ΔE*	Total colour change

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Peer-reviewed articles

- **Condón-Abanto, S.**, Arroyo, C., Álvarez, I., Brunton, N., Whyte, P., & Lyng, J. G. (2018). An assessment of the application of ultrasound in the processing of ready-to-eat whole brown crab (*Cancer pagurus*). Ultrasonics Sonochemistry, 40, Part A, 497-504.
- **Condón-Abanto, S**., Arroyo, C., Álvarez, I., Condón, S., & Lyng, J. G. (2016). Application of ultrasound in combination with heat and pressure for the inactivation of spore forming bacteria isolated from edible crab (*Cancer pagurus*). International Journal of Food Microbiology, 223, 9-16.
- **Condón-Abanto, S.**, Pedrós-Garrido, S., Cebrián, G., Raso, J., Condón, S., Lyng, J. G., & Álvarez, I. (2018). Crab-meat-isolated psychrophilic spore forming bacteria inactivation by electron beam ionizing radiation. Food Microbiology, 76, 374-381.
- **Condón-Abanto, S**., Raso, J., Arroyo, C., Lyng, J.G., Condón, S. and Álvarez, I. (2018). Evaluation of the potential of ultrasound technology combined with mild temperatures to reduce cadmium content of edible crab (*Cancer pagurus*). Ultrasonics Sonochemistry, 48, 550-554.

Articles submitted

- **Condón-Abanto, S.**, Raso, J., Arroyo, C., Lyng, J.G. and Álvarez, I. Quality-based thermokinetic optimization of ready-to-eat whole edible crab (*Cancer pagurus*) pasteurization treatments. Submitted to Food and bioprocess technology. (First review 4/7/2018).

Articles in preparation

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- **Condon-Abanto, S.,** Pedrós-Garrido, S., Alvarez, I., Whyte, P., Lyng, J.G. and Arroyo, C. Isolation, identification and thermal resistance of the microbiota present in ready-to-eat brown crab (*Cancer pagurus*) meat.

CONFERENCE AND WORKSHOP PRESENTATIONS

- **Condón-Abanto, S.,** Lian, F., Arroyo, C., Brunton, N., Whyte, P. and Lyng, J.G. Poster presentation: Efecto de la pasteurización del buey de mar (*Cancer Pagurus*) en el seno de un campo ultrasónico. SEM 2014. Zaragoza, Spain. (ISBN:978-84-941181-7-3)
- **Condón-Abanto, S**., Lian, F., Arroyo, C., Brunton, N., Whyte, P. and Lyng, J.G. Poster presentation: Predicción de la vida útil de buey de mar (*Cancer Pagurus*) pasterizado a 75 °C según las normas recomendadas por la FDA. SEM 2014. Zaragoza, Spain. (ISBN:978-84-941181-7-3)
- **Condón-Abanto, S**., Lian, F., Arroyo, C., Brunton, N., Whyte, P. and Lyng, J.G. Poster presentation: Effect of cooking assisted by ultrasound on the Irish edible crab. IFT15 annual meeting. July 2015. Chicago IL, USA.
- **Condón-Abanto, S.,** Sanz, J., Condón, S., Lyng, J.G., Raso, J., Álvarez, I. Poster presentation: High temperature-short time treatments in crab claws meat assisted by Pulsed Electric Fields. 2nd Worlds congress on electroporation and Pulsed Electric Fields in Biology, Medicine, Food and Environmental technologies. September 2017. Norfolk (VA), USA.
- **Condón-Abanto, S.,** Arroyo, C., Álvarez, I., Raso, J. and Lyng, J.G. Poster presentation: Assessment of the potential of ionizing radiation to inactivate bacterial spores present in brown crab (*Cancer pagurus*). 47th conference of the West European Fish Technologists' Association (47th WEFTA). October 2017. Dublin, Ireland.
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- **Condón-Abanto, S**., Lian, F., Arroyo, C., Whyte, P., Brunton, N. and Lyng, J.G. Oral presentation: Heat resistance of the most isolated spore-forming bacteria in ready-to-eat brown crab meat. Trans-Atlantic Fisheries Technology Conference, TAFT. October 2015. Nantes, France.
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- **Condon-Abanto, S**., Lian, F., Arroyo, C., Whyte, P., Brunton, N. and Lyng, J.G. Oral presentation: Effect of storage temperature on shelf-life and microbiological profiles of ready-to-eat whole brown crab. 46th conference of the West European Fish Technologists' Association (46th WEFTA). October 2016. Split, Croatia.
- **Condón-Abanto, S.,** Arroyo, C., Álvarez, I., Raso, J., Brunton, N. and Lyng, J.G. Oral presentation: Optimization of the second pasteurization step in ready-to-eat whole brown crab (*Cancer pagurus*). 47th conference of the West European Fish Technologists' Association (47th WEFTA). October 2017. Dublin, Ireland.

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ABSTRACT

The aim of the present study was to optimize and evaluate the potential of novel technological interventions in the production of ready-to-eat Irish edible crab (*Cancer pagurus*). The Thesis begins with a general overview of food preservation and main characteristics of edible crab including its significance for the Irish seafood industry. An initial study characterized the main microbiota present in raw and ready-to-eat brown crab and their thermal resistance. Results obtained showed the importance of *Bacillus* spp. and *Staphylococcus* spp. in these products. Characterisation of bacterial thermal resistance proved the effectiveness of recommended heat treatments to inactivate *Listeria monocytogenes* ($F_{70°C}^{7.5°C}$ = 2 minutes). However, the study also revealed that the most severe heat treatment currently recommended, which has *Clostridium botulinum* non-proteolytic type E as a target microorganism ($F_{90°C}^{8.6°C}$ = 57 minutes), is not sufficient to achieve a comparable inactivation (i.e. 6 Log_{10} cycles) of the most heat resistant bacterial spore isolated from crab samples namely, *Bacillus weihenstephanensis*. Following the microbial characterization studies, the potential for incorporating ultrasound to improve early stages in ready-to-eat crab production (i.e. the initial cooking step) was evaluated. The application of ultrasound during cooking enhanced the rate of heat transfer, allowing up to a 15% reduction in total cooking time. In addition, ultrasound also proved its efficacy for enhancing mass transfer from the crab to the cooking water. This improved crab cleaning during cooking would in turn allow the omission of the normal post cook cleaning process prior to packaging. Ultrasounds potential to enhance mass transfer from crab to the cooking water also prompted an investigation into its possible use to remove cadmium from crab. Results showed that ultrasound combined with mild temperatures has the capability to reduce the total cadmium content in edible crab by up to 22.8%. The results open the possibility for using ultrasound as alternative to resolve this issue for the crab industry. Following these studies the second heat treatment step (i.e. in-pack pasteurization) of ready-to-eat crab was optimized to minimize the impact of the treatment on the quality of the final product. Results showed that the colour of crab white meat was the parameter most affected by the heat treatment and therefore, a colour change kinetic for these heat induced changes was developed and used as a quality indicator for process optimization. Based on this study an optimal set of treatment conditions were proposed for the inactivation of *C. botulinum* non-proteolytic type E. However, based on the developed models the required heat treatment for a process which is solely thermal, would be too severe to retain a good quality. This situation would be further aggravated by the requirement for even more severe heat treatments if *B. weihenstephanensis* is considered as the target microorganism. Therefore, the use of alternative technologies (i.e. mano-sonication, mano-thermo-sonication and electron beam ionizing radiation) for the inactivation of the main bacterial spores isolated from brown crab was also evaluated. The use of ultrasound in combination with pressure and mild temperatures (i.e. mano-thermo-sonication) showed a synergistic effect in terms of bacterial spore inactivation, which in turn would allow a reduction in the total processing time by over a 80% while still maintaining a similar level of inactivation to heat only. The use of irradiation also proved to be an effective technology to inactivate bacterial spores while still remaining below the limit of 10kGy established by WHO. In addition radiation was the technology least affected by changes in bacterial species or treatment media composition. Overall,

the results of this thesis shows the potential for a number of alternative technologies and technical interventions to improve the processing of Irish edible crab and address present and future challenges in the production of these ready-to-eat products.

RESUMEN

El objetivo general de esta Tesis Doctoral fue optimizar y evaluar el potencial de diferentes tecnologías de procesado en la producción de buey de mar (*Cancer pagurus*) irlandés listo para el consumo. El primer capítulo de introducción general proporciona una visión global de las diferentes estrategias para la conservación de alimentos y de las tecnologías de procesado disponibles actualmente. Además, se lleva a cabo una revisión de la optimización del procesado de alimentos haciendo énfasis en los tratamientos térmicos. Finalmente, este capítulo resume una visión general del buey de mar, su importancia en la Industria Irlandesa y las prácticas actuales para su procesado.

Tras el capítulo de introducción, en primer lugar, se caracterizó la principal flora bacteriana responsable de la alteración del buey de mar listo para consumo y su resistencia frente a los tratamientos térmicos. De los resultados obtenidos, se deduce la importancia de *Bacillus* spp. y *Staphyloccocus* spp. como los principales microorganismos presentes en el buey de mar listo para el consumo. El estudio de caracterización termobacteriológica demostró que el tratamiento para inactivar *Listeria monocytogenes* ($F_{70°C}^{7.5°C}$ = 2 minutos), en este tipo de productos, es efectivo para inactivar todas las células vegetativas presentes de manera natural en el buey de mar. Sin embargo, el mismo estudio también reveló que el tratamiento más severo recomendado para inactivar *Clostridium botulinum* no proteolítico tipo E, en cangrejo, ($F_{90°C}^{8.6°C}$ = 57 minutos) no resultaba suficiente para alcanzar un nivel similar de inactivación (6 ciclos logarítmicos) de la bacteria esporulada más termorresistente aislada del buey de mar, *Bacillus weihenstephanensis*.

Tras la caracterización de la microbiota del buey de mar, se evaluó el potencial de incorporar la tecnología de ultrasonidos para mejorar el cocinado del buey de mar. Los resultados mostraron que la incorporación de ultrasonidos al cocinado del buey de mar mejoraba los fenómenos de transferencia de calor, lo cual permitió reducir el tiempo total del proceso hasta un 15%. Además, los ultrasonidos también probaron ser efectivos para mejorar los procesos de transferencia de masa producidos durante el cocinado mejorando así la limpieza de los cangrejos en el cocinado. Esto permitiría evitar la etapa de limpieza de los cangrejos antes del envasado que se realiza actualmente en el proceso industrial.

Dado el potencial de los ultrasonidos para mejorar los procesos de transferencia de masa durante el cocinado del cangrejo, se evaluó su uso para reducir la concentración de cadmio del buey de mar. Los resultados obtenidos probaron que la combinación de los ultrasonidos con temperaturas moderadas de tratamiento es capaz de reducir el contenido total de cadmio del buey de mar hasta un 22.8%, abriendo la posibilidad de utilizar esta tecnología para afrontar este importante reto en la producción de estos productos.

Tras la caracterización de las primeras etapas de la producción de buey de mar (cocción y lavado), se realizó un estudio de optimización del segundo tratamiento térmico, la pasteurización, basado en la cinética de cambio de calidad del producto. De los resultados obtenidos, se deduce que el color de la carne blanca es el parámetro de calidad que se ve más afectado debido al tratamiento térmico, por lo que se caracterizó su cinética de cambio de color, utilizándose como indicador para la optimización del proceso. En base a las ecuaciones desarrolladas en este estudio, el tratamiento térmico requerido para inactivar *B. weihenstephanensis* sería demasiado severo para retener una buena calidad en el producto final pasteurizado. Por este motivo, se evaluó el uso de tecnologías alternativas (Mano-Sonicacion, Mano-Termo-Sonicacion y radiaciones ionizantes aplicando electrones acelerados) para la inactivación de los esporos aislados del buey de mar. La combinación de ultrasonidos con presión y temperatura mostró un efecto sinérgico para la inactivación de las bacterias esporuladas, lo cual permitiría reducir hasta un 80% el tiempo total de procesado manteniendo unos niveles de inactivación adecuados. La irradiación también probó ser una tecnología efectiva para inactivar las bacterias esporuladas a dosis de tratamiento por debajo del límite establecido por la WHO de 10kGy. Además, la irradiación fue la tecnología para la inactivación de bacterias esporuladas menos afectada por cambios en la especie microbiana contaminante o en las condiciones del medio de tratamiento, lo cual reduciría el riesgo sanitario de los productos pasteurizados si se produjese un error al definir el microorganismo diana o en la composición del producto.

Los resultados de esta Tesis Doctoral muestran por tanto el potencial del uso de tecnologías de procesado alternativas a los procesos tradicionales, principalmente el calor, para mejorar la producción de buey de mar irlandés listo para su consumo y afrontar sus retos presentes y futuros.

Chapter 1

General introduction and literature review

Due to their composition, crustaceans such as brown crab are considered to be a healthy choice for consumers due to their high-quality protein, amino acid composition (Gökoðlu and Yerlikaya, 2003; Maulvault, *et al*., 2012) and their low saturated fat content (Barrento *et al*., 2008a, 2009b). However, its consumption is also associated with certain health risks such as high cholesterol levels in some edible parts, allergic reactions and contamination with toxic elements (Maulvault *et al*., 2012). Either way in case of edible crab (*Cancer pagurus*) a continuous increase in European landings has occurred over the last 60 years, increasing from 11,000 tonnes to over 48,000 tonnes, giving a total value of in excess of 57 million euros which endorses the increased interest in this crustacean. To date the three main European producers of edible crab are the United Kingdom, Ireland and France (Eurostat, 2018).

Edible crab production in Ireland started in 1980s and over the last three decades has suffered a significant increase, with Ireland being now one of the top three producers of this crustacean in the world with productions ranging between 5,000 and 8,000 tonnes per year, which has a net value of over 8 million Euros. In terms of production, edible crab is the second most exploited species in Ireland. This data shows the importance of this crustacean as a valuable commodity for the Irish fishing industry (BIM, 2014, 2017a).

Out of the total production of edible crab in Ireland, 42% is sold as fresh live crab and the remaining 58% is sold in various ready-to-eat formats. From processed crab only 1% is presented as transformed product with the majority simply presented as cooked whole crab, cooked claws or cooked meat (both white meat from crab appendages or brown meat from the carapace) which is either frozen or chilled (Eurostat, 2018). One of the main challenges for the crab industry is to reduce losses associated with transportation. In the case of exports of edible crab as fresh live product, losses of up to a 50% can occur from crab deaths or damage during transportation, while the export in a ready-to-eat format avoids such losses during transportation while also adding

value to the final product (Barrento, *et al.*, 2008a; 2010b; Uglow, *et al.*, 1986).

The main steps required in the production of ready-to-eat edible crab products are:

(1) Initial cooking (e.g. boiling water for 20-30 minutes): During this step a series of compounds (e.g. proteins and crab dirt) are released from the crab and attach to the carapace which makes a subsequent washing of the crabs prior to packaging essential.

(2) Cooling/washing: This step is necessary to complete the crab meat coagulation and is applied first using jets of water for dirt removal which is followed by cooling in a cold room. After cooling crabs are selected depending on their final destination to be sold as either whole cooked, extracted meats, claws, etc.

(3) Finally a second pasteurization step is commonly applied to products following their packagaing to ensure the microbiological safety of the final in-pack products.

Although these steps are generally common across the crab industry, the conditions used in each step are varying among companies leading to a wide range of variability in the quality of the final products.

Additionally due to the microbiological risks associated with this kind of product as mentioned, crustacean consumption is also associated with other health risks due to high cholesterol levels in some edible parts, allergic reactions and contamination with toxic elements (Maulvault *et al.*, 2012). Particularly, in edible crab high levels of Cadmium (Cd) have been reported, especially in the brown meat (Barrento *et al.*, 2009b, 2009c; Bolam *et al.*, 2016; Maulvault *et al.*, 2012, 2013). In the EU, the Cd content for crabs is only regulated for the white meat, which is located in the crab appendages (claws and legs), with 0.5 mg/kg being the maximum permitted limit (EU,

2006, 2011). However, several studies have warned of the risk of exceeding the total weekly intake of Cd set by EFSA (2009) (2.5 µg/kg body weight) in consumers from countries where brown meat is commonly consumed (Bolam *et al.*, 2016; Maulvault *et al.*, 2013; Noël *et al.*, 2011; Wiech *et al.*, 2017). As opposed to the EU, where there is no regulation concerning Cd levels in brown crab meat, some other countries such as China have recently started to impose limits considering all crab edible parts instead of white meat only. So, in a near future this problem could represent a great issue for the edible crab processing industry.

1.1 FOOD PRESERVATION

Most foodstuffs are derived from plants or animals. The inherent biological nature of such products means that once harvested or slaughtered these products undergo a series of changes, which modify the original characteristics of the product and ultimately these lead to spoilage. These changes can be physical or chemical in nature but can also be induced by the metabolic activity of ubiquitous microbial contaminants.

The ability to preserve foods and extend their shelf life has always been a key area that has developed with Human evolution. Since ancient times, and for many centuries, with no understanding of the underlying causes of food spoilage, a series of preservation methods were developed to ensure a constant availability of food. Many of these methods are still used today. The evolution of these preservation techniques came from experience which was in many instances influenced by the geographic locations of sub-populations. From these ancient times many food preservation methods including drying, smoking, salting, pickling, freezing (in cold climates) or fermentation (in warm climates where spoilage occurred at a faster rate) have evolved.

1.1.1 Food spoilage agents

Food spoilage agents are influenced by many external factors such as temperature, humidity, oxygen concentration, light and storage temperature

(Casp and Abril, 2003; Potter and Hotchkiss, 1995). Independent of the effect of these parameters on microbial growth/survival, significant changes can be also induced on microbial growth due to intrinsic characteristics of the food matrix. Overall, the main causes of food degradation can be classed as chemical or biological.

Chemical degradation. The two main reactions producing food alterations in this group are non-enzymatic browning and lipid oxidation/rancidity.

Non-enzymatic browning, also known as the Maillard reaction, involves a series of complex reactions between reducing sugars and proteins present in foods, which modify odour and flavour of foods, producing dark brown pigments with associated losses in protein (due to denaturation) and nutritional value (Pérez-Locas and Yaylayan, 2010). Additionally when these reactions are very intense toxic substances can potentially be produced (Cheriot *et al.,* 2009). The changes produced due to these reactions might be desirable, such as in the case of a roast appearance or flavour in meat but they can also be undesirable, as in case of a darkened colour or an off flavour in sterilized milk. The rate of development of Maillard reactions is influenced by a number of external factors with heat processing conditions (i.e. time and temperature) being the most important. Thus the Maillard reaction is a key factor in food processes such as cooking, pasteurization, sterilization and dehydration where products are exposed to high temperatures for long times (Martins *et al.,* 2000).

Another important chemical cause of food degradation is fat rancidity (Velasco *et al.,* 2010). These reactions are responsible for nutritional value losses and the formation of undesirable volatile compounds. The two main reactions occurring within this group are hydrolytic rancidity and oxidative rancidity. The first one is due to the action of lipases which releases fatty acids from triglycerides and phospolipids; while the second one refers to the action of oxygen and lipoxygenases on unsaturated fatty acids. These reactions are

also influenced by a number of external factors such as temperature, presence of catalysts, available oxygen concentration and the type of fatty acid among other things (Waraho *et al*., 2011).

Biological degradation. Biological deterioration is quantitatively more important than chemical due to the higher frequency of its occurrence and the severity of the consequences produced (i.e. food poisoning and/or spoilage). Within this group enzymes and microorganisms are the main causative agents though parasites and viruses are also of significant importance (Potter and Hotchkiss, 1995).

The enzymatic activity in all living cells is precisely controlled. In the case of foods, enzymatic activity continues after harvesting or slaughtering producing different changes in foodstuffs. Some of this enzymatic activity is desirable; leading in some cases to positive changes such as fruit maturation or meat tenderization. However, beyond certain limits continued enzymatic action leads to the decomposition of tissues which in turn facilitates further deterioration by microorganisms.

Food spoilage due to the action of microorganisms is a very variable phenomenon, since it is dependent on type and number of microbial species present which in turn depends on the type of food matrix, preservation conditions, including storage temperature and the presence or absence of oxygen (Blackburn, 2006). The action of microorganisms, due to their different metabolic processes, leads to the degradation of foods making them unfit for consumption. A wide range of microorganisms are related in different ways to foods, some of which are natural contaminants of foods while others are consciously or sub-consciously introduced in the food chain (Garg *et al*., 2016). The three main groups of microorganisms found in foodstuffs are those which (a) produce, preserve or develop characteristic flavours (i.e. processing aids), (b) cause food spoilage due to organoleptic changes or (c) are pathogenic and lead to food safety issues

Although, the total amount of food lost due to the action of both biological and chemical spoilage is uncertain, is estimated that one third of total global food produced for human consumption is lost or wasted (FAO, 2011). From these total losses, it is is estimated that about 25% of these losses is directly related to the activity of microorganisms (Petruzzi *et al*., 2017). Despite the losses produced by microorganisms these biological agents are of even greater concern for the food industry due to their potential as a serious public health hazard (Ravishankar and Bai, 2015).

In addition to the economic problems created by microbial spoilage, the presence of some of these microorganisms in addition to the presence of viruses and parasites is a constant threat to public health and a significant impediment to socio-economic development worldwide. It has been estimated that in 2010, the consumption of contaminated foods produced over 600 million illnesses and over 400,000 deaths worldwide (Havelaar *et al*., 2017). Figure 1.1A and 1.1B shows the percentages attributed to the different food contaminants (viruses, bacteria, parasites and chemicals).

As Figures 1.1A and 1.1B show, about 60% of the total illness and deaths related to food are produced by microorganisms, which emphasises the importance of this biological contaminant for the food industry. A wide range of bacteria can be hosted in food. The main vegetative threats are *Listeria* spp., *Salmonella* enterica, *Campylobacter* spp., *Escherichia coli*, *Shigella* spp., *Staphylococcus aureus*, *Vivrio cholera* and *Brucella* spp. while the spore forming bacteria of public health significance are *Clostridium botulinum*, *Clostridium perfringens* and *Bacillus cereus* (Havelaar *et al*., 2017). From these microorganisms, spore forming bacteria are of special interest in food technology due to their ability to generate forms which are resistant to food preservation processes.

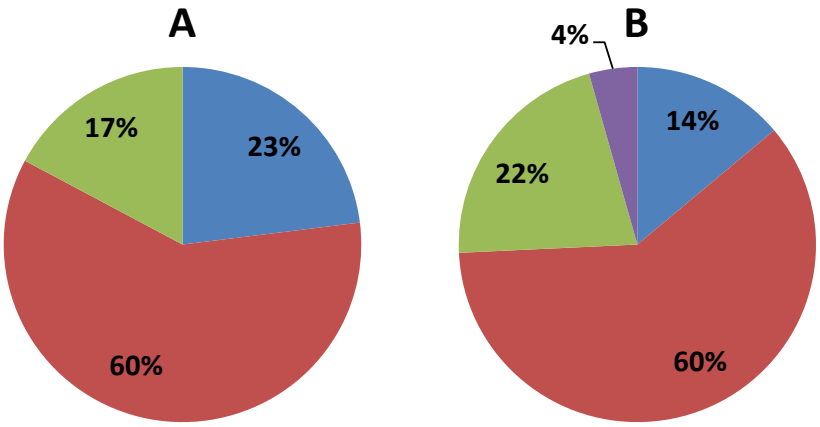


Figure 1.1: Percentage of (A) total foodborne illnesses and (B) total foodborne deaths produced by parasites (green), viruses (blue), chemicals (purple) and microorganisms (red). Data extracted from Havelaar *et al.*, (2015).

1.1.2 Food preservation strategies

In light of these biological and chemical challenges the main objectives of food preservation could be summarized as: (i) ensuring the product safety maintaining appropriate organoleptic conditions during its shelf-life; (ii) minimizing the impact of the preservation process on product quality (Blackburn, 2006). Thanks to current knowledge about the afore-mentioned food spoilage agents, their mechanism of action and the influence of other factors on their behaviour, the main strategies used for food preservation are (Rahaman, 2007a): Inhibition or Inactivation/elimination of the contamination. These preservation strategies constitute the basis of modern food technology and can be applied using different methods and technologies.

a. Inhibition (i.e. reducing microbial metabolic activity and the rate of enzymatic and chemical reactions). The inhibition methods used to reduce the microbial activity and enzymatic/chemical reactions include:

1) *Reduction of water activity (a_w)* by elimination of water or addition of solutes. The reduction of a_w in a food matrix prolongs the microbial growth lag phase, reduces the exponential growth rate and also the maximum microbial load attained at the stationary phase. The main advantages of this process is its simplicity and low cost. However, its principal disadvantage is its significant

impact on the organoleptic characteristics of the final product depending of the system used to reduce a_w (Rhaman and Labuza, 2007).

2) *Reduction of storage temperature.* This preservation method can be applied at two levels, chilling or freezing. Food chilling involves temperatures ranging from -1 to 15°C, and allows a shelf-life extension with reduced effects on food properties. The main inconvenience of chilling is the capability of certain pathogenic microorganisms, such as *Listeria* spp. and *Yersinia* spp., to grow at chilling temperatures (Walker and Betts, 2008). On the other hand, freezing leads to a reduction in product temperature, typically in the range of -18°C to -30°C. At these temperatures no microorganism is capable of reproducing and therefore chemical reactions become the dominant deterioration mechanism (Rhaman and Velez-Ruiz, 2007).

3) *Reduction of redox potential.* This method is based on the reduction of oxygen concentration, which in turn leads to a reduction or inhibition of aerobic microbial activity which in turn extends the shelf-life of food. The main disadvantage of this method is that the absence of oxygen promotes the development of anaerobic species, such *C. botulinum* (both proteolytic and non-proteolytic species) which is considered to be one of the most hazardous microorganisms in food, because of the severity of the associated poisoning. This method is applied during the packaging of food by: physical removal of air by vacuum packaging; chemical absorption of oxygen using oxygen scavengers; or by changing the gas atmosphere surrounding the food using inert or modified atmospheres (Gorris and Peppelenbos, 2007).

4) *Reduction of pH (Acidification).* The reduction of pH acts by destroying some enzymes located in cell envelopes and also by modifying the cytoplasmic pH, which in turn significantly reduces microbial development. From a technical perspective, food acidification can be achieved by adding acids to the food or by promoting the action of certain fermentative processes. The main advantage of this process is its simplicity and relatively low cost. However, similar to reductions in a_w, this process also produces significant changes on the properties and appearance of food matrixes (Rahaman, 2007b).

5) *Addition of bacteriostatic agents.* Traditionally this technique consisted of the addition of a wide range of chemical additives with antimicrobial properties, many of which were synthetic as opposed to natural compounds. Despite the efficacy of this method, current consumer perception of these additives has changed and nowadays its use as food preservation method is declining in popularity. Nowadays, the food industry is looking for more natural alternatives with antimicrobial activity, such as bacteriocins, organic acids, essential oils, etc., to replace the use of synthetic chemical additives (Smid and Gorris, 2007).

These preservation strategies are efficient at controlling microbial/chemical/enzymatic activity but do not eliminate them. They have advantages in terms of their simplicity or in terms of their low impact on the quality of the food material. However, the main limitation of these strategies is their inability to ensure food safety by eliminating the causative agents. Therefore, there effectiveness is highly dependent upon the initial quality of the raw material (Rahaman, 2007a).

b. Inactivation/elimination of contaminating sources (i.e. microorganisms or spoilage agents present in/on raw materials). Although the inhibition strategies can extend the shelf-life, are not able to eliminate the hazard. For this reason another group of preservation strategies which have the potential to eliminate the pathogens and spoilage agents, thereby ensuring foods safety, exists. Within these strategies, methods based on physical principles are the most important. Within that group thermal processing can be considered the most widely used (Rahaman, 2007a).

1.2. HEAT TREATMENTS FOR FOOD PROCESSING

The use of heat in food preparation dates back to the discovery of fire by man. In more recent times, it is fitting the person who is often credited as being the first food technologist is Nicolas Appert who came up with the concept of heating foods in sealed jars to extend their shelf-life in 1809

(Holdsworth and Simpson, 2007). Nowadays, heat still remains the most commonly used industrial method for food preservation (Ramesh, 2007a). The application of heat for this purpose is well understood and treatments can be defined using mathematical equations, which allow processors to predict the chemical changes and more importantly the extent of microbial inactivation. The term heat processing is used to encompass all technological processes which involve the use of heat for food preservation through microbial and/or enzyme inactivation. This group of processes includes pasteurization, sterilization and blanching (Casp and Abril, 2003).

In the case of pasteurization and sterilization the main objective is the microbial destruction for food preservation while in the case of blanching, some microbial inactivation is achieved, but these treatments have different primary objectives which are more related to enzyme inactivation.

1.2.1 Pasteurization

Historically this process is ascribed to Louis Pasteur and his experiments with wine in 1863. The term pasteurization could be defined as a heat process with the objective of eliminating vegetative pathogenic organisms and reducing the load of spoilage microbes present in foods (Casp and Abril, 2003). The intensity of pasteurization treatments is not sufficient to destroy all microorganisms present in foods and therefore this process has to be accompanied by additional preservation techniques (e.g. refrigeration, chemical preservatives or vacuum packaging) (Micali and Fiorino, 2016).

Compared to more severe heat processes (e.g. canning sterilization or ultra-high temperature sterilisation) pasteurisation treatments are generally recognised as having less impact on the organoleptic and nutritional characteristics of the treated product. In general terms, two different pasteurisation techniques have been defined depending on the treatments conditions (Micali and Fiorino, 2016):

a) *Low temperature-long time (LTLT),* which involves the application of temperatures below 70°C for quite long duration of time sometimes even hours (Ramesh, 2007a). These treatments are appropriate for products which have a low heat transfer coefficient (e.g. large solid foods) where it is impossible to get a rapid elevation of the coldest point of the product ("cold spot") to the target temperature using conventional heating methods.

b) *High temperature-short time (HTST), which* involves the application of temperatures between 70 and 100°C for much shorter time, only seconds (Holdsworth and Simpson, 2007; Ramesh, 2007a). In these treatments the temperature of the "cold spot" increases rapidly so the total treatment time is very short and therefore food properties are less affected in spite of the fact that the temperature is higher (see section 1.4.2).

1.2.2 Sterilization

Sterilization is a most severe heat treatment in comparison with pasteurization requiring temperatures of over 100°C usually in the range 115°C to 130°C. A commercially sterile product is a heat processed food which under reasonable storage conditions, will neither spoil nor endanger the health of the consumer. The main difference between pasteurisation and sterilization is their main objective. While pasteurization is focused on the destruction of vegetative cells, the objective of sterilization is the destruction of all living microorganisms (i.e. vegetative cells and bacterial spores) which are likely to grow and reproduce in foods when stored under normal ambient conditions (Ramesh, 2007b). Depending on the product pH two different intensities of sterilization are used. When the pH ≤ 4.5 the temperatures applied are generally lower (i.e. below 115°C), while in products with pH > 4.5 a minimum treatment of 3 minutes at 121.1°C (i.e. F_0 value of 3 min) or equivalent is required to achieve 12 decimal reductions of *C. botulinum* proteolytic type (Micali and Fiorino, 2016; Ramesh, 2007b). Depending on the temperature involved and whether the product is processed in a container or not, sterilization processes can be divided in two main groups:

a) *Traditional sterilization or Appertisation.* Earliest forms of this process (e.g. Nicolas Appert – Appertisation) involved the application of heat in open baths at temperatures of approximately 100-105°C (Borde, 2006). However, nowadays these treatments are applied in pressurized retorts at higher temperatures ranging between 105°C and 135°C and with products held at these temperatures for several minutes (e.g. canning). This sterilisation produces products with a great stability (even years at ambient temperature) although the original organoleptic properties of the food are quite significantly affected. This treatment is commonly applied to products which are packed in containers, where the heat transmission coefficient is low and it is impossible get a rapid temperature elevation at the "cold spot" in the product.

b) *Ultra-High Temperature (UHT) processes,* include treatments in which the temperature of the "cold spot" is increased very rapidly up to temperatures of 130-150°C and is maintained at this temperature for a very short period of time (often for just a few seconds) (Holdsworth and Simpson, 2007). Although the temperatures are higher than those used for canning, the original properties of food are less affected; although a residual enzymatic action can persist which gives products with lower stability compared to canned products. UHT processes can be applied by direct contact with the heating media (e.g. steam injection or steam infusion) or indirectly via a heat exchanger (e.g. plate or tubular) followed by an aseptic packaging step (Clare *et al.*, 2005; Morales *et al.*, 2000). UHT processing is generally considered more complex than canning due to the need for clean in place protocols and the greater complexity of the equipment involved.

1.3 NOVEL TECHNOLOGIES FOR FOOD PROCESSING

Increasing consumer demand for products with high nutritive and quality attributes has inspired a considerable amount of research in the area of minimal processing (Sun, 2014). However, this trend has raised new challenges for the food industry which cannot be easily solved using traditional preservation methods such as thermal treatments. As a result a branch of

research has emerged in the area of alternative technologies which have the objective of producing safe and stable products to satisfy current consumer demand. These alternative or new technologies are classified into two groups: (a) New heating methods or new thermal technologies and (b) non-thermal technologies.

Ohmic heating, Microwave heating and Radio-frequency heating are the main alternative thermal technologies considered for food processing. The main advantage of these technologies is their capability to produce a quick and homogenous volumetric heating with high energy efficiency. Whitin this group infrared heating is also included but mainly for surface heating of food products.

By contrast, the main advantage of novel non-thermal technologies is their capability to produce microbial inactivation at lower temperatures than traditional heat systems which in turn leads to the production of products with a greater quality retention and higher nutritional value. This is particularly true for heat sensitive foodstuffs. In addition, other investigations have found applications for these technologies in the area of enhanced heat and mass transfer processes and inducing structural changes in food (Chemat *et al.*, 2011; Oliveira *et al.*, 2017; Raso *et al.*, 2015). Within this group the technologies considered for food processing include: Ultraviolet Irradiation, High Intensity Light Pulses, High Hydrostatic Pressures, Cold Atmospheric Plasma, Pulsed Electric Fields, Ionizing Radiation and Power Ultrasound. Table 1.1 provides a brief overview of these technologies. During the development of this PhD Thesis only Ionizing Radiation and Power Ultrasound were used, thus these two technologies will be described in more detail.

Table 1. 1: Brief description of the novel thermal and non-thermal technologies for food processing.

Technology	Principle/mechanism	Main control parameters	Examples of applications	References
Ohmic heating	Joule heating produced by alternating electric current	Electric conductivity, treatment chamber design, current, voltage, temperature, frequency and flow properties (solid content, viscosity and pH)	Cooking, pasteurization, sterilization, water distillation, blanching, thawing, starch gelatinization, protein recovery	Jaeger *et al.*, 2016; Knirsch *et al.*, 2010; Lyng and Mackenna, 2013; sakr and Liu, 2014
Microwave heating	Use electromagnetic energy (at frequencies from300 MHz to 300 GHz) to generate heat in a food material due to the friction produced by the polarization of molecules (dipole water molecule agitation)	Dielectric properties (constant and loss factor), frequency, electric field intensity, food size, shape and composition; temperature; moisture content	Drying, baking, Thawing and tempering, pasteurization/sterilization, roasting, blanching	Chizoba Ekezie *et al.*, 2017; Guo *et al.*, 2017; Jiao *et al.*, 2018; Stefănoiu *et al.*, 2016
Radio-frequency heating	Use electromagnetic energy (at frequencies from 300 kHz to 300 MHz) to generate heat in a food material due to the friction produced by the polarization of molecules (Ion charge migration)	Dielectric properties (constant and loss factor), frequency, electric field intensity, food size, shape and composition; temperature; moisture content	Disinfestation, cooking, thawing, blanching, roasting, drying	Jiao *et al.*, 2018; Stefănoiu *et al.*, 2016; Varghese *at al.*, 2014
Infrared radiation	Use of electromagnetic radiation (range of wavelength from 0.78 to 1000 µm) for surface heating	wavelength, surface composition, water content, organic compounds (proteins, starches…)	drying, freeze drying, thawing, roasting, blanching, thawing, cooking, microbial inactivation	Jermann *et al.*, 2015; Pawar and Paratape, 2017; Rastogi, 2012
Ultraviolet irradiation	Use of electromagnetic radiation (between 200 and 300 nm) to produce Deoxyribonucleic acid (DNA) damage (formation of thymine dimers)	treatment time, fluence, turbidity, absorbance, rugosity, treatment temperature	liquid pasteurization/ sterilization, surface decontamination	Cebrián *et al.*, 2016; Fan *et al.*, 2017; Gayán *et al.*, 2014a, 2014b
High intensity light pulses	DNA damage (formation of thymine dimers), photo-thermal effect	pulse frequency and duration, treatment time, fluence, turbidity, absorbance, rugosity, treatment temperature	liquid pasteurization/ sterilization, surface decontamination	Ferrario *et al.*, 2013; Heinrich *et al.*, 2015; Kramer *et al.*, 2017; Gómez-López *et al.*, 2007; Oms-Oliu *et al.*, 2008

Table 1.1 continue

Technology	Principle/mechanism	Main control parameters	Examples of applications	References
High hydrostatic pressure	cell envelopes breakdown, oxidative damage	Treatment time, pressurization level, treatment temperature, food matrix composition (pH, aw, etc...)	pasteurization of liquid/solid foods, shelling for crustaceans, enhance mass transfer processes, enhance freezing and thawing processes	Cebrián *et al.*, 2016; Georget *et al.*, 2005; Mañas and Pagán, 2005; Oliveira *et al.*, 2017
Cold atmospheric plasma	DNA damage, cell envelopes breakdown and oxidative damages	Food matrix composition (aw, pH, nutrients, osmotic stability), processing variables (type of gas used, input voltage, mode of exposure-direct or indirect)	surfaces decontamination/ sterilization, enzyme inactivation, toxin degradation	Chizoba *et al.*, 2017; Pankaj and Keener, 2017; Pankaj *et al.*, 2018
Pulsed electric fields	Electroporation	electric field strength, pulse width and shape, number of pulses, frequency, temperature	pasteurization, enhance mass transfer processes, alter food structures, peeled, cooking	Blahovec *et al.*, 2017; Cebrián *et al.*, 2016; Raso *et al.*, 2015
Ionizing radiation	Use of electromagnetic waves at frequencies between 10^{17} and 10^{25} Hz which produce reactive oxygen species (ROS) and DNA damage	Type of radiation (Electron beam, X rays or Y rays), irradiation dose, penetration depth, temperature, pH and aw	Germination inhibition, disinfestation, parasites destruction, retard maturation and spoilage of vegetables, shelf-life extension of meat and fish, product sterilization	Dickson, 2001; Farkas, 2006; Mañas and Pagán, 2005
Power ultrasound	cavitation	Frequency, wavelength, temperature, pressure, vapour pressure of the medium	Cooking, freezing, drying, marinating, degassing, filtration, demoulding, defoaming, emulsification, oxidation, cutting, fermentation, microbial inactivation	Cebrián *et al.*, 2016; Chandrapala *et al.*, 2012; Chemat *et al.*, 2011, Zheng and Sun, 2006

1.3.1 Ionizing radiation (IR)

The use of ionizing radiation for food preservation can be considered as a re-emergent technology (Molins, 2001). Food irradiation 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; Jeong and Kang, 2017; Sarrías *et al.*, 2003) and assess its influence on food properties (Byun *et al.*, 2000, 2008; Lee *et al.*, 2001). Despite the fact that it shows great potential for food decontamination, the use of this technology at an industrial scale remains stalled in the field of the food processing in several countries, due to poor consumer perception of irradiated foods. This confused perception is generally a result of a lack of information, misinformation and misleading imagery and also the confusion among consumers of the difference between irradiated food and radioactive contamination.

Food ionization consists of the exposure of packaged or unpackaged foodstuffs to ionizing radiation for macro-organisms (e.g. insects, parasites) or microbial destruction in the food products (Odueke *et al.*, 2016). The three main sources of ionizing radiation for food processing, are gamma rays produced from cobalt (^{60}Co) (1.17 and 1.33 MeV) or Cesium (^{137}Cs) (0.662 MeV), generators of electron beam (max. energy 10 MeV) and X rays (max. energy 5MeV) (Codex Alimentarius Commission, 1984). Apart from their differing origins these irradiation sources have different energy and penetration depths. In food processing the usefulness of electron beam irradiation is limited by its low penetration depth while X rays are limited by their low energetic efficiency. By contrast, lambda rays (Y) have an elevated energy and penetration depth, which makes them more suited for processing larger food masses. However, the use of Y rays is limited because its main sources are radioisotopes.

Nowadays the main applications of this technology in the food industry are focused on the inhibition of vegetable germination; disinfection and disinsection in cereals, fruits, meats and fish; retardation of fruit and vegetable ripeness; shelf-life extension of raw meat and fish; pathogenic microbial inactivation; and sterilisation of spices and meals for immunocompromised people and also for space travel (Crawford and Ruff, 1996; Odueke *et al.*, 2016).

In food ionization the main parameter to take into account is the total dose absorved by the product. The irradiation dose is the total amount of energy absorbed by the irradiated product. The dose applied is measured in Gray (Gy) or kiloGray (kGy). In terms of energy 1 Gray equals to 1 Joule absorbed per kilogram of irradiated product. From a practical point of view three different ranges of doses exist for food processing: low, medium and high dose (Farkas, 2006; Molins, 2001; Odueke *et al.*, 2016):

- Radurization or low-dose treatments involve doses below 1kGy. These treatments are used for germination, inhibition, delay of ripeness, disinfestation and parasites destruction.
- Radicidation or medium dose treatments involve doses between 1 and 10kGy. These treatments are useful for the inactivation of spoilage and pathogenic vegetative cells but not for bacterial spores.
- Rappertization or high dose treatments involve doses over 10kGy. These treatments are focused on the destruction of microorganisms up to levels of sterilization.

The effects of ionizing radiations on biological material are the sum of its direct and indirect effects (Figure 1.2). The direct effect is a consequence of the physical changes produced on the molecules as a result of their absorption of energy. The energy absorption is proportional to the molecular weight which makes DNA one of the main target molecules due its larger size and complexity. For this reason the irradiation lethal dose

varies among different organisms depending on the complexity of their DNA (viruses (10-200kGy) Bacterial spores (>10-50kGy), Vegetative cells (>0.5-10kGy), Insects and parasites (>0.01-0.5kGy) and Mammals (>0.005kGy)). The indirect effect is related to the formation of reactive oxygen species (ROS) due to the direct action of radiation on cell components or the media surrounding, water for instance (Farkas, 2006; Lomax *et al.*, 2002; Sutherland *et al.*, 2000; Yokova *et al.*, 2008).

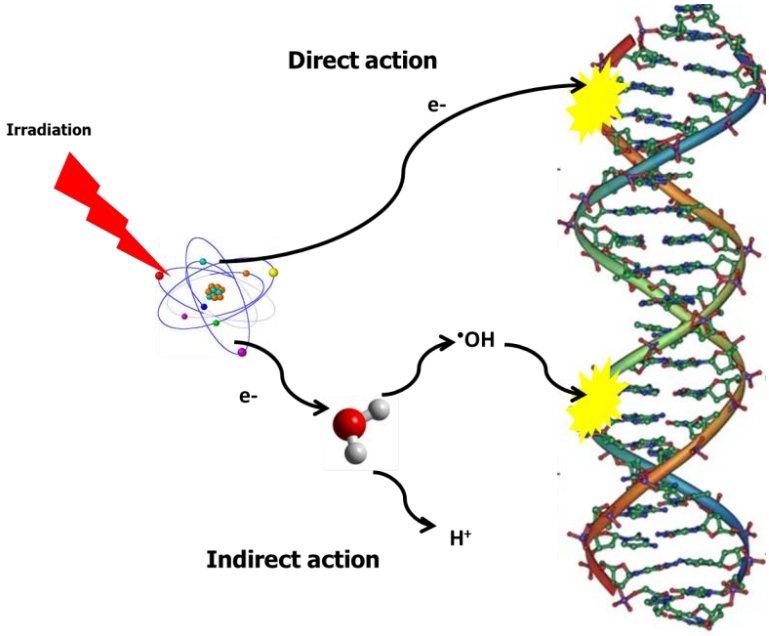


Figure 1.2: Scheme of the direct and indirect effects of irradiation on DNA

Despite the great potential of irradiation for food decontamination this process may also produce some undesirable changes on foodstuffs by both direct and indirect mechanisms; being water, proteins, other nitrogen compounds and aromatic compounds some of the most affected by irradiation treatments. The effects induced depend among other things on the irradiation dose, treatment temperature, oxygen availability and moisture. Some strategies to reduce the impact of these adverse effects on foodstuffs are the reduction of temperature (freezing temperatures), reduction of oxygen availability, addition of free radicals scavengers and dose reduction (Jay *et al.*, 2005).

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 10kGy 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). In the European Union (EU), only a total of 5,690.1 tonnes of food was irradiated in 2015, with Belgium being the most active country with a total of 3,916.9 tonnes of food irradiated in the same year (EU, 2016). Only dried aromatic herbs, spices and vegetable seasoning are authorised in the EU to be treated with ionizing radiation up to a maximum dose of 10kGy (EU, 1999). However, some countries within the EU have an extended list of food authorized to be treated with this technology. Table 1.2 shows the different foods and the maximum doses authorised in these EU countries.

Table 1.2: List of authorized food products and maximum irradiation dose in different EU countries (EU, 2009).

Product	Authorized at the given maximum overall average absorbed radiation dose (kGy)						
	BE	CZ	FR	IT	NL	PL	UK
Deep frozen aromatic herbs	10	10	10				
Potatoes	0,15	0,2		0,15		0,1	0,2
Yams		0,2					0,2
Onions	0,15	0,2	0,075	0,15		0,06	0,2
Garlic	0,15	0,2	0,075	0,15		0,15	0,2
Shallots	0,15	0,2	0,075				0,2
Vegetables, incl. pulses	1	1					1
Pulses		1			1		
Fruit (incl. fungi, tomato, rhubarb)	2	2					2
Strawberries	2	2					
Dried vegetables and fruits	1	1	1		1		
Cereals	1	1					1
Dried fruit		1					
Flakes and germs of cereals for milk products	10	10	10				
Flakes from cereals		1			1		
Rice flour	4	4	4				
Gum arabic	3	3	3		3		
Chicken meat		7			7		
Poultry	5	5	5				
Poultry (domestic fowls, geese, ducks, guinea fowls, pigeons, quails, and turkeys)	7	7					7
Mechanically recovered poultry meat	5	5	5				
Offal of poultry	5	5	5				
Frozen frog legs	5	5	5		5		
Dehydrated blood, plasma, coagulates	10	10	10				
Fish and shellfish (incl. eels, crustaceans and molluscs)	3	3					3
Frozen peeled or decapitated shrimps	5	5	5				
Shrimps					3		
Egg white	3	3	3		3		
Casein, caseinates	6	6	6				

BE, Belgium; CZ, Czech Republic; FR, France; IT, Italy; NL, Netherlands; PL, Poland; UK, United Kingdom.

1.3.2 Ultrasound (US)

Ultrasound technology consists of sonic waves with frequencies above the threshold of human hearing (16-18 kHz). These ultrasonic waves are defined and characterized by their frequency and their wavelength. Waves with frequencies between 20 and 40 kHz are defined as high-energy or

high-power ultrasound, whereas those whose frequency ranges between 40 kHz and 1 MHz are known as low-power ultrasound (Mason *et al.*, 1996). These two types of ultrasonic waves have very different applications. High-power ultrasound is used in the food industry for different purposes and is generally applied in processes which involve heat transfer, mass transfer or both. On the other side low-power ultrasound is used for quality analysis and process control purposes (e.g. sensors). Ultrasonic power is defined as the energy transmitted by the wave per second (W), ultrasonic intensity as the power per surface unit (W/cm^2) and ultrasonic power density as energy per volume of treated product (W/L) (Meullemiestre *et al.*, 2017).

When an ultrasonic wave is propagated through a liquid media, it creates alternating compression and expansion cycles. When the expansion cycle has the capacity to exceed intermolecular forces and to reduce the absolute pressure below vapour pressure, small bubbles are formed. Over the course of subsequent expansion/compression cycles, the bubbles expand and contract. A rectified diffusion process leads to the growth of bubbles until they reach resonance size. Then, over the course of the next acoustic cycle (which corresponds to one oscilation in Figure 1.3), the bubbles grow to a maximum size and then collapse. As a consequence of that implosion, molecules violently collide with each other, giving rise to shock waves and creating spots of very high temperature and pressure (Mason *et al.*, 1996). These extreme conditions usually induce water sonolysis, resulting in the emergence of highly reactive radicals. This phenomenon is known as transient cavitation, when generated by high power ultrasound. Cavitation is considered the main mechanism by which this form of ultrasound enhances heat and mass transfer phenomena (Kim *et al.*, 2004; Zhou *et al.*, 2002), though other effects such us acoustic streaming, which is a physical force that produces an additional mass flow (Solovchuk *et al.*, 2011) are also involved (Legay *et al.*, 2011). A scheme of the cavitation process is shown in Figure 1.3.

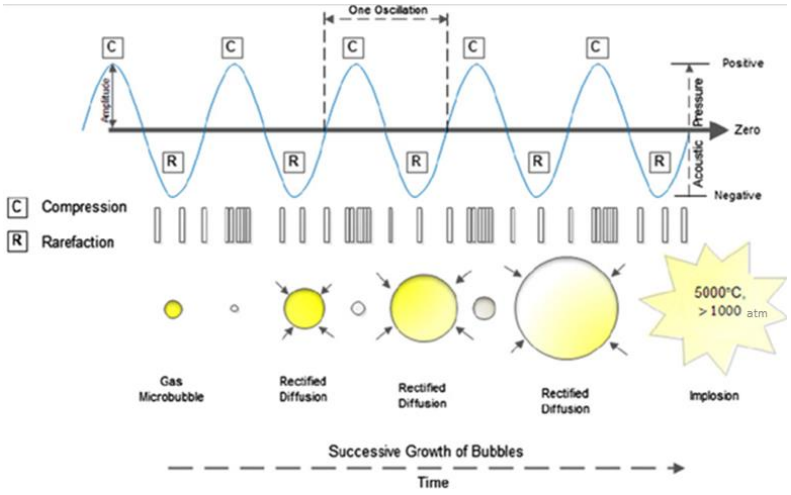


Figure 1.3: Scheme of the evolution of cavitation bubbles during the application of power ultrasound. Source (Adapted from Abbas *et al.*, 2013).

The effects of ultrasound on heat transfer has been extensively studied since the 1990s in model systems such as water, metal tubes, metal balls, etc. (Huamao *et al.*, 1997; Hyun *et al.*, 2005; Oh *et al.*, 2002; Zhou *et al.*, 2002). Furthermore its ability to enhance heat transfer in foods, mainly in processed meat products, during cooking processes (Alarcon-Rojo *et al.*, 2015; Reynolds *et al.*, 1978; Vimini *et al.*, 1983) has been investigated. Also the theoretical advantages of ultrasonic cavitation have been assessed in different processes related with the food industry, with cooking, marinating and extraction evaluated extensively (Alarcon-Rojo *et al.*, 2015; Cárcel *et al.*, 2007; Khan *et al.*, 2010; Luengo *et al.*, 2014; Ma *et al.*, 2009; Mason *et al.*, 1996; McDonnell *et al.*, 2014; Turhan *et al.*, 2013; Vimini *et al.*, 1983) and the usefulness of the application of ultrasound to enhance freezing, drying, degassing, filtration, demoulding, defoaming, emulsification, oxidation and cutting have also been explored (Ashokkumar, 2015; Chandrapala *et al.*, 2012; Chemat *et al.*, 2011).

Besides the applications related to heat and mass transfer processes, US technology is one of the new microbial inactivation technologies that has been suggested as an alternative to conventional heat treatments (US FDA,

2000). As mentioned before, today most authors (Condón *et al.*, 2011; Lee *et al.*, 2009; Wu *et al.*, 2015) agree that high power ultrasound inactivates cells via envelope breakdown as a consequence of shock waves produced due to cavitation phenomena. As a general rule the US resistance of bacterial cells decreases with size and is greater in coccoid-shaped bacteria (Alliger, 1975; Condón *et al.*, 2005). Furthermore, Gram-positive bacteria are usually more resistant than Gram-negative bacteria; with yeast and moulds presenting intermediate resistance (Condón *et al.*, 2011; Jambrak *et al.*, 2017; López Malo *et al.*, 2005), while bacterial spores are almost not affected by US treatments (Jambrak *et al.*, 2017; Sanz *et al.*, 1985). Most published data indicate that the bactericidal efficacy of ultrasound is low (Jambrak *et al.*, 2017; Lee *et al.*, 2013; Meullemiestre *et al.*, 2017). Therefore, most researchers have tried to improve the efficacy of the process by designing combined processes to enhance the overall lethal efficacy of ultrasound (Lee *et al.*, 2013; López-Malo *et al.*, 2005; Raso *et al.*, 1998a).

Some of the combined US processes proposed to date to increase the lethal effect of ultrasound have been classified (Chemat *et al.*, 2011; Piyasena *et al.*, 2003; Sala *et al.*, 1995) as thermosonication (TS, combination of ultrasound and heat), manosonication (MS, combination of ultrasound and pressure), and manothermosonication (MTS, combination of ultrasound, pressure and heat). The increment of the lethal effect of ultrasound by raising the temperature of the treatment media (TS) has been observed on bacterial vegetative cells (Lee *et al.*, 2009), yeast (Abid *et al.*, 2014; Bermudes-Aguirre and Barbosa-Canovas, 2012) and on bacterial spores (Garcia *et al.*, 1989; Milly *et al.*, 2007). It has been suggested that higher temperatures sensitize cell envelopes to shock waves originated by cavitation (Lee *et al.*, 2009; Wu *et al.*, 2015). In some cases the lethal effect of TS increases until reaching a maximum at a certain temperature following which the ultrasound effect decreases. This

decreasing behaviour has been associated with an increase of the vapour pressure of the medium (Knorr *et al.*, 2004; Sala *et al.*, 1995), which should facilitate cavitation, but while also reducing the intensity of cavitation implosion, as the vapour within the bubbles acts as a cushion (Alliger, 1975). When boiling temperatures are reached, cavitation ceases.

On the other side, the inactivation rate of vegetative cells due to MS increases when static pressure is raised (Lee *et al.*, 2009, 2013; Mañas *et al.*, 2000). An increase in hydrostatic pressure makes the formation and growth of bubbles more difficult and requires higher energy consumption for their production, but the resultant cavities release more energy when they implode, thereby increasing the lethal effect. When pressure is too high in comparison with ultrasonic power, cavitation formation is inhibited and transient cavitation ceases. This explains that the effect of pressure increases the bactericidal effectiveness of US until reaching a threshold, above which the effect of pressure progressively decreases until it vanishes (Guzel *et al.*, 2014; Pagán *et al.*, 1999b; Raso *et al.*, 1998a).

MTS process was designed in 1992 (Spanish Patent No. 9200686) with the aim of overcoming the limitations of TS and MS, while making use of their advantages. The authors hypothesized that pressure would increase cavitation intensity and compensate for changes in the vapour pressure of the medium as a result of heating, thereby permitting an advantageous use of thermosonication even at high temperatures (Sala *et al.*, 1995). Data published over the last 20 years confirms the validity of this hypothesis to inactivate vegetative microbial cells, spores and also enzymes.

1.4 PROCESS OPTIMIZATION

The previous section outlined in more detail the main technologies that were explored in the present PhD Thesis. The current section provides an overview about the area of process optimisation and their usage. Optimization is defined as the action of making the best or most effective

use of a situation or resource (Oxford dictionary, 2018). From the perspective of food processing, process optimization can be defined as the selection of the process parameters which will achieve a safe/stable product, while maintaining the best possible nutritional and organoleptic characteristics and maximizing the profit.

The origins of food process optimization can be attributed to the discoveries of Bigelow, Easty and Mayer in the 1920s, in the laboratories of the National Canners association in U.S. These researchers were investigating microbial destruction kinetics by heat. The modern concept of optimization, which combines microbial inactivation and food quality, was not developed until 1940s when Esselen proved the logarithmic destruction of vitamins subjected to heat, discovering the differences on the thermal sensitivities between microorganisms and vitamins. Nowadays the role of process optimization is to minimize product deterioration, maximize profits while, at the same time, ensure the hygienic and safety requirements imposed by national or international regulations. Nowadays, as a general rule in the food industry process optimization is dictated by food safety requirements (Valdramidis *et al.*, 2012) with quality having a secondary role.

Food process optimization requires an appropriate knowledge of the process and the variables involved on it. Figure 1.4 shows the main information and steps required to perform food process optimization. However, obtaining all required information is a slow and costly procedure which most of the times makes it a big challenge. A reasonable alternative is to predict, based on mathematical models the impact of the process variables on the characteristics of the product and optimize the process based on these predictions (Ling *et al.*, 2015; Stoforos, 1995).

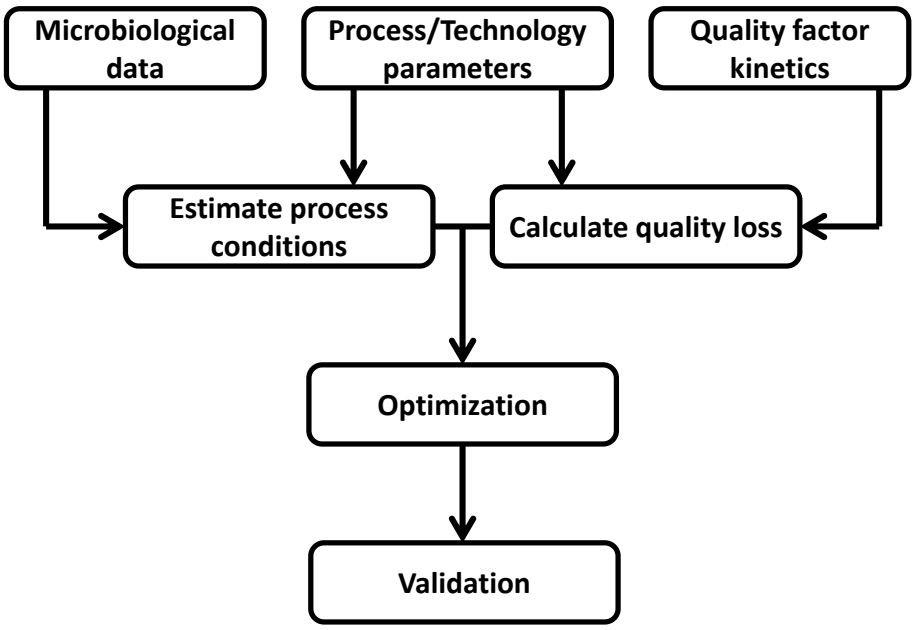


Figure 1.4: Schematic diagram of the key elements and steps involved in the optimization of a food process. Adapted from Stoforos, 1995

1.4.1 Modelling in the food industry

In the context of food processing, a mathematical model is defined as a method of simulating a real-life process using mathematical equations to forecast the future behaviour of a food subject to the process. The potential of modelling in the food industry is that it allows the defininition of how process variables affect product characteristics, based on mathematical equations, but with a reduced requirement for generating experimental data. An agreed criterion to classify predictive models does not currently exist. However, it is possible to classify models into groups based on the fundamentals of the mathematical approach using the classification proposed by Whiting and Buchanan (1993) who defined primary, secondary and tertiary models. These classifications are generally used for predictive microbial models but could be transferable to other aspects of food technology.

Within each of the aforementioned groups, based on its mathematical fundaments, predictive models are classified as **empirical models** or **mechanistic models**.

Empirical models fit a mathematical function to experimental data based on experience and observation. These models are interesting in practical situations due to the simplicity of describing experimental data, under specific conditions, through a convenient mathematical relationship. On the other side, mechanistic models are based on the rules that govern the process and force the adaptation of the model to the reality through a specific hypothesis. As general rule, mechanistic models predict more accurately the reality and allow extrapolation of predictions for a broader range of conditions and with higher confidence; although by contrast have lower correlation with the experimental data produced to generate the model than the empirical models.

Currently, mechanistic models are being used successfully in food engineering to describe heat and mass transfer process in food processing, but generally, the development of these models, requires a high investment to define the intrinsic and extrinsic characteristics that govern and influence the process, due to the complexity of many food matrixes. On the other side, the models which try to describe biological processes, such us microbial growth or inactivation, are mainly empirical or semi-mechanistic due to the lack of knowledge regarding all of the mechanisms involved.

Depending on its purpose, predictive models can be classified as **kinetic models** or **probabilistic models**. Kinetic models are especially useful in dynamic systems (i.e. systems in which the characteristics of the process evolve over time) and, once defined, allows the description of the status of the system in a specific moment during the process. These models have been extensively used to describe the effect of different variables on the growth and inactivation of microorganisms (van Boekel, 1996) as well the loss of product quality produced during processing (Ling *et al.*, 2015).

Probabilistic models are referred to stochastic processes and predict the outcome depending on the extent of product and process variability. These

models have been used to predict the probability of microbial growth due to physicochemical characteristics of a food system. This information could be useful in food production to define formulations, processing conditions, packaging and storage conditions (Roberts, 1997; Ross and McMeekin, 1995)

As it has been indicated, based on the classification proposed by Whiting and Buchanan (1993) predictive models are classified in **primary**, **secondary** and **tertiary**.

Primary models describe how a specific product characteristic evolves due to the action of a process. Generally these models are mathematical Equations which describe a kinetic over time under determined conditions. Examples include (a) the Gompertz Equation (Zwietering *et al.*, 1990) for microbial growth; (b) calculus relating to the determination of D_T values as a first order model for microbial inactivation and (c) zero, first and second order kinetics for quality degradation are just some examples of primary model types (Ling *et al.*, 2015).

Secondary models describe the behaviour of the parameters of the primary models depending on the ambient conditions which include factors such as temperature, pH or a_w. Some examples of secondary models are (a) the *z* value for microbial inactivation; (b) the Arrhenius or Belehradek Equations or (c) response surface Equations (Palop *et al.*, 1999).

Tertiary models integrate the relations obtained in primary and secondary models and allow for the prediction of outcomes while considering various influencing factors. The values of ambient factors are introduced into the secondary models in order to obtain Equations which calculate specific factors for the primary model, obtaining complex Equations which predict the response of the variable under study.

Theoretically to compose a good model it is necessary to know in detail all factors which potentially can influence the system under study and also the relevant interactions between these factors. The model will predict more accurately the reality when this information is known with higher detail and precision. However, sometimes part of this information is unknown or not considered because the required investment to obtain this additional information is too high and do not warrant an improvement of the model predictions.

The development of a mathematical model follows a series of steps which are well defined and can be described as follows:

Problem definition and experimental design:

This step consists in the identification of the problem or situation which needs to be simulated, optimized or controlled. But it is important to keep in mind that modelling is not the objective but it is a tool to get an objective (Annio and Russell, 1979). Once the problem has been defined, the experimental plan is designed. The purpose of these experiments is to solve the problem raised. To define a efficient experimental plan the following questions have to be answered: What are the required objectives and what are the factors which could influence these objectives?; which are the relevant characteristics of the sample to be processed?; what and how many are the ambient characteristics and their interactions which could influence the response?; which are the dependant (i.e. variables whose values depend upon the independent variable/s) and independent variables in the process and which is the most relevant Equation to describe the behaviours of the variable?; how will experimental data be collected?

Obtaining and analysis of the experimental data:

The amount of experimental data is crucial in the development of a mathematical model. A good experimental design should consider the costs of the methods and the optimal number of replicates. No guidelines exist to

describe what is the appropriate number of replicates to produce a robust model. From a statistical point of view it is necessary to have a higher number of observations than parameters. However, the final number of observations depends on different factors such as the objective of the model, the type of model or previous knowledge of the considered variables within the model. In any case the total number of observations should be always as large as possible as the greater the amount of data the more robust the resultant model will be (Hyndman and Kostenko, 2007).

Other important factors which affect the robustness of the model are the distribution of the experimental points within the experimental design, this being the determinant for the optimal estimation of the dependant variables. In some cases the experimental points have to be equidistant within the range under study while, in other cases the experimental points have to be grouped in specific values within the range under study where greater effects are expected (Walker and Jones, 1993).

Generation of a model and data fitting:

Once the experimental data has been obtained, the primary model is fitted to the data, which generally describes the kinetics of the reaction under study. The second step is to correlate the parameters obtained for the primary model with other external factors using secondary models (i.e. to describe through a mathematical Equation the effect of the external factors on the parameters of the primary model). Finally when primary and secondary models are combined a tertiary model is produced. Generally, simple models or "parsimonious models" provide better predictions and their range of application is greater, since they are based on general assumptions (Baranyi *et al.*, 1996). By contrast, complex models require the fitting of many different parameters and their range of action is limited to specific scenarios under defined conditions.

Validation of a model:

The last step in the generation of a mathematical model is its validation. This is the most important step since it will determine the applicability and usefulness of the model. In this procedure the values generated by the model are compared to measured values. If the predicted values do not align well to the real values, it becomes necessary to revisit the first step again in order to improve the accuracy of the model. Two types of validations exist:

1) *Internal validation*, which consists of the comparison of values generated by the model *versus* the values used to generate the model or other values obtained under similar conditions.

2) *External validation*, which consists of the comparison of the values generated by the model *versus* data obtained *ex professo* for the validation, in different conditions to which was used during the development of the model, within the range under study.

The comparison between real and predicted values can be performed in two different ways: graphically or mathematically. In the graphical evaluation the predicted values are represented *versus* measured values generating a simple graph (Figure 1.5 shows an example of a validation graph), in which the less accurate predictions (i.e. outliers) are easily and quickly detected.

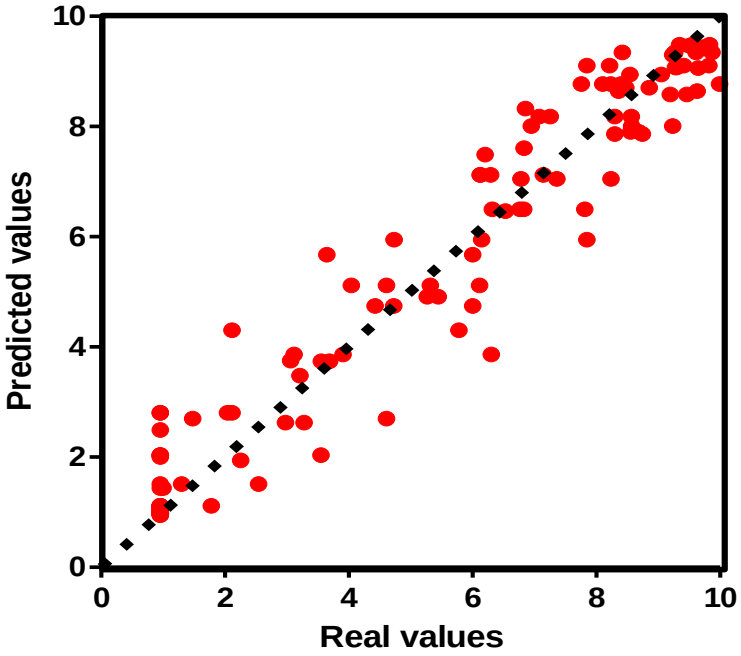


Figure 1.5: Example of a validation graph. Dashed line represents the bisectrix which correspond to a perfect fit.

The dashed line in Figure 1.5 represents the perfect fit. The points furthest from it represent the least accurate predictions. From Figure 1.5 it is also possible to define if the model overestimates or underestimates its predictions. If the points are located above the line, the model would overestimate on its predictions while if the points are located in the area under the line the model would underestimate on its predictions. This procedure is commonly done in conjunction with the analysis of residual errors which indicates the differences between predicted and real values.

Although the graphical evaluation is simple and useful, a mathematical analysis is also necessary in order to evaluate the robustness and goodness of the model fit. The most common used parameters are:

a) *Coefficient of determination (R^2).* This parameter provides a measure of how well real values are represented by the model, based on the proportion of total variation of outcomes explained by the model. It is calculated as shown in Equation 1.1.

$$R^2 = \left(\frac{\sum_{i=1}^{n}(X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2 \sum_{i=1}^{n}(Y_i - \bar{Y})^2}} \right)^2 \qquad \text{(Eq. 1.1)}$$

Where X_i represents real values values, $\bar{X}$ the average of the real values, Y_i represents predicted values, $\bar{Y}$ the average of predicted values and n is the total number of observations. R^2 values close to 1 indicate a better correlation between predicted and observed values.

b) *Root mean square error (RMSE).* This parameter quantifies the quadratic error of the differences between predicted and real values. It is calculated as shown Equation 1.2.

$$RMSE = \sqrt{\frac{\sum(X_i - X_0)^2}{n}} \qquad \text{(Eq. 1.2)}$$

Where X_i represents predicted values, X_0 observed values and n is the total number of observations. Small values of this parameter involve higher certainty in the models prediction. However, models with high *RMSE* values still can predict accurately as this parameter depends on the order of magnitude of the values predicted and observed.

c) *Standard error of prediction (SEP).* This parameter is similar to *RMSE,* but quantifies the error in relative terms, so its value is not dependent on the magnitude of the data. It is calculated as shown Equation 1.3.

$$SEP = \frac{100}{\bar{X}_0} \sqrt{\frac{\sum(X_i - X_0)^2}{n}} \qquad \text{(Eq. 1.3)}$$

Where $\bar{X}_0$ represents the average of the observed values and the others are defined in *RMSE*.

d) *Bias factor (B_f) and Accuracy factor (A_f).* These parameters were defined to assess the goodness of fit of the predictive models in a rapid way and provide indicators of the reliability of the models (Ross, 1996). These parameters are calculated following Equations 1.4 and 1.5 respectively.

$$B_f = 10^{\left(\frac{\sum \log(X\ pred./X\ obs.)}{n}\right)} \qquad \text{(Eq. 1.4)}$$

$$A_f = 10^{\sqrt{\left(\frac{\sum (\log(X\ pred./X\ obs.))^2}{n}\right)}} \qquad \text{(Eq. 1.5)}$$

Where Xpred. represents the predicted values, Xobs. represents the observed values and n is the number of observations.

The B_f indicates if the model overestimates or underestimates in its predictions. Values of $B_f<1$ indicates that observed values are smaller than the predicted and values of $B_f>1$ indicates that the observed values are bigger than the predicted. In the case of the perfect fit of the data, B_f is equal to 1. Big values of A_f indicates less accuracy in the predictions while, when the correlation between observed and predicted values is perfect A_f becomes equal to 1. Ideally the value of A_f should be 1 but generally increases between 0.1 and 0.15 for each variable, included in the model, are accepted (i.e. A_f values between 1.2 and 1.3 are acceptable for a model which considers two or more variables) (Ross *et al.*, 2000).

To date one of the main examples of the application of modelling to the processing of the food industry is the design and optimization of heat processes.

1.4.2 Heat process optimization

The American canning industry began sometime after 1810 and is considered to be the origin of modern food technology. A greater understanding of the canning process was developed due to the findings of Louis Pasteur in 1865 and further advances in knowledge of microbial inactivation and vitamin degradation by heat during 1920s and 1940s. As mentioned previously, the primary objective of food processing is the production of safe products which will not pose a potential hazard to public health. Therefore, the first step in the optimisation of a process is to evaluate and characterise the effect of that process on the main target microorganisms present in the product to be treated (Stofors *et al.*, 1995).

Form the experiences of Bigelow and Esty in 1920 it was concluded that microbial inactivation by heat follows a first-order kinetic at a constant temperature. Although, the techniques have evolved during the years and other inactivation models have been reported to describe microbial inactivation by heat (Cebrián *et al.*, 2017), still nowadays the findings of Bigelow and Esty are being used in the food industry for the design of thermal processes. Due to the exponential reduction of microbial population by heat, when the logarithm of microbial count is represented *versus* treatment time a straight line is observed as shown in Figure 1.6.

This new knowledge gave rise to the concept of the D_T value, which can be defined as the time necessary, at a constant temperature, to reduce the microbial count by one Log_{10} cycle (i.e. to reduce the total microbial population by 90% or by a factor of 10). This parameter can be calculated as the negative inverse of the slope of the line which describes the microbial inactivation expressed as Log of microbial numbers *vs* treatment time. This concept could be considered as the primary model used to define thermal processes.

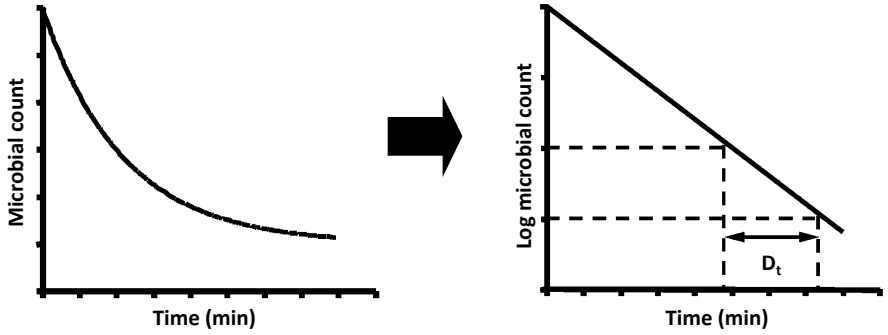


Figure 1.6: Effect of treatment time on the microbial count subjected to a heat treatment at a constant temperature. Source (Cebrián *et al.*, 2017).

In addition Bigelow (1921) demonstrated the exponential effect of the treatment temperature on the D_T value, which gave rise to the concept of the *z* value. This concept is defined as the required temperature rise (°C) to bring about a 1 log change in the D_T value (i.e. reduce or increase the D_T by 90% or by a factor of 10). Similar to the calculation of the D_T, *z* values can be calculated graphically as the inverse of the slope of the line correlating the Log_{10} of D_T values and the treatment temperature (Figure 1.7).

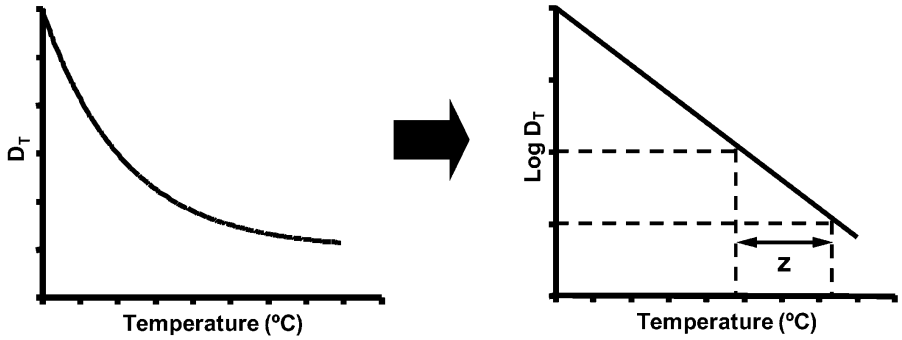


Figure 1.7: Effect of treatment temperature on the microbial inactivation rate subjected to a heat treatment. Source (Cebrián *et al.*, 2017).

The concept of the *z* value can be considered as a secondary model used to define a thermal process. But, contrary to the observations with D_T values, the concept of the *z* value has been proven to be a more robust and useful model to correlate the treatment temperature with the parameters of other primary models used to describe the heat inactivation curves, examples including the Weibull distribution (Fernández *et al.*, 2007; Hassani *et al.*, 2005) or the Geeraerd Equation (Arroyo *et al.*, 2011).

D_T and z are correlated by Equation 1.6 (This Equation could be considered as a tertiary model used to calculate the impact of a thermal process in terms of microbial inactivation), which allows the definition of the D_T values at any treatment temperature (T) by knowing a reference D_T of the target microorganism and also its z value. These concepts form the basis of classical thermobacteriology and forms basis of heat process optimization.

$$D_T = D_{Tref} 10^{(T_{ref} - T)/z} \quad \text{(Eq. 1.6)}$$

Similar to what has been described for microbial inactivation, the kinetics of heat induced chemical reactions in foods can be studied and modelled. However, the key reaction which defines the quality of the product varies from product to product and from consumer to consumer. For example with cooked beef, tenderness is generally the key factor affecting consumer acceptance but consumers' attitude vary in terms of the choice of well-done or rare steak. For milk, colour and nutritional value may be key factors while in juices the flavour and enzyme inactivation may be key reactions. Additionally, a general model to describe these degradation kinetics (i.e. primary model) does not exist and different order of reactions has been observed (i.e. zero-order, first-order, pseudo-first-order, second-order kinetics) (Ling *et al.*, 2015).

Despite the differences in primary models between microbial inactivation and quality degradation, process optimization is possible because the effect of treatment temperature remains constant (i.e. primary model parameters vary exponentially due to temperature changes). So an equivalent to z value, known as Q_{10} value, can be defined. Conceptually, this parameter relates to quality changes and has the same meaning as z value for microbial inactivation; though Q_{10} values for chemical reactions are generally higher than those reported for z, (e.g. 10ºC for microbial inactivation *vs* 30ºC for chemical reactions) which is the basis of thermal

process optimization. This difference in z values between chemical and microbial inactivation is exploited in UHT processing where a 30°C increase in processing temperature may produce a 1000 fold increase in the rate of microbial inactivation but only a 10 fold change in the rate of chemical reactions.

The development of kinetic models for both, microbial inactivation and quality degradation are two of the main components in the optimization of heat processes. However, the applicability of these tertiary models is limited to treatments performed at constant temperatures, while in most heat processes within the food industry the product temperature is not constant but varies throughout the process (Holdsworth and Simpson, 2007). Therefore, the last required information, in the optimization of a heat process is the characterization of the time temperature profile of the product in order to integrate the combined effect of all temperatures attained during the treatment time.

The time temperature profile of a foodstuff during heat processing consists of, in its simplest form, a heating, holding and cooling cycle and can be determined experimentally, by placing thermocouples within a product, or can be estimated mathematically by predictive models (Stoforos, 1995). The advantage of the experimental procedure is that the results obtained will be more robust, for subsequent calculations, but in contrast, the applicability of the data is solely focused on the specific conditions used to obtain the data. Additionally, data collection is a costly and time consuming procedure. On the other side the use of mathematical models to predict the time temperature profile during processing has the advantage that it allows the definition and evaluation of different process variables (the number and nature of those variables is dependent on the type of model used) but also the relatively reduced amount of experimental data required to generate the model. By contrast, the conclusions extracted have higher uncertainty.

Once all the required information has been obtained the next step in heat process optimization is to calculate the process conditions in terms of time and temperature to achieve a certain limit of microbial inactivation and quality characteristics (Figure 1.4). To do this, two equivalent parameters are generally described: F_T^z for microbial inactivation and C_T^z for quality changes (Holdsworth and Simpson, 2007). Conceptually, *F* and *C* values are similar than D_T but, these two values represent the equivalent treatment time at a reference temperature to reach a certain level of microbial inactivation or quality level respectively. Results calculated for a process, in terms of *C* value, are generally compared to a target *F* value which represents the time required to achieve a target Log_{10} reduction of the most pertinent pathogen in the product. Although, the C_T^z value is also critical in terms of product quality and nutritional value it is less frequently considered, as the main focus is product safety. These two values represent the whole thermal effect accrued during the heat process.

Figure 1.8 shows as example the theoretical graph for the optimization of a thermal process related to microbial inactivation (i.e. F_T^z) and two different quality parameters (i.e. C_T^z values). In the graph, the black line represents the minimum equivalent time-temperature combinations necessary in order to achieve an adequate level of microbial inactivation. The blue line represents the minimum equivalent time-temperature combinations required in order to achieve a minimum quality target and the red line represents the maximum tolerable equivalent time-temperature combinations in order to keep a specific quality attribute under a defined limit. Based on Figure 1.8, the optimum conditions in this example are those which accomplish all product requirements, represented by the green area. In the example, only one microbiological and two quality variables have been considered, but in this type of optimization graph it is possible to include even more lines representing other variables such as enzymatic inactivation or even the economic cost of the process.

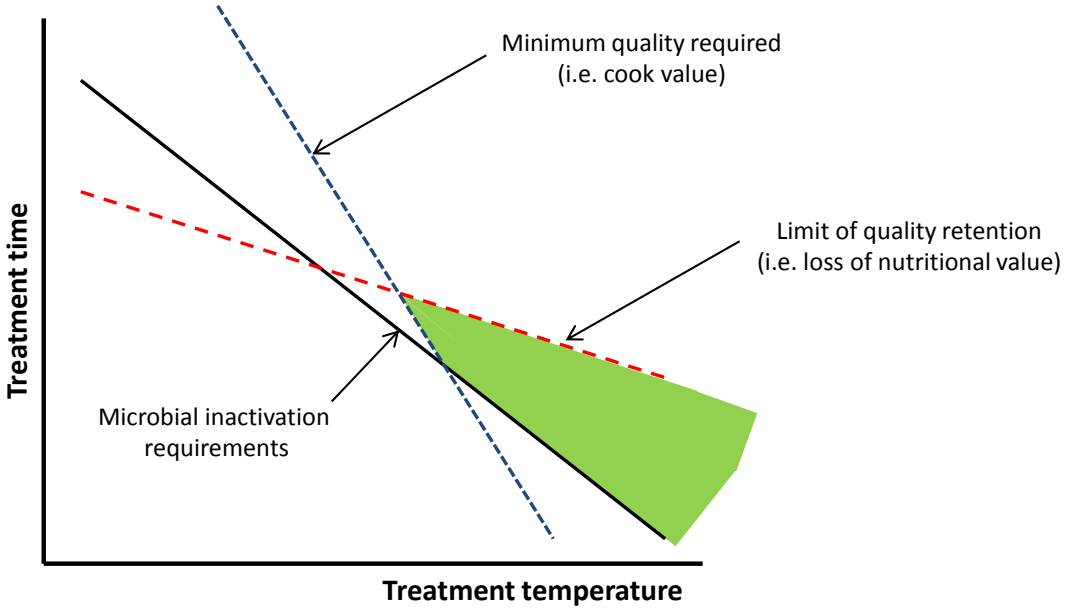


Figure 1.8: Theoretical lines for the minimum microbial inactivation required (black line), the maximum quality degradation accepted (red line) and the minimum quality required (blue line) depending on the treatment temperature. The green area represents the time-temperature combinations which accomplish all product requirements (safety and quality in this example).

1.5 EDIBLE CRAB-BROWN CRAB (*CANCER PAGURUS*)

The development of the present PhD thesis is part of a larger project focused on Irish brown crab (*Cancer pagurus*). Therefore, the following section describes the particular characteristics of this foodstuff and its production process.

Cancer pagurus, commonly known as edible crab or brown crab, is a species of crab found in the North Sea, North Atlantic Ocean and in the Mediterranean Sea. It is a robust crab of a reddish-brown colour, having an oval carapace with a characteristic "pie crust" edge and black tips to the claws (Figure 1.9). *C. pagurus* is a nocturnal predator, targeting a range of molluscs and crustaceans. The maximum age of brown crab is around 20 years and the maximum sizes registered for the carapace width are 265mm for males and 225mm for females and the maximum registered weight is 3kg. For fishing the minimum size accepted, of this crab, is 130-140mm depending on the region it is captured and measured perpendicular to the anteroposterior midline of the carapace (Council regulation (EC) No.

850/98, as ammended). This corresponds to a weight of about 350 grams, and it takes about 5 or 6 years for crab larvae to reach this size.

Figure 1.9: Female exemplar of edible crab (*Cancer pagurus*)

Like all decapods an edible crab has ten appendages attached to its thorax, the first being a pair of claws. In case of edible crab both appendages and carapace contain edible tissues with commercial interest. Muscle meat or white meat, is mainly located in claws and legs but also some of this meat type is located in the thorax. Brown meat is obtained exclusively from the carapace of the female exemplars and mainly consists of hepatopancreas and gonads. The composition of each meat type varies between crabs but also depends on the maturity stage, season and fishing area. In case of white meat the total fat content ranges from 0.2 to 0.6% while the protein content varies between 16.4 and 20.5%. On the other side, in brown meat the total fat varied between 3.1-3.6% and protein content from 19.2-25.5%; while for hepatopancreas the fat content ranges between 10.0 and 16.6% and protein content ranges from 9.4 to 15.8% (Barrento *et al.*, 2009b, 2010a).

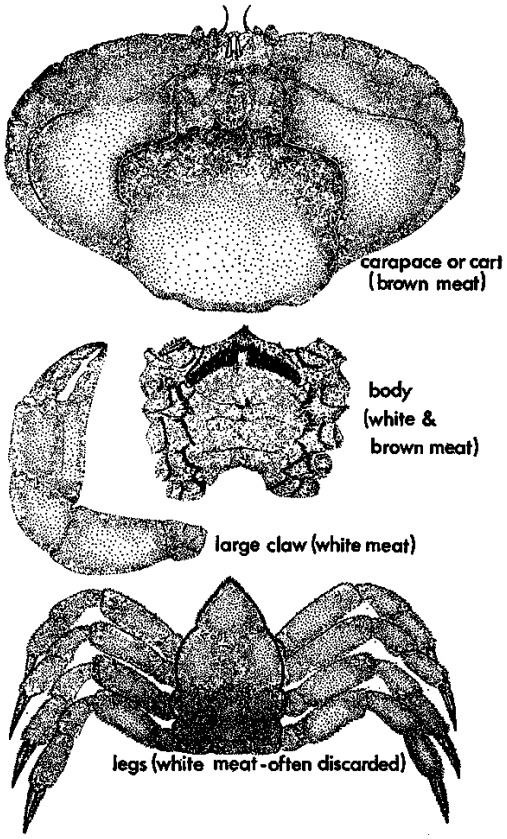


Figure 1.10: Edible parts of brown crab (*Cancer Pagurus*). (Edwards and Early, 1980; FAO, 1980)

Due to its composition, crustaceans in general and brown crab in particular are considered as a healthy choice for consumers due to their high-quality protein, amino acid composition (Gökoðlu and Yerlikaya, 2003; Maulvault *et al*., 2012) and their low saturated fat content (Barrento *et al*., 2008b, 2009b). Although, most of these studies consider that the benefits of crustaceans are related to white meat only, in case of edible crab, also brown meat is highly appreciated and consumed especially in southern European countries such as Spain and Portugal (Bolam *et al*., 2016; Maulvault *et al*., 2013). For this reason, both male and female crabs are exploited commercially.

The main fishing areas of brown crab are the western coasts of the north Atlantic, from Scandinavia to Morocco, particularly around the British and Irish coasts. Generally adult crabs are mainly fished in depths of less than 50m, and waters at temperatures from 5 to 16°C. Though, the fishing

season spans all year long, the peak season in the brown crab fishery is from April to November when the meat yield is the highest and mature females are targeted. As mentioned before, due to its high nutritional and organoleptic quality, consumers have an increasing interest in crustaceans over the last decades. More specifically in case of edible crab this has manifested itself as an increase in European landings.

Figure 1.11 shows the evolution of landings of edible crab in Europe over the last 65 years. As can be observed a continuous increase in landings of this crustacean has occurred continuously over this period which has increased from 11,000 tonnes in the 50s to over 48,000 tonnes in 2013, giving a total value of in excess of 57 million euros. To date the three main European producers of Edible crab are the United Kingdom, Ireland and France. However, the Netherlands, Spain and Portugal also have landed a certain amount of this product though volumes are at a lower level. Table 1.3 shows the production of the main producers and the value in Euros of Edible crab in 2013 (Eurostat, 2018).

Table 1.3: Volume and value of landings of edible crab in the main producers countries in 2013.

Country	Tonnes of edible crab	Value (€)
United Kingdom	25,881	39,417,302
Rep. of Ireland	5,745	8,575,990
France	5,780	8,246,373
Netherlands	711	582,528
Spain	85	190,070
Portugal	1	2,562
Total	38,203	57,014,825

Source Eurostat 2018

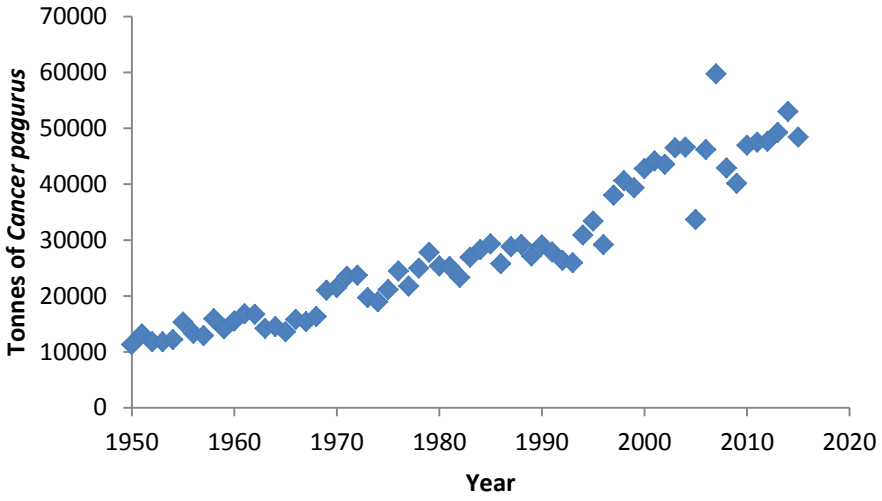


Figure 1.11: Volume of landings (Tonnes) of edible crab (*Cancer pagurus*) in Europe over the last 65 years. Source: Eurostat 2018.

The fishing of edible crab in Ireland started to develop in the 1960s, with the first processing plants based in Donegal in 1968-1969, though at this stage the landings of this crustacean were small (BIM, 2005). However, the real development of edible crab production in Ireland started in 1980s and over the last three decades has suffered a spectacular increase up to 2004, when a total of 13,690 tonnes of edible crab landings were registered (Figure 1.12) (Eurostat 2018). From 2004 to date a decrease in landings has been registered, although Ireland is still one of the top three producers of this crustacean worldwide with productions ranging between 5,000 and 8,000 tonnes per year, which has a net value of over 8 million Euro. In terms of production edible crab is the second most exploited crustacean species in Ireland. These data shows the importance of edible crab as a valuable commodity for the Irish fishing industry (BIM, 2014, 2017b).

The latest Figures show that of the total production of edible crab in Ireland, 42% is sold as fresh live crab and the remaining 58% is sold in various ready-to-eat formats (Eurostat, 2018). From processed crab only 1% is presented as transformed product while the major part is presented as cooked whole crab, cooked claws or cooked meat, either frozen or chilled (Eurostat, 2018). Either way, from the total Irish production of edible

crab only 16% is commercialized in Ireland while the other 84% is exported. The main three importers of Irish edible crab are France (49%), Spain (18%) and China (9%) while the remaining 24% is exported to other countries (BIM, 2017b).

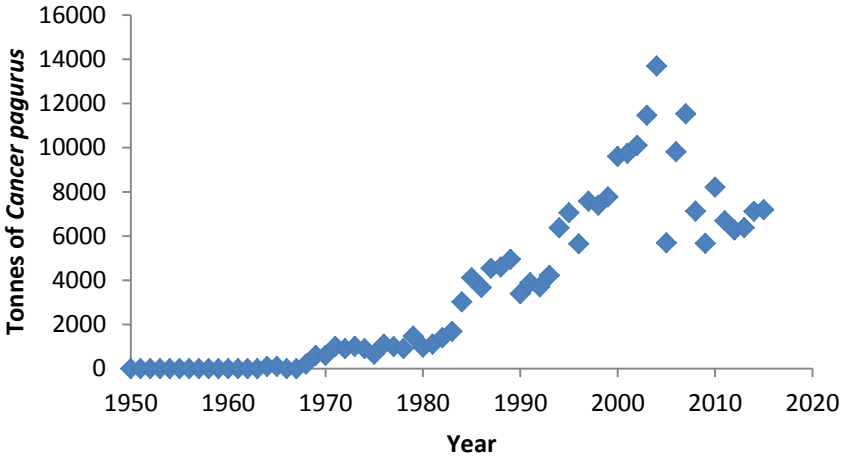


Figure 1.12: Volume of landings (Tonnes) of edible crab (*Cancer pagurus*) in Republic of Ireland over the last 65 years. Source Eurostat, 2018.

As mentioned above, most of the Irish production of edible crab is exported. The main challenge during export is to reduce losses associated with transportation. In case of the exports of edible crab as fresh live product, losses of up to a 50% can occur due to crab death or damage during transportation, while the export in a ready-to-eat format avoids such losses during transportation and also adds value to the final product (Barrento, *et al.*, 2008a, 2010b; Uglow, *et al.*, 1986).

Figure 1.13 shows a flow diagram including the main steps and processes required for the production of ready-to-eat cooked whole crab and different crab products. The figure also includes a previous step, the transport of product to the processing plant. This has been included because, as mentioned before, if the transportation of live crabs is not carried out properly, this could incur losses, which in turn will determine the final destination of the crabs (i.e. whole cooked, claws only or meat

separate) and is the main reason why an initial classification at the beginning of the process must be performed.

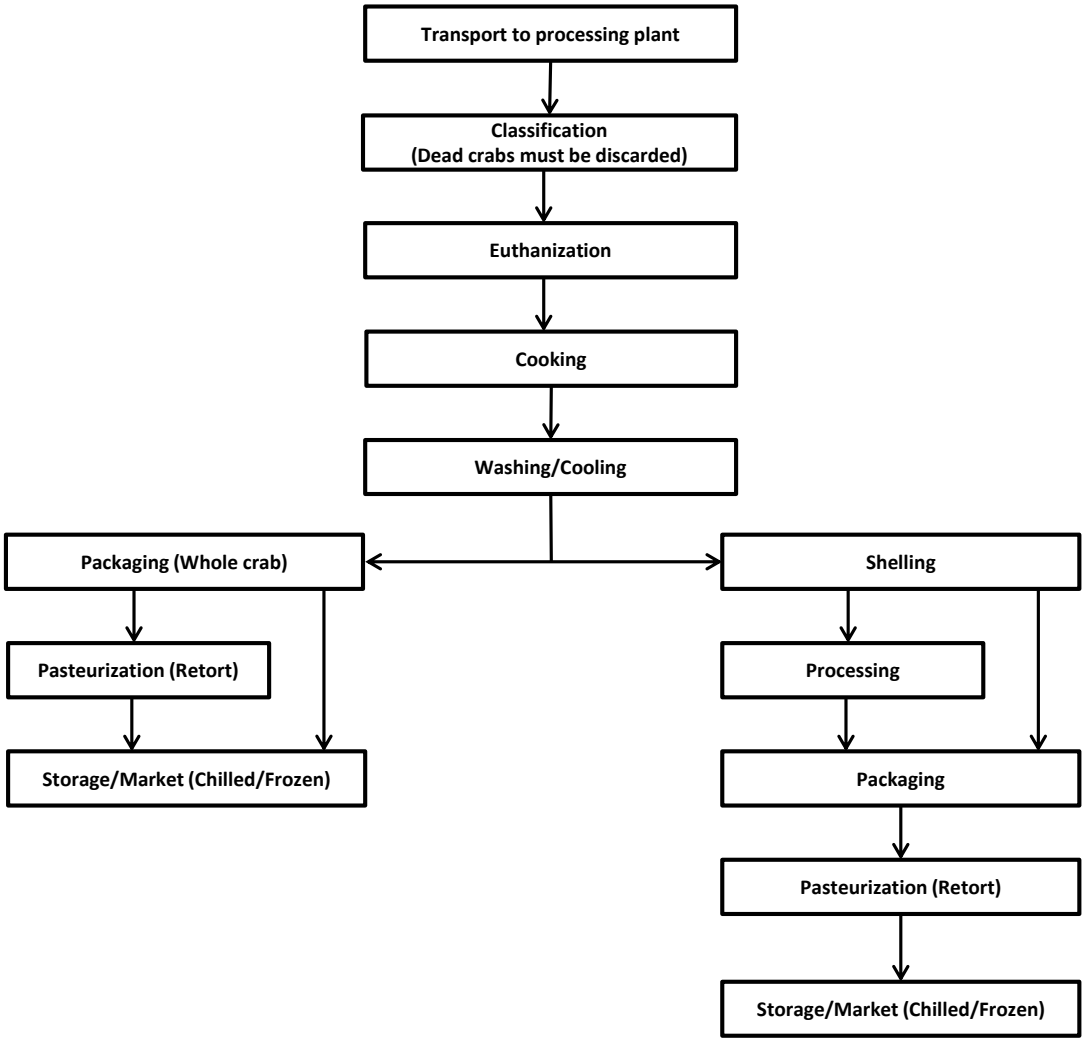


Figure 1.13: Flow diagram of the main steps followed for the production of ready-to-eat edible crab and crab products.

After classification and prior to processing, crabs are euthanized. This operation is to avoid crabs losing their claws but also to comply with ethical principles (Baker, 1955). The most common practice for crabs slaughter in the industry is by thermal shock with chilled fresh water (death occurs after 3-5 hours at 10 °C) or warm water (38-49°C crabs death occurs in about 30 minutes) (Edwards, 1979). More recently, electrical stunning has been also

suggested as a suitable alternative for crab euthanization, which in turn would improve the meat quality (Roth and Øines, 2010).

Immediately after sacrifice crabs are cooked. This is an essential step to develop the specific organoleptic attributes but also to facilitate an adequate detachment of edible tissues from the cell (Codex Alimentarius, 1983; Tinker and Learson, 1972). The most common cooking methods currently used in the crab industry are steam cooking or immersion in boiling water. Steam cooking is generally applied under pressure in batch in retorts at temperatures of about 105°C. Cooking by immersion is performed in vessels with water at 98-100°C with 2-3% added salt. When comparing both cooking types, immersion represents a lower cost of installation, increases the meat yield and removes undesirable compounds (Baxter and Skonberg, 2008; Hong *et al.*, 1993). On the other side, steam cooking would improve organoleptic properties and the retention of characteristic aromatic compounds, but in turn would increase the production of amines, generally associated with off-flavours (Dima *et al.*, 2012; Ward *et al.*, 1983). Though, these are the most commonly used conditions for cooking crabs in the industry a wide range of cooking conditions, including temperatures ranging from 70 to 120°C and times from 3 to 25 minutes, have been evaluated at lab scale on different quality attributes for different crab species (Anacleto *et al.*, 2011; Dima *et al.*, 2012; Lorentz *et al.*, 2014; Maulvault *et al.*, 2012; Ulmer, 1964; Ward *et al.*, 1983).

Although certain microbial inactivation is achieved during the initial cooking the primary objective of this step is the coagulation of proteins and the development of characteristic flavours. In addition, the subsequent steps of washing/cooling and manipulation can introduce potential recontamination which in turn could suppose a risk for public health.

After the first cooking a washing/cooling step is performed. Washing is a very important step since it is the point at which the cook loss deposits

are removed. During cooking a series of compounds are released from the crab, mainly proteins. These released compounds attach to the carapace during cooking, as shown in Figure 1.14, which makes it essential to wash the crabs prior packaging. For this reason the first part of cooling is carried out using water jets, for cleaning and finalised in a cold room. The entire process has a duration of approximately 3-4 hours. On the other side, cooling is very important because allows the completion of meat coagulation, which facilitates its removal from the carapace and avoids losses (Edwards, 2001).

Figure 1.14: Example of crabs during cooking (left) and immediately after cooking with no washing (right).

Immediately after cooling crabs, which will be sold as ready-to-eat cooked whole are packaged for sale (premium products) or frozen. Most commonly, a second heat treatment is applied, to ensure the microbiological safety of the product. Crabs which are destined for manufacture of crab based products or packaged crab meat are transferred to a meat picking area. At this stage, white meat, from the body claws and legs and brown meat, from the body of female crabs, is removed. The main inconvenience of this process is that the main procedure to remove the meat is hand picking, although compressed air jets are sometimes used to assist removal of meat from the legs (Edwards, 2010). Either way this step takes up a significant portion of processing time. Finally, when the various

types of meat have been removed, they are packed together or separately, depending on the intended final destination and pasteurised. In more elaborated products, which require a greater level of processing (e.g. pate, chowder, bouillion, etc.) a second pasteurization is mandatory due to the high risk of contamination/recontamination (Adams, 2000; Dima *et al.*, 2015; Ghazala and Trenholm, 1996).

Due to the nature of crab edible tissues (high water activity, moderate salinity and moderate pH) it provides an excellent environment for the development of various pathogens, which can be associated with foodborne diseases due to improperly processed/preserved/stored low-acid chilled foods. According to the U.S. Department of Health and Human Services of the Food and Drug Administration (FDA, 2011) the most important foodborne non-spore-forming pathogens, associated with these kinds of products are *Listeria monocytogenes, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio cholerae, Escherichia coli, Salmonella* spp.*, Shigella* spp.*, Staphylococcus aureus, Campylobacter jejuni* and *Yersinia enterocolitica* (FDA, 2011; Gram and Huss, 1996).

For all of these vegetative bacteria, *Listeria monocytogenes* is considered as the target microorganisms in pasteurisation treatments, since it is generally considered to be the most heat resistant vegetative cell and is able to multiply at chill temperatures (FDA, 2011). *L. monocytogenes* may be transmitted to humans via contaminated foods (Brackett and Beuchat, 1990). Its development is dependent on intrinsic factors such as the product pH, water activity and salt content and extrinsic factors such as cooking/storage temperatures and gas atmosphere and packaging type (FSAI, 2005). *L. monocytogenes* is able to grow in a range of pH from 4.2 to 9.5 and at temperatures from -1.5°C to 45°C and at a_w of up to 0.9. It is documented that they are able to survive in environments down to -18°C and pH values as low as 4.3 (FSAI, 2005).

Pasteurized crabmeat has been shown to be an adequate support for the growth of *L. monocytogenes* at 1°C and temperature rises up to 5°C dramatically increase its growth rate (González *et al*., 2013). Multiple potential reasons for the presence of *L. monocytogenes* have been identified, insufficient thermal processing, cross contamination from raw crab, factory environment or food-handlers. According to Regulation (EC) No 2073/2005 ready-to-eat foods able to support the growth of *L. monocytogenes*, such as ready-to-eat edible crab and derived products, must comply with the following food safety criterion: absence of the pathogen in 25 g of product before the food has left the immediate control of the food business operator (FBO) who has produced it. However, if the FBO is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life, then this latter criterion would apply (100 cfu/g throughout the shelf-life).

For pasteurisation treatments, achieving a six logarithmic reduction of the target microorganism is accepted as an adequate level of inactivation to avoid foodborne disease (FDA, 2011). In the case of *L. monocytogenes*, its prevalence in raw seafood is estimated as 1 to 10^3 colony forming units per gram (CFU/g) in up to 8% of these products. Only 1% of these products are contaminated with levels greater than 10^3 CFU/g while levels greater than 10^6 CFU/g are not observed. However, for the majority of seafood (91%), the contamination level of *L. monocytogenes* is generally lower than 1 CFU/g (FDA, 2011). If this pathogen is considered as the target microorganisms for pasteurization the recommended treatment is a $F_{70°C}^{7.5°C}$ of two minutes or an equivalent treatment, in order to achieve a proper inactivation level of *L. monocytogenes* i.e. in excess of 6 Logs (CCFRA, 1996; CFA, 2006; ECFF, 1996; FDA, 2011).

Additionally to the vegetative cells enumerated before, ready-to-eat crab based products and seafood in general, represent a serious hazard due

to the presence of pathogenic spore forming bacteria, namely: *Clostridium perfringens*, *Clostridium botulinum* and *Bacillus* spp. which are the main three spore forming bacteria associated with seafood products (FDA, 2011). Among these, group II (non-proteolytic) *Clostridium botulinum* types B, F and specially type E, are of particular concern to the seafood industry. *C. botulinum* non-proteolytic type E is known to be a naturally occurring marine organism which can also grow at refrigeration temperatures as low at 2°C (Corner *et al*., 1989) and it has been also associated with human botulism cases (Lindström *et al*., 2006). In addition, it has been reported that the prevalence of group II (non-proteolytic) *C. botulinum* type E in fish and other seafood may be as high as 40-70% (Baker, 1990; Huss *et al*., 1974; Johannsen, 1963). However, despite its high prevalence in fish and seafood, its counts in raw foods are typically not very high, varying from less than 1 spore/kg (Baker, 1990; Lindström *et al*., 2006; Tanasugarn, 2006) to 10^2 spores/kg (Hielm *et al*., 1998; Lindström *et al*., 2006). For all these reasons *C. botulinum* non-proteolytic type E is considered as a major concern when designing a pasteurization process for ready-to-eat seafood based product (FDA, 2011; Silva and Gibbs, 2010). Generally as it is considered for *L. monocytogenes,* a pasteurization process which ensures a 6 Log_{10} reduction of the population of spores of *C. botulinum* non-proteolytic type E is considered adequate for any of these product types (FDA, 2011).

Considering the thermal resistance of *C. botulinum* non-proteolytic type E the most recommended heat treatment to be applied is an $F_{90°C}^{7or10°C}$ of ten minutes, or equivalent considering z of 10°C for treatment temperatures ≥90°C or 7°C for treatment temperatures <90°C (CFA, 2006; ECFF, 2006; FDA, 2011). These treatments are recommended for all ready-to-eat refrigerated products packed in the absence of oxygen, including fish and fishery products. However, in case of crab meat-based products, more severe heat treatments have been proposed. It is known that the presence

of the enzyme lysozyme improves the recovery of heat-treated *Clostridium* spp. spores and therefore, a higher heat resistance is observed for this species (Peck and Fernandez, 1995; Scott and Bernard, 1982). Moreover, it has also been reported that this enzyme is present in fish and seafood at level of up to 200 µg/g (Lie *et al*., 1989; Lund and Notermans, 1992). For this reason, two more severe alternative treatments were proposed by the FDA for crab meat $F_{90^oC}^{9^oC}$ = 31 min (Blue crab, *Callinectes sapidus*) and $F_{90^oC}^{8.6^oC}$ = 57 min (Dungeness crab, *Metacarcinus magister*) to achieve a satisfactory reduction of *Clostridium botulinum* spores in crab meat (FDA, 2011; Peterson *et al*., 1997, 2002).

Additionally to the microbiological risks mentioned, crustacean consumption is also associated with other health risks due to high cholesterol levels in some edible parts, allergic reactions and contamination of toxic elements (Maulvault *et al*., 2012). Particularly, in edible crab high levels of Cadmium (Cd) have been reported, especially in brown meat (Barrento *et al*., 2009b, 2009c; Bolam *et al*., 2016; Maulvault *et al*., 2012, 2013). Noël *et al*. (2011) reported Cd levels from 0.78 to 27.6 mg/kg of wet weight (ww) and from 6.38 to 61.8 mg/kg (ww) in the brown meat of common crabs (*Cancer pagurus*), captured in open or coastal areas of the Atlantic North East respectively, landed in Ireland and United Kingdom. Also, Bolam *et al.* (2016) analysed Cd levels in the brown meat of *Cancer pagurus*, sampled throughout the UK, and observed Cd concentrations between 0.11 and 26 mg/kg (ww) and Maulvault *et al.* (2012) reported Cd concentrations between 5.0 to 8.4 mg/kg (ww) in the brown meat of crabs caught in the Scotland coasts. Besides, Wiech *et al.* (2017) reported Cd concentrations from 5.7 to 12 mg/kg (ww) after different processes and significantly higher Cd levels of up to 44 mg/kg (ww) in crabs from Northern Norway.

In the EU, the Cd content for crabs is only regulated for the white meat from crab appendages (claws and legs); with 0.5 mg/kg being the

maximum permitted limit (EU, 2006, 2011). However, several studies have warned of the risk of exceeding the total weekly intake of Cd set by EFSA (2009) (2.5 µg/kg body weight) in consumers from countries where brown meat is commonly consumed, such as in Spain and Portugal (Bolam *et al.*, 2016; Maulvault *et al.*, 2013; Noël *et al.*, 2011; Wiech *et al.*, 2017). The main challenge in this regard is that the processing techniques currently used in the crab industry hardly affect the original concentration of Cd present in the crab (Maulvault *et al.*, 2012; Wiech *et al.*, 2017), which is directly dependant on the fishing area where they originated from. There is not specific EU regulation regarding the Cd content in crab's brown meat, tough other countries such as China have started to regulate those. Therefore, in a near future Cd could represent a great issue for the edible crab processing industry.

Considering the background presented in this literature review regarding food preservation, process optimization and edible crab the main objectives of the present PhD Thesis could be summarize in:

1.6 OBJECTIVES

The main objectives of the present PhD Thesis were:

- **To evaluate the effect of the storage temperature on the shelf-life in raw crab, commercial crab products and ready-to-eat whole crab cooked under mild temperatures and packed under vacuum.**
- **To identify the main microorganisms present at the end of the microbiological shelf-life of raw crab, commercial crab products and ready-to-eat whole crab cooked under mild temperatures and packed under vacuum.**
- **To determine the thermal resistance of the main microorganisms present in raw crab, commercial crab**

products and ready-to-eat whole crab cooked under mild temperatures and packed under vacuum.

- **To evaluate the effectiveness of the heat treatments recommended by U.S. Food and Drug Administration for this type of products.**
- **To evaluate the potential advantages of the incorporation of ultrasound technology in the initial cooking step of edible crab to improve heat and mass transfer processes (e.g. reduce cooking time, cleaning and cadmium reduction).**
- **To optimise the second pasteurization step applied in the production of ready-to-eat edible crab using a quality index degradation kinetic approach.**
- **To evaluate the potential use of alternative technologies (i.e. mano-sonication, mano-thermo-sonication and ionizing radiation) to inactivate the main spore forming bacteria isolated from ready-to-eat edible crab.**

Chapter 2

Microbial characterization of edible crab (*Cancer pagurus*)

From this chapter two articles are in preparation for their submission.

Condón-Abanto, S., Lian F., Arroyo, C., Álvarez, I. and Lyng, J.G. Effect of storage temperature on microbial shelf-life and microbiological profiles of ready-to-eat Irish whole brown crab (*Cancer pagurus*). In preparation

Condón-Abanto, S., Pedrós-Garrido, S., Alvarez, I., Whyte, P., Lyng, J.G. and Arroyo, C. Isolation, identification and thermal resistance of the microbiota present in ready-to-eat brown crab (*Cancer pagurus*) meat. In preparation

2.1 ABSTRACT/RESUMEN

ABSTRACT

In the past, the brown crab (*Cancer pagurus*) market mainly consisted of fresh live product. However, the opening of new markets (e.g. China and USA) has now created a demand for ready-to eat products. This in turn creates an opportunity to add value but also affords manufacturers the chance to improve existing commercial processes (e.g. use of milder heat treatments to produce a higher quality product). However, an important consideration in all of this is product shelf-life following these new processes and the microbiota present in these types of product. For this reason, one of the aims of the present study was to assess the effect of storage temperature on the shelf-life and microbiological profiles of ready-to-eat whole crab cooked under mild temperatures and packed under vacuum. A tertiary model based on the modified Gompertz Equation and the root square model, for mesophilic bacterial growth at different storage temperatures was developed. The end of shelf-life was considered as the point where the mesophilic bacterial load reached 6 Log_{10}-cycles. Once the shelf-life was determined and the effect of the storage temperature was quantified, a deeper study of the natural microbiota present in raw and cooked brown crab meat and in some commercial brown crab products was performed. Finally, the thermal resistance of the main microorganisms identified in crab meat samples were determined, in terms of D_T and z values and the effectiveness of the recommended heat treatments by U.S. Food and Drug Administration for this type of products was assessed.

Shelf-lifes of 362, 257, 204 and 145 hours for white meat and 492, 340, 265 and 183 hours in case of brown meat, were calculated under storage temperatures of 2, 5, 7 and 10°C respectively. A linear relationship was found between the storage temperature (°C) and the Log_{10} of shelf-life regardless of the meat type, though white meat was found as the limiting component which defined the shelf-life of ready-to-eat brown crab. In all cases a bigger heterogeneity of microbiota was detected in the case of brown meat. In raw

meat the main microbial groups detected were *Pseudomonas* spp., then *Bacillus* spp. which was present in a similar concentration to *Staphylococcus* spp*.* In cooked crab the microbial groups present in the greatest proportion were *Bacillus* spp. and *Staphylococcus* spp*.,* although their proportions varied depending on the storage temperature. The 18 bacterial genus and 31 species isolated were identified by 16S rRNA sequencing. *Carnobacterium divergens* and *Shewanella baltica* were the most frequently isolated non-spore forming species while *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* were the most frequently detected spore forming bacteria. In the thermal resistance characterization study, *Kocuria atrinae* showed the highest level of resistance among all vegetative cells, though the recommended treatment for *Listeria monocytogenes* ($F_{70^oC}^{7.5^oC}$ = 2 min) proved to be effective to inactivate this microorganism. For spore forming species, the most severe treatment recommended to inactivate *Clostridium botulinum* ($F_{90^oC}^{8.6^oC}$ = 57 min in Dungeness crab (*Metacarcinus magister*)), proved to be sufficient to achieve a 6 Log_{10} reduction of *B. mycoides* and *P. psychrodurans* but not *B. weihenstephanensis.* Overall, this research provides valuable information necessary to design processes for the production of safe cooked brown crab products and highlights the importance of *Bacillus* spp. in this crustacean.

RESUMEN

Tradicionalmente el mercado del buey de mar (*Cancer pagurus*) ha consistido en un producto fresco vivo. Sin embargo, la aparición de nuevos mercados (como por ejemplo China y Estados Unidos) ha creado una demanda de este producto listo para el consumo. Esta tendencia ha creado la oportunidad de añadir valor al producto y ofrece a los productores la oportunidad a los productores de mejorar los procesos ya existentes (como por ejemplo el uso de bajas temperaturas de cocinado). Sin embargo, la creación de este nuevo tipo de productos requiere evaluar tanto su vida útil como la flora bacteriana presente en ellos. Debido a esto, uno de los objetivos de este estudio fue evaluar el efecto de la temperatura de almacenamiento en la vida útil y el perfil microbiológico del buey de mar listo para comer, cocinado a baja temperatura y envasado al vacío. Para ello, se desarrolló un modelo terciario basado en la ecuación modificada de Gompertz y el modelo de la raíz cuadrada, para describir el crecimiento de las bacterias mesófilas durante el almacenamiento del buey de mar a distintas temperaturas. Considerando que el final de la vida útil de producto se alcanzaba cuando el recuento de mesófilos alcanzaba un valor de 6 ciclos logarítmicos. Una vez definida la vida útil del producto y cuantificado el efecto de la temperatura de almacenamiento sobre esta, se realizó un estudio en profundidad de la flora microbiana presente en el buey de mar crudo, cocinado a baja temperatura y de diferentes productos comercializados a base de cangrejo. Finalmente, se determinó la resistencia al calor (utilizando los valores D_T y z) de las principales bacterias aisladas e identificadas de las diferentes muestras de cangrejo y se evaluó la efectividad de los tratamientos de pasteurización recomendados por la *Food and Drug Administration* de los EE.UU. para este tipo de productos.

Se determinó una vida útil de 362, 257, 204 and 145 horas para la carne blanca y de 492, 340, 265 y 183 horas para la carne marrón para unas temperaturas de almacenamiento de 2, 5, 7 y 10°C respectivamente. El logaritmo de la vida útil se correlacionó linealmente con la temperatura de

almacenamiento independientemente del tipo de carne, aunque la carne blanca mostró un deterioro más rápido por lo que fue el factor limitante para definir la vida útil del buey de mar cocinado a baja temperatura listo para el consumo. En todos los casos, se observó una mayor heterogeneidad en la flora bacteriana presente en la carne marrón. En las carnes de cangrejo crudas, los grupos bacterianos identificados en mayor proporción fueron *Pseudomonas* spp., *Bacillus* spp. y *Staphylococcus* spp. En la carne de cangrejo cocinada, los principales grupos bacterianos identificados fueron *Bacillus* spp. y *Staphylococcus* spp., aunque la proporción en la que se detectaron vario dependiendo de la temperatura de almacenamiento. De todas las colonias aisladas, se identificaron 18 géneros y 31 especies bacterianas diferentes utilizando la secuenciación del ARNr 16S. De los identificados, *Carnobacterium divergens* y *Shewanella baltica* fueron las bacterias no esporuladas más frecuentes y *Bacillus mycoides*, *Bacillus weihenstephanensis* y *Psychrobacillus psychrodurans* las más frecuentes de las bacterias esporuladas. En el estudio de termorresistencia, *Kocuria atrinae* fue identificada como la bacteria no esporulada más resistente al calor de las aisladas del cangrejo, y se demostró que el tratamiento térmico recomendado para inactivar *Listeria monocytogenes* ($F_{70^oC}^{7.5^oC}$ = 2 min) es suficiente para alcanzar niveles de inactivación adecuados de esta bacteria. En el caso de las bacterias esporuladas se demostroó que el tratamiento térmico más severo recomendado para inactivar *Clostridium botulinum* ($F_{90^oC}^{8.6^oC}$ = 57 min en (*Metacarcinus magister*)) es efectivo para inactivar 6 ciclos logarítmicos de *B. mycoides* y *P. psychrodurans* pero no de *B. weihenstephanensis*. Este estudio aporta información valiosa para el diseño de procesos de producción, microbiológicamente seguros, para productos de buey de mar cocinados y recalca la importancia de *Bacillus* spp. en este tipo de productos.

2.2 INTRODUCTION

Crustacean products are widely appreciated worldwide for their taste but also as a good source of valuable nutrients and for their low saturated fat content (Barrento *et al.*, 2009a). In particular, brown crab (*Cancer pagurus*) is one of the most commonly consumed crustaceans in southern European countries (Eurostat, 2009), but more recently the opening of new markets such as the United States and China (Edwards and Early, 2001) has lead to an increase in landings of these crustaceans in the last years (FAO, 2015). Traditionally the market for crustaceans has been dominated by fresh live products. However, the increase in demand of brown crab has opened a new market of ready-to-eat (RTE) products, from whole cooked crab to prepared meals based on these crustaceans. Over the last 30 years, landings of brown crab have doubled in Europe while Ireland has experienced a ten-fold increase making this country one of the top three producers of brown crab in Europe (FAO, 2011-2017). Two-thirds of the Irish crab landings are exported from which 58% is exported as processed products (Eurostat, 2009). This method of export allows the extension of shelf-life but also avoids loses associated with the transport of fresh live crabs (Barrento *et al.*, 2008a; Uglow *et al.*, 1986).

In Ireland, the production of ready-to-eat brown crab meat products generally involves a first cooking step followed by washing/cooling, meat extraction and a final second 'in-pack' pasteurization step (Edwards and Early, 2001). However, harmonized process conditions among producers do not exist, as they use their own processing protocols in order to achieve the minimum criteria set by the US Food and Drug Administration (FDA) for the control of *Clostridium botulinum* (non-proteolytic type E) of 10 minutes at 90°C or *Listeria monocytogenes* of 2 minutes at 70°C (FDA, 2011). This lack of standardisation can result in variability among what should be reasonably similar products from different processors. Additionally, the natural bacterial population present in brown crab and ready-to-eat brown crab products have not been previously investigated. Also, another challenge of major concern is that this type of

minimally processed products are potentially a new niche for heat resistant microorganisms, such as psychrotrophic spore forming bacteria, which could have negative effects on their microbiological safety and quality (Stenfors and Granum, 2001). As a result, this knowledge gap limits the ability of processors to identify optimal conditions to control microbial hazards while maintaining satisfactory organoleptic properties of brown crab and derivate products.

Ii is widely recognised that microbial growth is the major cause of food spoilage; more specifically the growth of specific microorganisms associated with the raw material known as specific spoilage organisms or SSO (Gram and Dalgaard, 2000). For fish and seafood a number of these SSO have been identified and characterized (Gram and Huss, 1996; Gram and Dalgaard, 2000; Gram *et at*., 2002; Jay *et al*., 2000). However, this scientific knowledge is based in fresh fish and seafood mainly and scarce or outdated information exists about the SSO in processed shellfish products. The situation is even more limited in case of RTE brown crab (Faghri *et al*., 1984; Lee and Pfeifer, 1975; Linton *et al*., 2003, Ward *et al*., 1977). Predictive microbiology is a very useful tool widely used to determine and characterize microbial growth and inactivation and to define the shelf-life of foodstuffs. However, the predictive microbiology software available is also limited in terms of the number of microbial species and strains it can model (Dabadé *et al*., 2015; Dalgaard *et al*., 1997; Koutsoumanis *et al*., 2006).

Due to the limited amount of information on SSO regarding RTE brown crab, one of the objectives of this research was to evaluate the natural microbiota present in Irish brown crab and to build a mathematical Equation to predict the shelf-life of RTE brown crab at different temperatures, based on total viable counts. A characterization of the main microbial groups present in cooked crabs at the end of shelf-life and the possible effects of storage temperature on these microbial groups were also assessed. Once the shelf-life was determined the next objective was to identify the bacteria isolated from the various samples, at the end of their shelf-life, using 16S rRNA sequencing and finally to characterize the thermal resistance (D_T and z values) of the main

bacteria isolated. This data was then used to evaluate the effectiveness of the reference heat treatments for *L. monocytogenes* and *C. botulinum* non-proteolytic type Eas recommended by the USDA and FDA for this type of products.

2.3 MATERIAL AND METHODS

2.3.1 Raw material

In this research female brown crabs (*Cancer pagurus*) landed in the coasts of Ireland in winter 2015 with weights ranging from 400 to 600g were used as raw material. Crabs were obtained from a local fishmonger and maintained at 4ºC in dry conditions for a maximum of 48h, prior to analysis as raw products. In case of lab-cooked samples, immediately after sacrifice crabs were cooked in a thermostatic bath (Guyson mod. KS MK3 525, North Yorkshire, UK) at 75°C for 45 min. In a study presented later in Chapter 3 it is demonstrated that this cooking process (75°C for 45 min) achieved a $F_{70^oC}^{7.5^oC}$ of 2 min in the crab cold-spot, which corresponds to the FDA recommended heat treatment for ready-to-eat seafood products considering *L. monocytogenes* as the target microorganism (FDA, 2011). Additionally, raw crab meat and six different commercial crab meat products obtained in different supermarkets in Dublin during February and March of 2015 were also used in this study. The characteristics of these samples are described in Table 2.1.

Table 2.1: Summary of commercial crab products evaluated in this study

Sample code	Product type	Packaging	Shelf-life (days)
A	Picked ready-to-eat white meat	modified atmosphere	30
B	Ready-to-eat whole claws	vacuum	30
C	Picked ready-to-eat white meat	vacuum	30
D	Picked ready-to-eat white meat	vacuum	30
E	Picked ready-to-eat white meat	vacuum	30
F	Picked ready-to-eat white meat	vacuum	30

2.3.2 Crab sample preparation

2.3.2.1 Microbiota in raw crab

For the study of the microbiota present in raw meat crab, a total of twelve crabs from three different batches were evaluated. Immediately after their euthanasia (Roth and Øines, 2010) brown meat (located inside crab's carapace) and white meat (located mainly in claws and legs) were carefully removed under aseptic conditions in a laminar flow cabinet (Faster, Mod. Bio 48. Ferrera, Italy). Aliquots of ten grams of each type of meat were prepared and used for microbiological analysis. Each crab was sampled at least in duplicate.

2.3.2.2 Microbial shelf-life experiments

For shelf-life experiments, batches of fifteen crabs were cooked at 75°C for 45 min and packed under vacuum. Different batches were stored at 2, 5, 7 and 10°C for up to a maximum of 30 days. During the shelf-life study, at preselected days, one sample from each storage temperature was taken from the fridge and both brown and white meats were picked under aseptic conditions in a laminar flow cabinet. Three samples of 10 grams each were prepared from each type of meat to perform the shelf-life experiments. The end of the shelf-life was considered when total viable mesophilic counts (TVC_m) reached 6 Log_{10} CFU/g (ICMSF, 1986).

2.3.2.3 Microbiota in lab-cooked and commercial crab products

For the study of the microbiota present in lab-cooked crab at the end of the shelf-life, twelve crabs in total from three different batches were used. Crabs were cooked as described in Section 3.3.1 in three different batches with 4 crabs per batch. After cooking, each batch was divided into two groups. One group was stored at 4°C for 12 days and the other group was stored at 10°C for 6 days. These storage periods correspond to the end of the shelf-life of the product calculated from the shelf-study results obtained in this chapter. For commercial crab products, commercially sourced samples were stored in a

regular refrigerator (Whirpool mod. WMT552) (7±2°C) up to the end of their shelf-life (date indicated in the label by the producer).

After storage, meats from lab-cooked crabs were extracted aseptically and aliquots of ten grams of each type of meat were prepared and used for microbiological analysis. Each sample was sampled at least in duplicate. In case of commercial samples, on the last day of their shelf-life, samples were opened and two aliquots of 10 grams each were prepared and used for microbial analysis.

2.3.3 Microbial analyses and growth media

For the microbiological analyses, samples were taken from the refrigerator on the last day of their shelf-life. Ten grams of crab meat of each sample were diluted in 90mL of Maximum Recovery Diluent (MRD) (Oxoid, Hampshire, UK) and mixed thoroughly for 2 min with a stomacher (model 400 circulator, Seward Stomacher, UK) at 300rpm. Subsequently, ten-fold dilution series were prepared in MRD and aliquots were plated on a range of different agars to evaluate the main microbial groups present in the samples. The agars used in this investigation are summarized in Table 2.2.

For the enumeration of colonies grown in/on the plates at least two replicates of each sample were performed, considering only the plates with counts between 10 and 300 CFU. Experiments were performed in triplicate on different working days.

Table 2. 2: Culture media and incubation conditions used during this investigation.

Agar	Brand	Bacterial Group	Incubation temp. (°C)	Incubation time (h)	Atmosphere	Observations
TSA	Oxoid	TVCm	25	48	Aerobic	+0.5% NaCl/ pour plate
TSA	Oxoid	TVCp	10	168	Aerobic	+0.5% NaCl/ pour plate
L.H.	recipe	TVCp	10	168-240	Aerobic	Pour plate
CFC	Oxoid	*Pseudomonas* spp.	24	48	Aerobic	Spread plate
VRBGA	Oxoid	*Enterobacteriaceae*	37	48	Aerobic	Double layer/pour plate
MRS	Oxoid	Lactic acid bacteria	25	72	Anaerobic	Pour plate
BP	LAB	*Staphylococcus* spp.	35	24	Aerobic	Spread plate
PEMBA	Oxoid	*Bacillus* spp.	37	24	Aerobic	Spread plate
Iron agar Lyngby	Oxoid	H_2S producing bacteria	30	24	Aerobic	Pour plate/ Black colonies
SPS	Sigma-Aldrich	*Clostridium* spp.	37	48	Anaerobic	Black colonies
Oxford	Oxoid	*Listeria* spp.	30	48	Aerobic	Whitish colonies/ Pagadala *et al.*, 2012

TSA, Tryptic Soy Agar; L.H, Long and Hammer's Agar; CFC, Cetrimide Fusidin Cephaloridine agar; VRBGA, Violet Red Bile Glucose Agar; MRS, Man Rogosa Sharpe agar; BP, Baird-Parker agar; PEMBA, Bacillus cereus agar base supplemented with polymyxin B; SPS Sulfite Polymyxin Sulfadizine agar. TVC_m, Total Viable Mesophilic Counts; TVC_p, Total Viable Psychrophilic Counts.

2.3.4 Isolation and identification

After enumeration, a number of well defined colonies were randomly selected from the different agar plates. Each colony was used to inoculate a test tube containing 5mL of Tryptic Soy Broth (TSB) (Oxoid) supplemented with 0.6% (w/v) of Yeast Extract (YE) (Oxoid) and 0.5% (w/v) of NaCl (TSB-supplemented) and incubated at 25°C under either aerobic or anaerobic conditions until turbidity was detected. Following incubation, a loopful from each tube was streaked on a plate of Tryptic Soy Agar (TSA) (Oxoid) supplemented with 0.6% (w/v) of Yeast Extract (YE) (Oxoid) and 0.5% (w/v) of NaCl (TSA-supplemented), and incubated for 24–72h at 25°C under aerobic or anaerobic conditions. A single colony was then used to inoculate test tubes containing 5mL of TSB-supplemented and re-incubated in similar conditions. Once turbidity was detected, 0.1 mL from the tubes were transferred into a 200mL flask containing 50mL of TSB-supplemented and incubated as before. Bacterial populations were examined periodically by microscopy using a Neubauer chamber. When bacterial cultures reached the stationary phase of growth (i.e. stable counts for 3 consecutive days), two stock cryovials were prepared, coded and stored at -80°C.

A total of 92 visually-distinct types of colonies, 28 from raw crab meat (both white and brown), 40 from cooked meats and 24 from commercial products were cultured for identification. For the identification work using 16S rRNA sequencing, bacterial populations were recovered by inoculating 5mL TSB-supplemented tubes with a loop-full from the cryovials and incubated at 25°C under either aerobic or anaerobic conditions until turbidity was detected. Growth was sub-cultured from each tube, by plating onto a TSA-supplemented media and incubated at 25°C under either aerobic or anaerobic conditions for 24-72h. A single colony was then transferred into a 5mL TSB-supplemented tube and incubated at 25°C under the conditions described previously. When turbidity was detected, 0.1mL was then transferred in to 50mL TSB-supplemented. Once the stationary phase was reached (using a Neubauer

chamber) two aliquots of 1mL were prepared in sterile Eppendorf tubes and a stock cryovial from each culture was prepared. One of the aliquots was stored at 4°C and the other one was used for 16S rRNA sequencing.

Samples for 16S rRNA sequencing were prepared as follows. A 1 mL aliquot of each bacterial culture was centrifuged at 10,000rpm for 5 min at 4°C in an Eppendorf centrifuge (model 5417 R, Eppendorf AG 22331, Hamburg, Germany). The supernatant was discarded and the pellet was re-suspended in sterile distilled water inside a laminar flow cabinet. This washing process was repeated twice. After washing, pellets were re-suspended in 500µL of lysis buffer (Fisher Scientific, New Hampshire, United States) and 200µL were transferred to a 96 deep-well plate and sealed. The lysed cells were sent under refrigeration to a commercial laboratory (Eurofins genomics Ltd.) for the sequencing. Sequences (forward and reverse) for each bacterial culture were received in AB1 and SEQ file formats and compared against the US National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.5 Heat resistance experiments

After the identification of the microbial species isolated, their heat resistance was evaluated.

2.3.5.1 Culture preparation and treatment media

Microorganisms were first resuscitated from the stock cryovials as described in section 2.3.4. Then, a 0.1mL aliquot of an overnight pre-culture was inoculated into 200mL Duran bottles containing 50mL of sterile TSB-supplemented containing 6mm diameter sterile glass beads (approximately 30g) to prevent the formation of cell aggregates. These were incubated at 25°C in a shaking thermostatic bath at 130rpm (mod. LSB18, Grant instruments Ltd., Cambridge, UK) until cultures reached the stationary phase. All microorganisms were incubated for 48h except for *Staphylococcus* spp. which required 72h to reach the stationary phase.

2.3.5.2 Heat treatments

In this study, thermal resistance experiments were performed in pH 7.0 McIlvaine citrate-phosphate buffer (Dawson *et al.*, 1974) which is similar to the pH of crab meat. Firstly, the most heat resistant species within the same genus was determined by means of the end-point method. The procedure used was as follows: 1mL of the stationary phase cultures was re-suspended in tubes containing 9mL of citrate-phosphate buffer in order to achieve an initial count of approximately 10^8 CFU/mL. Tubes were then submerged in a thermostatic bath (mod. LSB18, Grant instruments Ltd., Cambridge, UK) at 60°C for 90 seconds and immediately cooled in iced water. Finally, aliquots of each serial dilution were pour plated in TSA-supplemented agar and incubated at 25°C for 72 h. Further incubation times did not provide higher counts (data not shown).

Once the most heat resistant microbial species within the same genus was determined (those showing lower Log_{10} reductions after the 60ºC/90s treatment), their decimal reduction time values (D_T) and z values were determined using a thermoresistometer TR-SC (Condón *et al.*, 1989, 1993). This instrument consists of a 350mL stainless steel vessel containing the treatment medium and equipped with an electrical heater and a refrigeration coil system for maintaining a constant temperature, an agitation device to ensure uniform distribution and temperature homogeneity of the medium and inoculum and ports for injecting the microbial suspension and for sample extraction. Once the target temperature attained stability (±0.1°C), 0.2mL of the corresponding microbial suspension was injected with a sterile syringe into the treatment medium. After inoculation, 0.1mL samples were collected at different times and immediately pour plated in TSA-supplemented agar. Survival curves (log_{10} number of survivors *versus* treatment time) were obtained at different temperatures ranging from 54 to 69°C and the corresponding D_T values (min) were calculated. In order to calculate z values (°C), four temperatures were evaluated for each bacterial culture. All thermal experiments were carried out at least in triplicate on different working days.

2.3.6 Data and statistical analysis

2.3.6.1 Shelf-life determination

Gompertz Equation modified by Zwietering *et al.*, (1990) (Equation 2.1) was fitted to the TVC_m growth at different temperatures (2, 5, 7 and 10°C):

$$y = A * exp\left\{-exp\left[1 + \frac{\mu_{max} * e}{A} * (\lambda - t)\right]\right\} \qquad \text{(Eq. 2.1)}$$

where, y indicates the Log_{10} of the count reached at the different times, A indicates the increased Log_{10} cycles from initial count (y_0) to stationary phase (y_{max}) and t is the storage time expressed in hours; μ_{max} represents the maximum growth rate (h^{-1}) observed in the linear part of the growth curve, λ represents the duration of the lag phase (h) and e equals to 2.718.

The estimated values for λ and μ_{max} were further expressed as a function of the storage temperature by using the Square Root Model (Ratkowsky *et al.*, 1982) (Equations 2.2 and 2.3):

$$\sqrt{\mu_{max}} = b_\mu * (T - T_{\min\mu}) \qquad \text{(Eq. 2.2)}$$

$$\sqrt{\frac{1}{\lambda}} = b_\lambda * (T - T_{\min\lambda}) \qquad \text{(Eq. 2.3)}$$

where b is a constant, T (°C) is the storage temperature and T_{min} is the respective theoretical minimum temperature of growth (°C), estimated by extrapolation of the regression line when $\sqrt{\mu_{max}}$ or $\sqrt{\frac{1}{\lambda}} = 0$.

All models were fitted to the data by using a non-linear regression and least square approach with GraphPad PRISM® 5.0 software (GraphPad software, Inc., San Diego, CA, USA). The same software was used to perform the statistical analyses: *t*-test and one-way ANOVA followed by the *post hoc* Tukey test. Differences were considered significant when $P \leq 0.05$.

2.3.6.2 Thermal resistance determination

Survival curves at a constant temperature (T) were obtained by plotting the Log_{10} fraction of survivors (survivors after a certain treatment time *t* divided by the microbial concentration at *t*=0) *versus* the treatment time (*t*, min). In cases where the profile of the survival curve appeared as a downward concave shape, the Geeraerd log-linear regression plus shoulder model was used (Geeraerd *et al.*, 2000). To fit the model to the survival curves, the Geeraerd and Van Impe inactivation model fitting tool (GInaFiT) was used (Geeraerd *et al.*, 2005). The model (Equation 2.4) describes the survival curves through two parameters: the shoulder length (*Sl*), defined as the time preceding the exponential decay, and the inactivation rate (*kmax*) which corresponds to the slope of the exponential portion of the survival curve.

$$N_0 = N_0 e^{-k_{max}\, t}\left(\frac{e^{k_{max}\, Sl}}{1+\left(e^{k_{max}\, Sl}-1\right)e^{-k_{max}\, t}}\right) \qquad \text{(Eq. 2.4)}$$

where N_0 represents the initial count.

For survival curves with log-linear profiles, Bigelow and Esty model (Bigelow & Esty, 1920), which is also included in the GInaFiT software, was used to calculate the exponential inactivation rate (*kmax*) in min^{-1}. The traditional decimal reduction time value (D_T value) in minutes was then calculated with Equation 2.5.

$$D_T = 2.303/k_{max} \qquad \text{(Eq. 2.5)}$$

The *z* value (°C), which represents the temperature increase required to reduce D_T values by 10 fold was then calculated from the negative inverse of the regression line of the Thermal Death Time curves (TDT) obtained when Log_{10} D_T values are represented versus treatment temperature.

Based on the thermal resistance parameters (D_T and z values), an equivalent treatment to reduce 6 Log_{10} cycles of the microbial population were also calculated and compared with those proposed for *L. monocytogenes* in case of vegetative cells and *C. botulinum* in case of spores, based on the recommended treatments by FDA for fishery products (FDA, 2011). t-test and one-way ANOVA were performed with the GraphPad PRISM 5.0 software (GraphPad software, Inc., San Diego, USA) and differences were considered significant if $P \leq 0.05$. The standard deviations (*SD*) are given in the Figures as error bars.

2.4 RESULTS AND DISCUSSION

2.4.1 Microbiological profile of raw crab meats

Experimental counts for crab raw fresh meats obtained on the different specific agars are shown in Figure 2.1. Overall, the counts obtained in this research for crab raw meats are in accordance with those previously reported for different raw unprocessed shell fish, which are between 2.5 and 3 Log_{10} cycles (Gornik *et al.*, 2011; Robson *et al.*, 2007). Differences in counts between the two types of meat (i.e. white *vs* brown) were observed for all bacterial groups. Interestingly, higher counts were detected in brown meat. Faghri *et al.*, (1984) reported similar differences in counts depending on the crab's tissue (muscle, gills and hemolymph), obtaining lower counts in crab muscle, which corresponds to white meat than in case of gills and hemolymph. These higher counts in brown meat can be explained by the fact that crabs do not have a closed structured circulatory system so the haemolymph, located inside the crab body, could be a reservoir of bacteria (ICMSF, 2005). In both types of crab meat the psychrophilic counts were higher than for mesophilic bacteria. In case of mesophilic bacteria the counts were 1.2 and 2.5 Log_{10} cycles for white and brown meat, respectively, while for the psychrophilic bacteria the counts in PCA (Plate Count Agar, Oxoid) were 1.8 and 3.0 Log_{10} cycles. However, when a specific agar was used for marine psychrophilic bacteria (L.H agar) the counts were 2.1 and 3.7 Log_{10} cycles in white and brown meat, respectively, i.e. 0.3

and 0.7 Log_{10} cycles higher compared with the non-selective PCA. This mismatch was also observed by Broekaert *et al.*, (2011) who reported increases in the counts between 0.3 and 1.1 Log_{10} cycles when L.H agar was used instead of PCA.

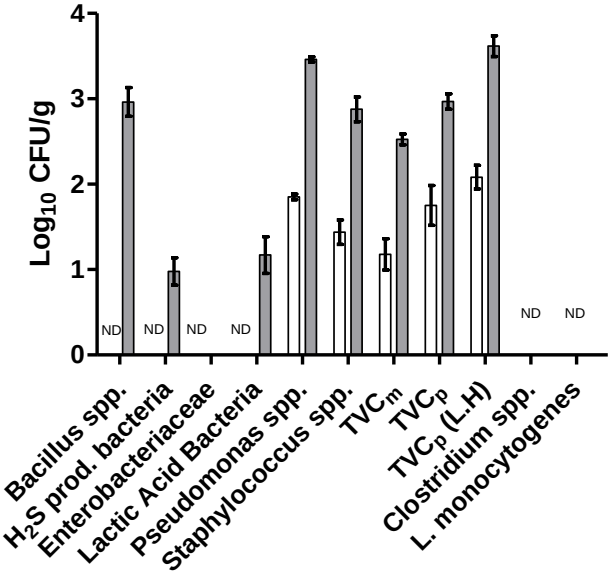


Figure 2.1: Log_{10} counts obtained in different selective and non-selective agars (see Table 2.2) for white (white bars) and brown (grey bars) raw crab meat. TVCm, Total viable mesophilic counts; TVCp, Total viable psychrophilic counts; L.H, Long and Hammer agar. ND = not detectable.

In case of white meat, only *Pseudomonas* spp. and *Staphylococcus* spp. were detected and the counts were 1.8 and 1.4 Log_{10} cycles, respectively. The heterogeneity of the flora detected in brown meat was larger than in white meat. Also in brown meat *Pseudomonas* spp. was the genus detected in a higher proportion with a total count of 3.5 Log_{10} cycles while *Staphylococcus* spp. and *Bacillus* spp. counts were 2.9 and 3 Log_{10} cycles respectively. The main difference observed between meats were the significant counts observed for *Bacillus* spp. (2.9 Log_{10} cycles) and the presence of H_2S producing bacteria (0.9 Log_{10} cycles) and LAB (1.2 Log_{10} cycles) in brown meat.

Overall, results obtained in this section are in agreement with most published data on different crab species and shellfish. But interestingly, our study also revealed a significant presence of *Bacillus* spp. in brown meat though

not in white meat. Some authors have also reported the presence of this spore forming bacteria in the intestinal content of *Dungeness crab* (Lee and Pfeifer, 1975). Linton *et al.*, (2003) also described the presence of *Bacillus* spp. in different shellfish after a high hydrostatic pressure process, though some other authors did not find this microorganism in raw shellfish (Boziaris *et al.*, 2011; Faghri *et al.*, 1984; Gornik *et al.*, 2011). The presence of *Bacillus* spp. could be expected since different authors have reported its presence but mainly in processed products (Gram and Huss, 1996; Linton *et al.*, 2003). This could be attributable to the low counts found in raw products, which will make them unable to compete with other bacteria present in higher proportions. Nonetheless, the fact that they are spore-forming bacteria with a "far-from-negligible" resistance to conservation processes gives them an important role in the spoilage of processed seafood products. The absence of H_2S producing bacteria and LAB in white meat is also in accordance with the study of Boziaris *et al.*, (2011) with lobsters. Although these two bacterial groups along with the *Enterobacteriaceae* family are considered of significant relevance in the degradation of fresh fish (Gram and Huss, 1996; Gram and Dalgaard, 2002; Jay, 2000), their importance in brown crab seems to be minor due to the lower counts observed here compared to reports on other species. It is also interesting to note that in this research, neither *Listeria* spp. nor *Clostridium* spp. were detected in any of the samples analysed. This could be attributable to insufficient sample size or due to the small counts and relatively low prevalence of these microorganisms in crab as indigenous bacteria (Fledhusen, 2000; Rocourt *et al.*, 2000; Pagadala *et al.*, 2012).

2.4.2 Microbiological shelf-life of cooked crabs

In this section the effect of the storage temperature on the shelf-life of crabs cooked at a mild temperature (75°C) for the required time to apply at least an equivalent $F_{70^oC}^{7.5^oC}$= 2 min (45 min) as determined in Chapter 3, was evaluated. To determine the effect of storage temperature on the shelf-life of this seafood product, the Gompertz Equation modified by Zwietering *et al.*,

(1990) (Eq. 2.1) was used as primary model to describe the growth curves of TVC. The square root model (Ratkowsky *et al.*, 1982) (Eq. 2.2 and Eq. 2.3) was subsequently used to describe the relationship between the maximum growth rate (μ_{max}) and the lag phase (λ) with the storage temperature.

2.4.3 Microbial growth modelling (Primary model)

Figure 2.2 shows the growth curves obtained at 2, 5, 7 and 10°C for TVC_m over the storage time expressed in hours, for white meat (Figure 2.2A) and brown meat (Figure 2.2B). As it can be observed in both Figures, the main difference between the curves is the duration of the lag phase at the different storage temperatures (i.e. lower storage temperatures produced longer lag phases). Figure 2.2 also shows that the maximum count reached (9.5 ± 0.8 Log_{10} cycles) is independent of the storage temperature and the meat type. Similar results were observed by Koutsoumanis (2001) with Mediterranean gilt-head seabream where the storage temperature did not affect the N_{max} (parameter describing the maximum count obtained) observed. Dalgaard *et al.*, (1997) also reported no effect of the storage temperature (between 0°C and 15°C), and CO_2 concentration (from 0 to 100%), on the N_{max} reached in packed cod. Due to this, N_{max} was considered constant and equal to 9.5 Log_{10} cycles.

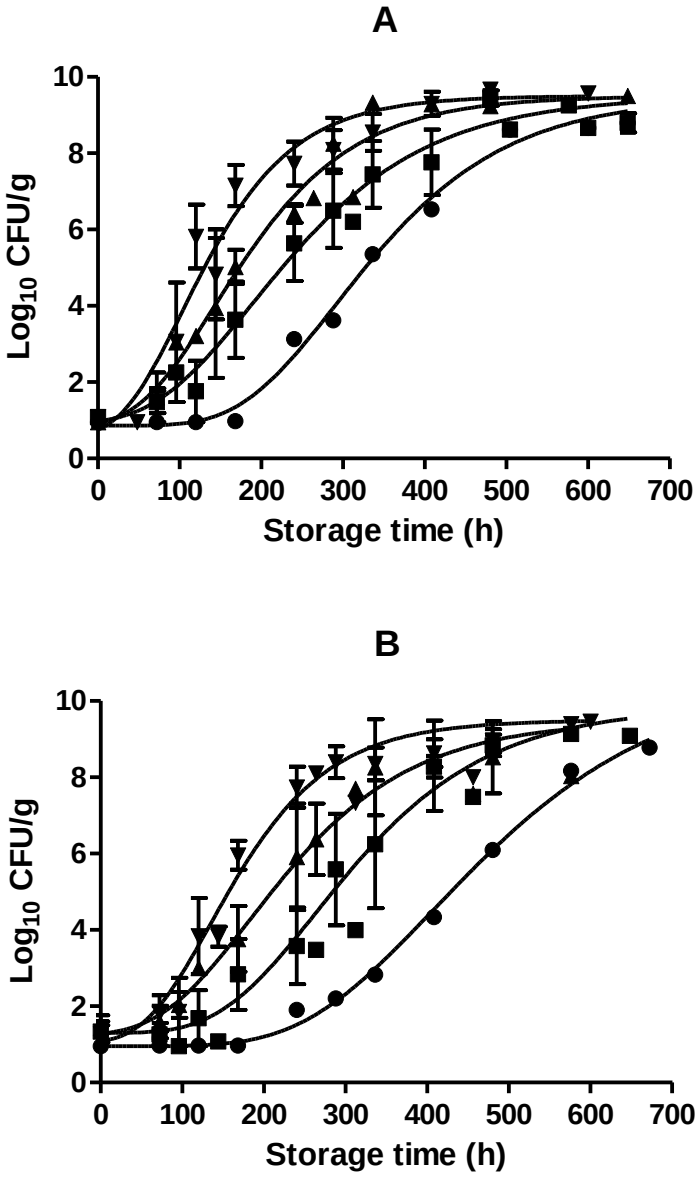


Figure 2.2: Evolution of TVC_m over storage time (hours) at 2°C (●), 5°C (■), 7°C (▲), and 10°C (▼) for white (A) and brown (B) meat obtained from ready-to-eat whole brown crabs cooked (75°C for 45 minutes) and packed under vacuum.

The main two model parameters, μ_{max} and λ, obtained for white and brown meat are shown in Table 2.3. For both meat types when the storage temperature was reduced, an increase in the lag phase (λ) and a reduction in the growth rate (μ_{max}) were observed. In the case of the growth rate (μ_{max}) no significant differences were observed between meats at any storage

temperature, indicating that the differences between meat compositions have a minor relevance in terms of the growth rate of the microorganisms present after cooking. However, significant differences between the growth rates were detected depending on the storage temperature as it is well described in the literature (Churchill *et al.*, 2016; Dabadé *et al.*, 2015; Dalgaard, 1995; Dalgaard *et al.*, 1997; Koutsoumanis, 2001; Taoukis *et al.*, 1999). The growth rates calculated in this research for TVC_m at temperatures from 2 to 10°C ranged from 0.022 to 0.043 (h^{-1}). These values are in accordance with those reported by Churchill *et al.*, (2016) for TVC_p in salmon stored at temperatures between 0 and 15°C. However, other authors have reported slower growth rates in different fish and fishery products stored at different temperatures (Dabadé *et al.*, 2015; Dalgaard *et al.*, 1997; Koutsoumanis and Nychas, 2000; Koutsoumanis, 2001; Taoukis *et al.*, 1999). The discrepancies observed between the model parameters obtained in this research and those reported in the literature could be attributed to the fact that the major part of that research was performed in fresh fish for specific spoilage bacteria (SSO) and, in some cases, the microorganism of interest was inoculated. On the contrary, this research was focused on the natural microbiota present in cooked crab so it is not surprising to observe these discrepancies.

Regarding the lag phase (λ), Table 2.3 shows significant differences based on the storage temperature, as for μ_{max}, but also based on the crab meat type. In case of brown meat, λ ranged from 248 to 53 h while in the case of white meat λ ranged from 148 to 28h for temperatures of 2 and 10°C, respectively. λ values calculated for white meat were similar to those reported by *Dabadé et al.*, (2015) for *Pseudomonas* spp. and *Carnobacterium* spp., in cooked shrimps. Contrary to the observed for μ_{max} values, the λ calculated for brown meat were 1.6, 1.8, 1.5 and 1.9 fold higher than those calculated for white meat at 2, 5, 7 and 10°C respectively. This could be attributed to the different composition of the two meats. While white meat is mainly composed of protein and structural lipids, brown meat is rich in storage lipids required for the animal physiological needs (Maulvault *et al.*, 2012). Moreover, the moisture content reported in

white meat is about 1.5 fold higher than in brown meat according to published data (Maulvault *et al.*, 2012) and data obtained in this thesis (Chapter 3). Considering that λ is a transition period during which cells adapts to their new environment (Koutsoumanis, 2001; Robinson *et al.*, 1998), the differences in the meat composition such as the higher protein content as well as the moisture content in the case of white meat could explain the differences detected on λ between meats. Other possible explanations for these differences could be the different microbiota present on each type of meat, or even a combination of the previous hypothesis. In any case, the results obtained seem to indicate that once the adaptation has been done, meat composition is no longer relevant in terms of microbial growth.

Table 2.3: Lag phase (λ) in hours and maximum growth rate (μ_{max}) in hours^{-1} estimated with the modified Gompertz Equation (Zwietering *et al.*, 1990) (Eq. 2.1)for total viable counts in white and brown crab meat from ready-to-eat whole brown crab pack under vacuum and stored at 2°C, 5°C, 7°C and 10°C.

T (°C)	Meat type	$\lambda \pm SE$ (h)	$\mu_{max} \pm SE$ (h^{-1})	R^2	RMSE
2	W	$148^{a} \pm 11.4$	$0.027^{a} \pm 0.002$	0.99	0.30
	B	$248^{b} \pm 8.4$	$0.022^{a} \pm 0.001$	0.99	0.14
5	W	$79^{c} \pm 4.3$	$0.027^{b} \pm 0.004$	0.77	1.56
	B	$146^{d} \pm 4.8$	$0.026^{b} \pm 0.006$	0.73	1.65
7	W	$49^{e} \pm 2.6$	$0.034^{c} \pm 0.004$	0.91	0.95
	B	$73^{f} \pm 4.2$	$0.028^{c} \pm 0.004$	0.87	1.05
10	W	$28^{g} \pm 2.4$	$0.043^{d} \pm 0.005$	0.87	1.16
	B	$53^{h} \pm 1.8$	$0.038^{d} \pm 0.004$	0.89	0.99

T, storage temperature; λ, Lag phase; μ_{max}, maximum growth rate; *SE*, standard error; R^2, determination coefficient; *RMSE*, root mean square error; W, white meat; B, brown meat. For each column, different subscriptletters show significant differences ($P \leq 0.05$).

2.4.4 Effect of storage temperature (Secondary model)

The effect of temperature on the model parameters λ and μ_{max} was also studied using the Root Square Model (Ratkowsky *et al.*, 1982) (Eq. 2.2 and Eq. 2.3). This equation was selected as a secondary model because it is simple and

contains parameters (described in Section 2.4) that are easily comparable with values reported by other authors (Dalgaard *et al.*, 1997). Table 2.4 shows the calculated parameters b (i.e. the constatnt) and T_{min} (i.e. theoretical minimum temperature) for white and brown meat for both the maximum growth rate (μ_{max}) and the lag phase (λ). For μ_{max}, b values were 0.0058 and 0.0061 and T_{min} values were -24.9 and -21.1, with R^2 of 0.87 and 0.90, for white and brown meat, respectively. No significant differences were found between those values for the μ_{max} in the two types of meat. Therefore, the effect of temperature on μ_{max} remained constant regardless of the meat type. b and T_{min} values for μ_{max} are close to those reported by Churchill *et al.*, (2016) for the TVC_p in Atlantic salmon stored at temperature ranging from 0 to 15 °C. However, other studies focused on the natural *Pseudomonas* spp. load have reported higher values for both b and T_{min} from the modelling of μ_{max} with respect to the storage temperature in different fish species; Koutsomanis *et al.*, (2000) reported a b value of 0.0211 and T_{min} of -10.65 for the growth of *Pseudomonas* spp. in Mediterranean gilt-head seabream stored from 0 to 15°C. The same author in a different study (Koutsomanis, 2001) also reported values of 0.0193 and -11.8 for b and T_{min}, respectively, for the same microbial species in the same fish type. Taoukis *et al.*, (1999) also reported higher values of b and T_{min}, than those obtained in this work, for *Pseudomonas* spp. (0.0018 and -11.36, respectively) and *Shewanella putrefaciens* (0.0212 and -11.14, respectively) in Mediterranean Boque stored at different temperatures. Dabadé *et al.*, (2015) reported values of b = 0.021 and T_{min} = -12.6 for *Pseudomonas psychrophila* and b = 0.032 and T_{min} = -4.2 for *Carnobacterium maltaromaticum* in cooked tropical shrimps. These differences among studies for the parameters of the secondary model for μ_{max} could be attributable to the water from which the different fish and shellfish were captured. It is widely accepted that the main microorganisms present in fish and fishery products largely depend on the environment they originate from (Gram *et al.*, 1996, 2002; Jay, 2000). Therefore, it seems reasonable to say that the storage temperature affects the survival and growth of the specific microbiota present in fish from different

water in different ways. This would explain the higher values obtained in experiments with fish from tempered water (Mediterranean Sea and tropical waters) than those with fishery species from cold waters (North Atlantic) as it is the case of the brown crab used in the present work and also in salmon. Besides, most of those studies were performed for specific microorganisms, sometimes inoculated, which could mask the natural behaviour, due to microbial competence with the natural flora. If the initial equilibrium is altered, by adding higher concentrations of one specific microbial species, the growth conditions will be different producing discrepancies in the growth parameters. This hypothesis would be supported by the fact that the parameters calculated in this work and those reported by Churchill *et al.*, (2016) for the growth of TVC are similar while other studies performed with SSO reported bigger values (Dabadé *et al.*, 2015; Koutsomanis *et al.*, 2000; Koutsomanis, 2000; Taoukis *et al.*, 1999).

Table 2.4: Parameters of the square root model (Ratkowsky at al., 1982) (Eq. 2.2 and Eq. 2.3) for the maximum growth rate (h^{-1}) and Lag phase (h) for total viable counts growth in/on ready-to-eat brown crab packed under vacuum

Meat type	parameter	Value	R^2	*RMSE*
White	b_μ	0.0058 ± 0.0011	0.87	0.024
	$T_{min\mu}$	-24.9		
	b_λ	0.0140 ± 0.00051	0.99	0.053
	$T_{min\lambda}$	-3.4		
Brown	b_μ	0.0061 ± 0.0013	0.90	0.024
	$T_{min\mu}$	-21.1		
	b_λ	0.0096 ± 0.0013	0.96	0.042
	$T_{min\lambda}$	-4.4		

R^2, determination coefficient; *RMSE*, root mean square error.

From the modelling of the effect of storage temperature on the lag phase (λ), b values of 0.014 and 0.0096 and T_{min} values of -3.4 and -4.4 were calculated for white and brown meats, respectively (Table 2.4). Significant differences were found between the values of the two model parameters for both types of meat, meaning that storage temperature influences the lag phase

differently depending on the type of meat. *b* values, but not T_{min}, reported here are in the range of those reported by Koutsuomanis (2001) and Koutsoumanis *et al.*, (2001) for *Pseudomonas* spp. in gilt-head seabream. The differences between the calculated *b* values and those reported in the literature would be attributable, as in case of μ_{max}, to differences in the microbiota present in the product. Nevertheless, the differences found between b_λ values for the crab meat types would need further explanation. In this research the shelf-life model was built from the results obtained from a mild cooked crab. As shown in Chapter 3 the crab's cold spot is located in the abdomen. Due to the different heat penetration rates observed, between crab parts, significant differences on the total *F* value applied exists between the two types meats, so a difference on the microbiota selected after the treatment could exist. Although, the differences between meat compositions could also contribute, as has been discussed previously.

2.4.5 Shelf-life determination (Tertiary model)

Finally, based on the parameters extracted from the primary (modified Gompertz Equation) and secondary (Root Square Model) models, two different final Equations were developed to calculate the microbial growth in white (Eq. 2.6) and brown meat (Eq. 2.7).

$$y = A \times exp\left\{-exp\left[1 + \frac{[0.0058\times(T-(-24.9))]^2\times e}{A} * \left[\left(0.014 \times \left((T-(-3.7))\right)\right)^2 - t\right]\right]\right\} \quad \text{(Eq. 2.6)}$$

$$y = A \times exp\left\{-exp\left[1 + \frac{[0.0061\times(T-(-21.1))]^2\times e}{A} * \left[\left(0.0096 \times \left(T-(-4.4)\right)\right)^2 - t\right]\right]\right\} \quad \text{(Eq. 2.7)}$$

Where *y* indicates the Log_{10} cycles of the counts, *A* indicates the difference between the counts (Log_{10} cycles) in the stationary phase (y_{max}) and the initial counts (y_0), *t* is the storage time expressed in hours and *T* represent the

storage temperature (°C). Figure 2.3 shows the comparison between observed and predicted values calculated from Equations 2.6 and 2.7 as well as R^2 and *RMSE* as indicators of the accuracy of the tertiary Equations for white (A) and brown (B) meat. Based on Equations 2.6 and 2.7 the shelf-life of white and brown meat were calculated, considering 6 Log_{10} cycles as the end of the shelf-life. A log-linear relationship was found between the shelf-life and the storage temperature for both meat types as Dabadé *et al.*, (2015) previously reported for tropical shrimps.

Table 2.5 shows the Equations which describe the effect of the storage temperature on the shelf-life of each meat type. In this case, no differences were found between the slopes obtained for each meat type meaning that the relative effect of the storage temperature on the shelf-life was independent of the type of meat.

From the Equations shown in Table 2.5, a shelf-life of 12 and 16 days were calculated at 4°C, while 6 and 7 days were defined as the shelf-life limit at 10°C for white and brown meat, respectively. According to these results, the ready-to-eat edible crab (*Cancer pagurus*) shelf-life seems to be limited by the white meat, when crabs are processed in a single step cooking at mild temperatures.

Table 2.5: Equations which define the shelf-life of ready-to-eat whole brown crab packed under vacuum as a factor of the storage temperature.

Meat type	Equation
White	Log_{10} *Shelf-life* (h) = -0.0497 * T + 2.6588
Brown	Log_{10} *Shelf-life* (h) = -0.0537 * T + 2.8001

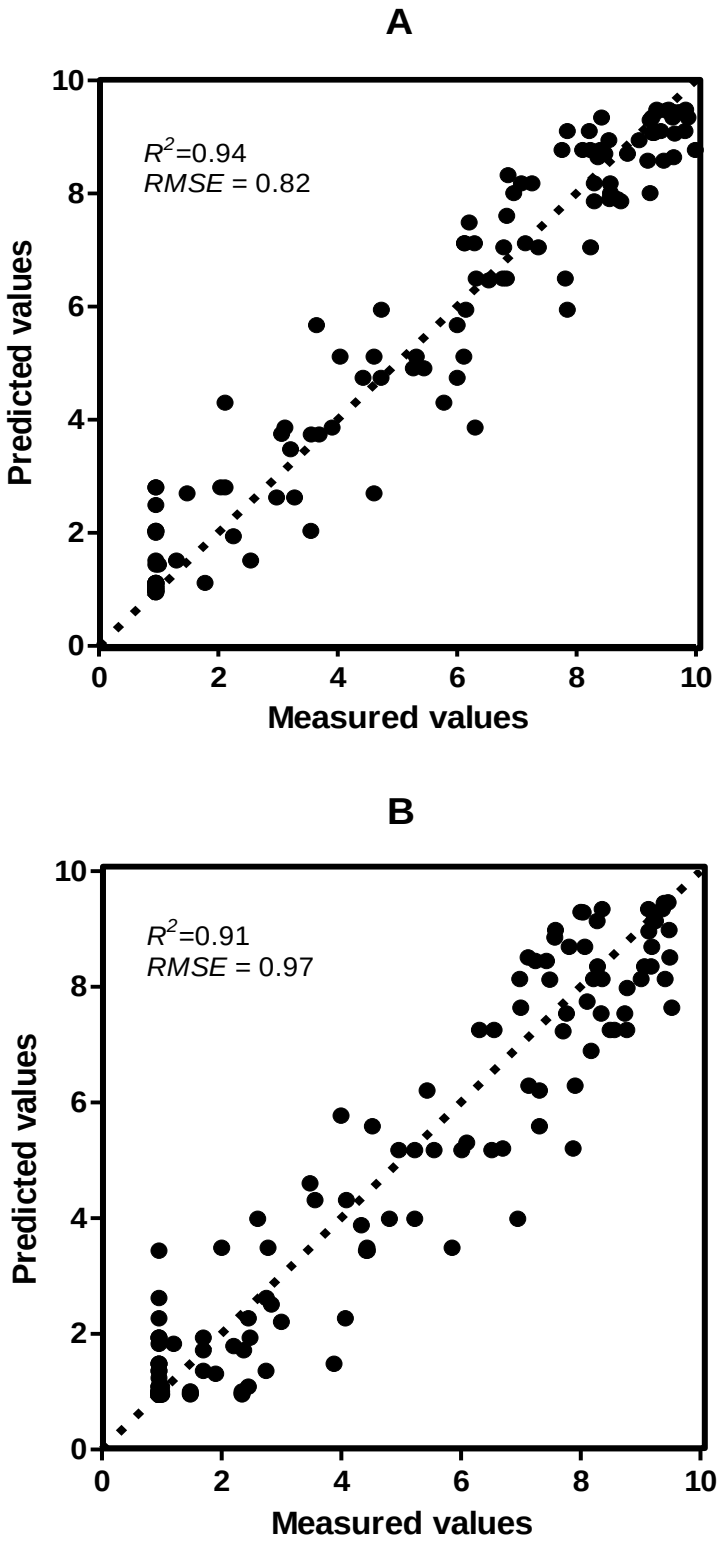


Figure 2.3: Measured Vs predicted counts of TVC_m at all storage temperatures in white (A) and brown (B) meat (estimated from Eq. 2.6 and 2.7)

2.4.6 Effect of storage temperature on microbial flora present in cooked crab

Based on the developed model (Section 2.4.5), a study of the main microbiota present at the end of the shelf-life i.e. after 12 days at 4°C and after 6 days at 10°C was performed. Similar to raw meats (Figure 2.1), Figure 2.4 shows the microbiological profiles for cooked crab at the end of shelf life, using different selective and non-selective agars (Table 2.2) for white (A) and brown (B) crab meat. In general, no differences between counts were observed at 4 and 10°C in the case where the same bacterial group was detected at both temperatures; except for *Bacillus* spp. in white meat and *Staphylococcus* spp. in brown meat. This result supports the effectiveness of the model developed in the previous section.

The maximum counts registered for TVC_m (Figure 2.4) were 5.3 and 4.8 Log_{10} CFU/g in white meat and 5.9 and 6.0 Log_{10} CFU/g in brown meat at 4 and 10°C, respectively. As it was observed with the raw meats (Figure 2.1), in the case of cooked crab meats, the psychrophilic bacteria counts were higher and the highest counts were detected in L.H agar (6.1 and 4.9 Log_{10} CFU/g in the case of white meat and 6.4 and 6.1 Log_{10} cycles in the case of brown meat). For white meat (Figure 2.4A), H_2S producing bacteria were only found in the meat stored at 10°C in a level of 2.7 Log_{10} CFU/g. In the case of *Pseudomonas* spp. no differences were detected between the absolute counts at 4 and 10°C (2.5 Log_{10} CFU/g). Similarly, no differences between counts were observed between *Staphylococcus* spp. counts at 4 and 10°C (3.4 Log_{10} CFU/g). Only in the case of *Bacillus* spp. significant differences were observed in counts 3.9 Log_{10} at 10°C and 5.3 Log_{10} at 4°C. For brown meat (Figure 2.4B), H_2S producing bacteria, *Enterobacteriaceae* and LAB (Lactic Acid Bacteria) were only detected when the meat was stored at 10°C. The presence of *Staphylococcus* spp. was dependent on the storage temperature: at 4°C this bacteria count was 3.2 Log_{10} cycles and 5.7 Log_{10} cycles for samples stored at 10°C. On the other

side, in case of *Bacillus* spp., no significant differences were detected between the counts at 4 and 10°C (between 5.5 and 6 Log_{10} CFU/g).

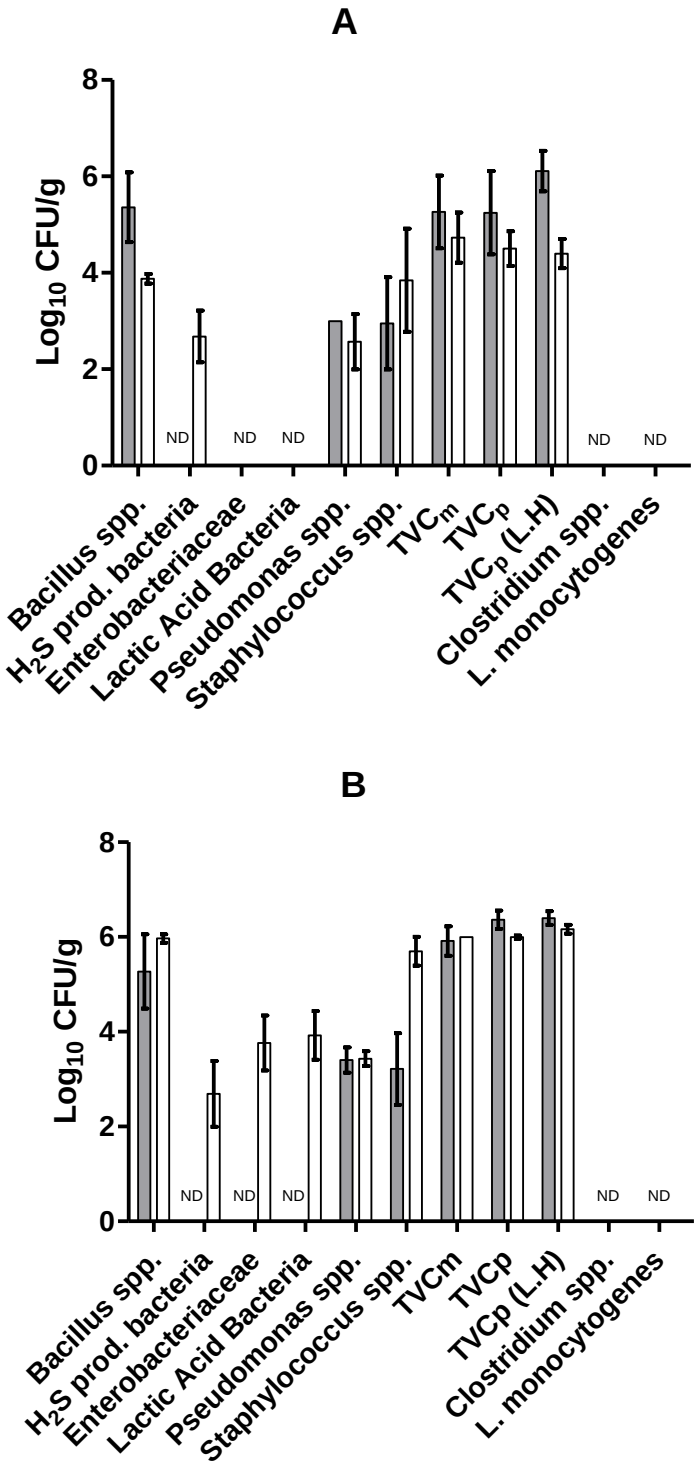


Figure 2.4: Log_{10} counts obtained for *Bacillus* spp., H_2S producing bacteria, *Enterobacteriaceae*, Lactic Acid Bacteria, *Pseudomonas* spp., *Staphylococcus* spp., Total Viable Counts, Total Psychrophilic Aerobic Counts and total psychrophilic bacteria (L.H) for white (A) and brown (B) meat of cooked crab store at 4°C (grey bars) and 10°C (white bars) at the end of the shelf-life (calculated from Eq. 2.6).

Based on the results of this section, *Bacillus* spp. proved to be the species of major significance in ready-to-eat whole brown crab followed by *Staphylococcus* spp. especially at higher storage temperatures. The presence of other SSO bacteria, which is considered of importance, was practically non-existent. Ward *et al.*, (1977) also reported the presence of *Bacillus* spp. in pasteurized crab meat stored at 3.3 °C. However the same authors also reported an important relevance of *Pseudomonas* spp., which has not been detected in this research. Lorentzen *et al.*, (2014) also reported the importance of H_2S producing bacteria and *Pseudomonas* spp. in cooked King crab clusters stored at 4°C. The presence of *Pseudomonas* spp. as important SSO in cooked brown crab was also reported by Anacleto *et al.*, (2011). But other authors have also reported the importance of *Bacillus* spp. in processed fish and fishery products (Gram and Huss, 1996; Linton *et al.*, 2003). However, this is the first work which shows the importance of *Staphylococcus* spp. as species of significance in a processed seafood stored at 10ºC. The discrepancies observed with previous studies, where the presence of *Staphylococcus* spp. has not been highlighted, could be attributable to different raw materials, the temperatures used in the experimental plan or the different treatments applied. In the present study a thermal treatment specifically designed to inactivate *L. monocytogenes* in seafood, based on the US FDA recommendations, was applied. This would explain the low counts for vegetative bacteria cells and the strong presence of *Bacillus* spp. as those are able to produce heat resistant spore forms. It is also noteworthy to mention that in the majority of the published studies the thermal treatment conditions applied are not reported hence the differences in the microbiota could be also associated to the different thermal treatments applied.

2.4.7 Bacterial groups present in lab-cooked and commercial samples of ready-to-eat brown crab

Once a suitable model to describe the shelf-life of ready-to-eat brown crab meat samples depending on the storage temperature has been defined, the

microbiota of a number of crabs cooked, as described in section 3.2, packed under vacuum and stored at 7±1°C were analysed on the last day of their shelf-life (defined based on previous results). Additionally, the microbial groups of a number of commercial samples (see Table 2.1) were also analysed at the end of their shelf-life, as indicated in the label. The percentages calculated in this section are calculated based on the presumptive counts in specific agars using Equation 2.8.

$$\text{Representativity } \% = \frac{\text{Count in specific agar}}{\text{Count in non-specific agar } (TVC_m)} \times 100 \qquad \text{(Eq. 2.8)}$$

For laboratory processed crab meat stored for 7 days (i.e. the end of their shelf-life as defined by the equations developed in section 2.4.5), the maximum TVC_m counts observed were 5.1 and 7.1 $\log_{10}$ CFU/g for white and brown meat, respectively. In contrast to raw meat, the highest counts were detected for TVC_m instead of TVC_p. The proportions of the different bacterial groups detected are presented in Figure 2.5. For white meat, the main genera detected were: *Bacillus* spp. (14%), *Staphylococcus* spp. (13%), *Pseudomonas* spp. (0.7%) and H_2S producing bacteria (0.1%), while undefined bacterial groups represented 71% of the total microbiota, showing the limitations of presumptive counts in selective agars. For brown meat, unidentified bacterial groups made up 9%, with *Bacillus* spp. most frequently detected (59.4%) followed by *Staphylococcus* spp. (29.7%). Furthermore, *Enterobacteriaceae*, LAB and *Pseudomonas* spp. were detected in low proportions corresponding to 0.3%, 0.5% and 0.2%, respectively. As for raw crab meat, H_2S producing bacteria, *Listeria* spp. and *Clostridium* spp. were not detected in either white or brown cooked meats samples.

The TVC_m counts enumerated in commercial samples at the end of their shelf-life were 8.3, 2.1, 6.8, 5.4, 6.7 and 3.6 Log_{10} CFU/g for samples A, B, C, D, E and F, respectively (see Table 2.1). These results highlight substantial variability in TVC_m counts among commercial crab products. The highest counts were detected in particulate white meat packed under modified atmosphere

conditions, where the main bacteria detected were H_2S producing bacteria (77.6%), followed by *Bacillus* spp. (4.9%) and *Pseudomonas* spp. (3.8%), while LAB and *Staphylococcus* spp. represented only 1% of the total microbial concentration, as shown in Figure 2.5. Ready-to-eat whole crab claws (sample B) were found to have the lowest counts at the end of shelf-life. In samples C and F no specific bacterial groups were detected with the selective agars used. *Staphylococcus* spp. represented 20% of the flora in product E, while *Pseudomonas* spp. and H_2S producing bacteria were detected in proportions of 16.5% and 5% respectively in sample D (Figure 2.5).

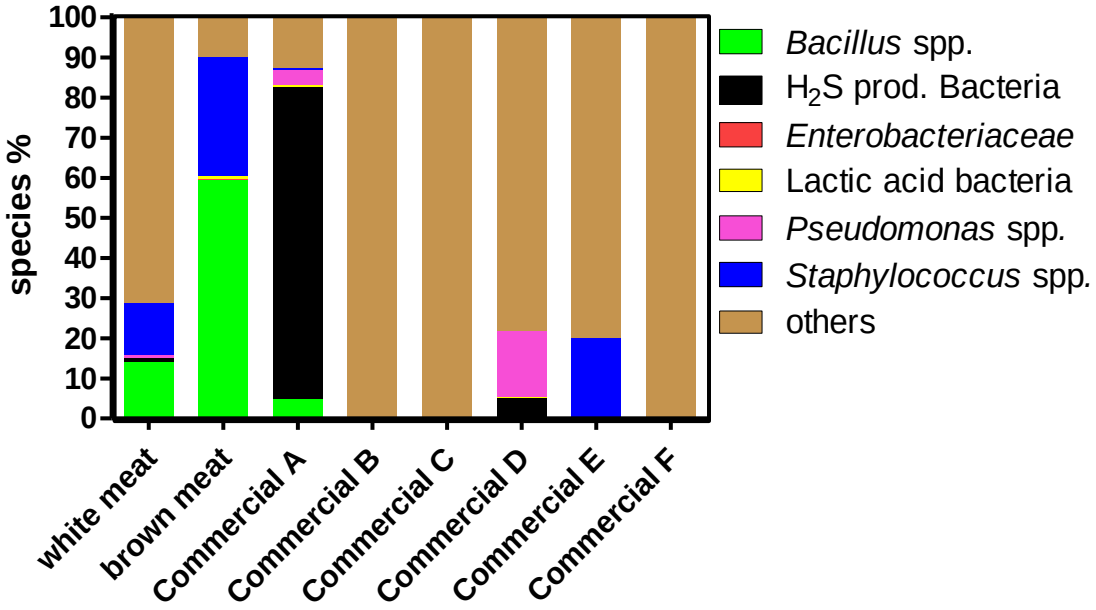


Figure 2.5: Relative percentage of in lab-cooked white and brown crab meat and various commercial samples at the end of their shelf-life.

The heterogeneity observed among the commercial samples may be related to the different processes used by individual companies within the crab processing sector. Differences in the microbiota present in meat cooked in a single step under controlled conditions versus commercial could be related to the fact that commercial crab products are pasteurised after packaging and they are generally designed to target non-proteolytic *Clostridium botulinum* type E (Edwards and Early, 2001). Therefore, the low presence of spore-forming bacteria??? is not surprising and this is likely the reason why neither *Listeria* nor *Clostridium* were detected.

2.4.8 Bacterial species isolated in brown crab

Table 2.6 shows the main bacterial species identified using 16S rRNA sequencing in crab and crab products. Interestingly, in raw crab meat 14% of the isolates identified were confirmed as *Shewanella baltica* in both brown and white meat even though no H_2S producing bacteria were detected in iron Lyngby agar, which is expected to isolate this species as it is a sulphite producing bacteria. However, the detection of this bacterium is not surprising given that they are considered a common specific spoilage organism (SSO) in seafood. *Carnobaterium divergens* was identified in 28.5% of the bacterial isolates and was the most frequently isolated bacterium irrespective of crab meat type even though the LAB counts (the group to which it belongs) were very low in MRS agar. The remaining bacterial species isolated from crab meat represented from 3.5 to 7% of the total bacteria isolated in raw crab meats and included several species of *Staphylococcus*, *Pseudomonas*, *Psychrobacter*, *Kocuria* and different genera such us *Enterococcus*, *Dietzia*, *Vibrio*, *Planococcus*, *Brochotrix*, *Oerskovia*, *Chryseobacterium*, *Corynebacterium*, *Providencia* and *Pseudoalteromonas*.

For commercial samples, a number of vegetative cells were identified, though *Staphylococcus* spp., *Carnobacterium* spp. and *Corynebacterium* spp. were the dominant species identified. In case of spore-forming bacteria, only *Psychrobacillus psychrodurans* and *Bacillus weihnestephanensis* were identified, with *P. psychrodurans* being the most prevalent in commercial samples. For crab meats cooked in a single step under laboratory conditions, a number of vegetative genera were also detected including *Pseudomonas* and *Staphylococci*. For spore-forming bacteria, *Bacillus mycoides*, *B. weihenstephanensis* and *P. psychrodurans* were the most prevalent species recovered with proportions of 35%, 30% and 7.5% identified, respectively.

Table 2.6: Vegetative bacterial species isolated from cooked ready-to-eat brown crab (*Cancer pagurus*) processed under laboratory conditions and various commercial products and identified by 16S rRNA sequencing.

Vegetative Cells		**Sample Type**				
Bacterial genus	species	White meat		Brown meat		Commercial meat
		Raw	Processed	Raw	Processed	Processed
Staphylococccus	*equorum*	1	1	1	-	-
	capitis	1	1	-	-	-
	xylosus	1	-	-	-	3
	fluorettii	-	-	-	-	1
Shewanella	*baltica*	2	1	1	-	1
Carnobacterium	*divergens*	4	1	4	1	2
	funditum	-	-	-	-	1
Enterococcus	*aquimarinus*	-	-	-	-	1
Dietzia	*maris*	-	-	1	-	-
Pseudomonas	*deceptionensis*	-	1	-	-	-
	lundensis	-	1	-	1	-
	fluorescens	-	-	-	1	-
	synxantha	-	-	1	-	-
	psychrophila	-	1	-	-	-
Psychrobacter	*fozii*	1	-	1	-	-
	maritimus	-	-	1	-	-
	articus	-	-	1	-	-
	faecalis	-	-	1	-	-
Vibrio	*anguillarum*	-	-	1	-	-
Planococcus	*halocryophilus*	-	-	1	-	-
Brochotrix	*thermosphacta*	-	-	-	1	-
Oerskovia	*turbata*	-	-	1	-	-
Kocuria	*salsiccia*	-	-	1	-	-
	atrinae	-	-	-	-	1
Chryseobacterium	*humi*	-	-	1	-	-
Corynebacterium	*testudinoris*	-	-	-	-	3
Providencia	*heimbachae*	-	-	-	-	1
Pseudoalteromonas	*elyakovii*	-	-	1	-	-
Spore forming						
Bacillus	*mycoides*	-	9	-	5	-
	weihenstephanensis	-	8	1	4	1
Psychrobacillus	*psychrodurans*	-	-	-	3	9

Overall, the results of this study support the hypothesis that bacterial spores are an important bacterial group in processed ready-to-eat seafood products as other authors have reported previously (Gram and Huss, 1996; Linton *et al*., 2003). However, this is the first study identifying the main spore forming species in ready-to-eat brown crab (*B. mycoides, B. weihenstephanensis* and *P. psychrodurans*) and non-spore forming bacteria of which *Carnobacterium divergens, Staphylococcus xylosus* and *Corynebacterium testudinoris* were the most isolated. The detection of *S. baltica* and four different species of *Pseudomonas* are also worth noting as they are considered important SSO in seafood due to their high spoilage potential (Gram and Dalgaard, 2002).

2.4.9 Thermal resistance of the main vegetative cells isolated from ready-to-eat brown crab

Once the main bacteria were identified, their thermal resistance was determined in McIlvaine citrate-phosphate buffer (pH 7.0). Initially, the thermal resistance at 60°C of each species was evaluated in order to determine the most heat resistant isolates within the same genus. Figure 2.6 shows the inactivation achieved when stationary phase cultures were treated at 60°C for 90 seconds. No differences in heat resistance were observed among the various *Staphylococcus* spp. isolated (Figure 2.6A). Among the *Pseudomonas* spp. and *Carnobacterium* spp. isolates, *P. lundensis* and *C. divergens* (III) showed the highest heat resistance, respectively, and were selected for further investigation (Figure 2.6B, 2.6C). In the case of the two *Shewanella* spp. isolates, *S. baltica* (I) was confirmed as the most heat resistant. No differences in heat resistance were observed among the three *Corynebacterium* spp. isolates (Figure 2.6E). In addition, the thermal resistance of *Providencia heimbachae*, *Kocuria atrinae*, *Enterococcus aquimarinus* and *Brochotrix thermosphacta* were also evaluated since these microorganisms were isolated from laboratory cooked crab meat samples in this study.

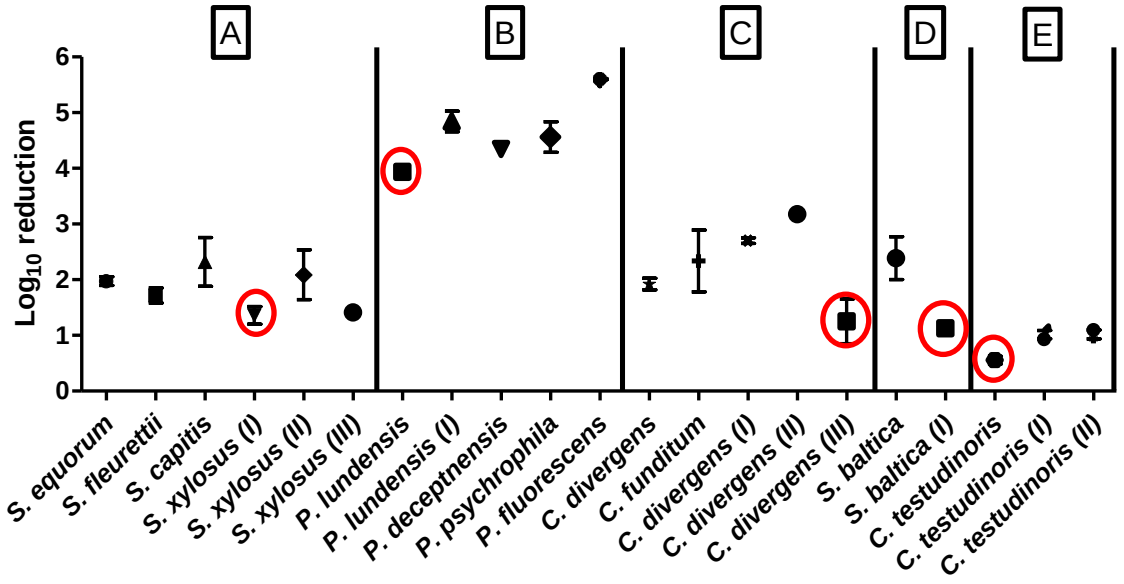


Figure 2.6: Log_{10} reductions in the populations of *Staphylococcus* spp. (A), *Pseudomonas* spp. (B), *Carnobacterium* spp. (C), *Shewanella* spp. (D) and *Corynebacterium* spp. (E) following a heat treatment at 60°C for 90 seconds.

Table 2.7 shows the D_T values of all microorganisms tested over a range of temperatures from 54 to 69°C as well as the *z* values (°C) for the vegetative cells which showed higher heat resistance within each bacterial genera isolated. The table also presents the shoulder length (*Sl*) values for *Kocuria atrinae,* which was the only bacteria isolated that showed shoulders in its survival curves. When comparing D_T values, *Carnobacterium divergens* and *Shewanella baltica* showed the highest heat resistance within the range of temperatures used in this study, with D_{60} values of 2.18 and 2.2 min respectively, followed by *Kocuria*, *Enterococcus*, *Staphylococcus* and *Pseudomonas* ($D_{60°C}$ values of 1.8, 1.73, 1.1 and 1.1 min, respectively). *Corynebacterium testudinoris*, *Providencia heimbacheae* and *Brochotrix thermosphacta* were found to be the most heat sensitive with $D_{60°C}$ values of 0.47, 0.12 and 0.05 min, respectively. But this is the first time that thermal resistance of the natural microbiota of ready-to-eat edible crab has been reported. Spinks *et al.*, (2006) reported $D_{60°C}$ values between 2.2 and 0.06 minutes, which are in the range of D_T values calculated in this investigation, for various pathogenic and non-pathogenic bacteria commonly isolated in food and different water sources. These authors also

calculated *z* values which ranged between 4.3 and 6.4°C, which are in accordance with the *z* values calculated for the microorganisms studied in the present work (4.3–7.5°C, Table 2.7).

Differences in D_T and *z* values observed between studies may be a result of bacteria studied in the present work having been isolated from ready-to-eat crab stored under refrigeration, which may have selected for mainly psychrophilic species which could affect their thermal resistance. This hypothesis could be supported by the fact that the D_T and *z* values calculated in our research and those calculated from the data reported by Spinks *et al.*, (2006) are in agreement since the microorganisms investigated in the latter study were isolated from rainwater systems which are also likely exposed to a range of relatively low temperatures (12–25°C). Moreover, incubation temperature can affect thermal resistance of microorganisms with lower temperatures generally associated with lower heat resistance (Mañas *et al.*, 2003; Pagán *et al.*, 1999a; Spinks *et al.*, 2006), which could also explain the differences detected among different studies.

Since the thermal inactivation curves for *Kocuria atriane* showed shoulders, the heat resistance of this microorganism cannot be directly compared with the other species. As a result, 6*D* values i.e. time needed to achieve 6 Log_{10} reductions in the microbial population were calculated for all species investigated. Figure 2.7 illustrates the 6*D* values at 60°C as a reference temperature for all microorganisms. Although *K. atrinae* did not display relatively higher D_T values (Table 2.7), when the effect of the shoulder length was considered in the thermal resistance calculation (6*D* values), *K. atrinae* became the most heat resistant among all the microorganisms studied and therefore should be considered when defining a heat treatment.

Table 2.7: Thermal resistance (D_T and z values) of a selection of non-spore forming bacterial species isolated from lab-cooked crab meat.

Microorganism	T (° C)	*Sl* (min)	D_T (min)	*z* (° C)	R^2	*RMSE*
Providencia heimbachae	55	-	0.55 (0.01)	5.5 (0.22)	0.98	0.06
	58	-	0.19 (0.04)			
	60	-	0.12 (0.02)			
	61	-	0.04 (0.19)			
***Staphylococcus xylosus* (I)**	55	-	15.10 (0.05)	4.3 (0.30)	0.99	0.02
	57	-	4.90 (0.08)			
	60	-	1.10 (0.28)			
	63	-	0.20 (0.68)			
Carnobacterium divergens (III)	58	-	3.49 (0.01)	5.6 (0.47)	0.97	0.07
	60	-	2.18 (0.08)			
	62	-	0.91 (0.13)			
	64	-	0.32 (0.36)			
Pseudomonas lundensis	56	-	5.17 (0.02)	5.4 (0.17)	0.99	0.05
	58	-	1.97 (0.04)			
	60	-	1.10 (0.13)			
	62	-	0.36 (0.45)			
Shewanella baltica (I)	58	-	3.99 (0.04)	5.5 (0.31)	0.99	0.03
	60	-	2.02 (0.12)			
	62	-	0.86 (0.15)			
	64	-	0.33 (0.86)			
Kocuria atrinae	60	4.4 (0.44)	1.80 (0.14)	6.6 (0.38)	0.99	0.02
	63	1.0 (0.14)	0.68 (0.29)			
	66	0.28 (0.06)	0.30 (0.50)			
	69	0.04 (0.04)	0.11 (0.65)			
Corynebacterium testudinoris	58	-	1.39 (0.07)	6.5 (0.03)	0.97	0.06
	60	-	0.47 (0.48)			
	62	-	0.31 (0.44)			
	64	-	0.15 (0.88)			
Enterococcus aquimarinus	56	-	5.27 (0.03)	7.5 (0.43)	0.99	0.02
	58	-	3.25 (0.07)			
	60	-	1.73 (0.09)			
	62	-	0.85 (0.18)			
Brochotrix thermosphacta	54	-	0.45 (0.32)	6.5 (0.37)	0.93	0.09
	56	-	0.13 (0.69)			
	58	-	0.08 (0.43)			
	60	-	0.05 (0.05)			

T, temperature (°C); *Sl*, shoulder length (min); D_T, decimal reduction time value (min).
R^2, determination coefficient related and *RMSE*, root mean square error from the TDT curves to calculate *z* values. Standard deviations are shown in brackets.

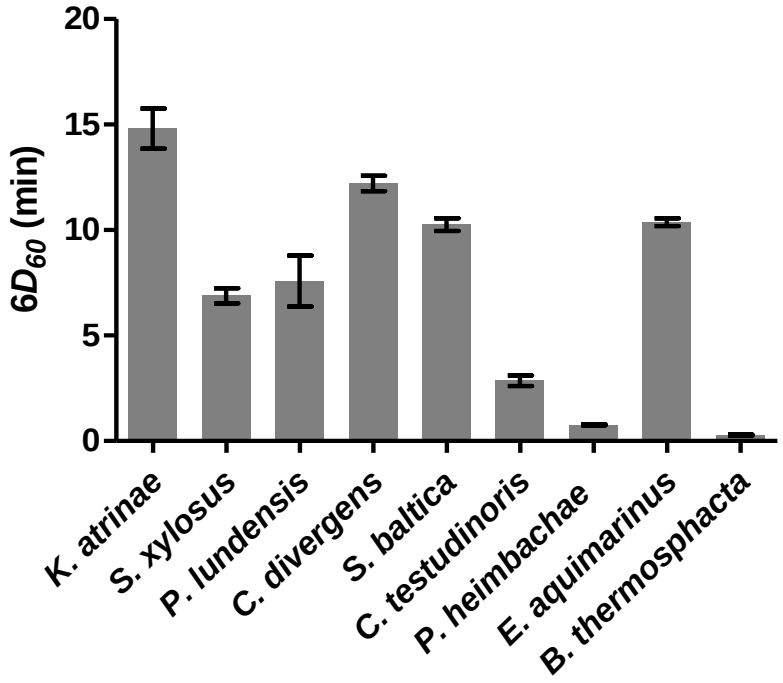


Figure 2.7: Time (min) necessary to reduce the population of different species by 6 Log_{10} cycles (6*D* values) in pH 7 McIlvaine citrate-phosphate buffer at 60°C.

The presence of shoulders in thermal inactivation curves has been reported by several authors in various treatment media and for different microorganisms. These observations have been attributed to limitations or artefacts related with the methodology used, to heterogeneity within microbial populations or to cell damage and repair phenomena (Arroyo *et al.*, 2011; Asselt and Zwietering, 2006; Hassani *et al.*, 2005). The same methodology was used for all microorganisms in this study limiting the influence of methodological artefacts and no aggregates were observed in cultures by microscopy. Hence, it is possible that the presence of a shoulder in *K. atrinae* could be related to a distribution of resistances within the population, or due to damage and repair phenomena. In any case, the results obtained in this research highlight the importance of considering deviations from linearity in microbial inactivation kinetics when designing a thermal process. Failure to consider these deviations could lead to selecting inappropriate target organisms which in the worst case scenario could invalidate the designed process.

2.4.10 Heat treatments for ready-to-eat brown crab

For processed seafood, *Listeria monocytogenes* as a vegetative microorganism, and *Clostridium botulinum* non-proteolytic type E as a spore forming microorganism, are widely accepted as the main target microorganisms to define heat treatments; and a reduction of 6 Log_{10} cycles of their populations is considered sufficient to ensure safe end products (FDA, 2011). To evaluate the effectiveness of the reference heat treatments for both vegetative cells and spore-forming bacteria on the natural flora identified in brown crab in this study, the thermal death time curves (TDT) were calculated in order to achieve 6 Log_{10} reductions (FDA, 2011). The reference TDT lines for *L. monocytogenes* and the most heat resistant vegetative cell isolated in this research (*Kocuria atrinae*) were compared (Figure 2.8). In parallel, Figure 2.9 shows three different TDT lines, based on the recommended treatments by FDA for *C. botulinum,* compared to the TDT lines calculated for the three spores isolated from processed brown crab, which are described in chapter 6 in this thesis.

For vegetative cells (Figure 2.8), a slight difference between the slopes (*z* value) was observed between the two microorganisms. However, the recommended treatment for *Listeria monocytogenes* ($F_{70}^{7.5}$= 2 min) is more than 3-fold higher than the treatment required to achieve the same inactivation level (6 log_{10}) in *Kocuria atrinae*. Therefore, if the treatment defined for *L. monocytogenes* (FDA, 2011) is to be applied to brown crab, an inactivation of more than 18 log_{10} cycles of *Kocuria atrinae* would be achieved.

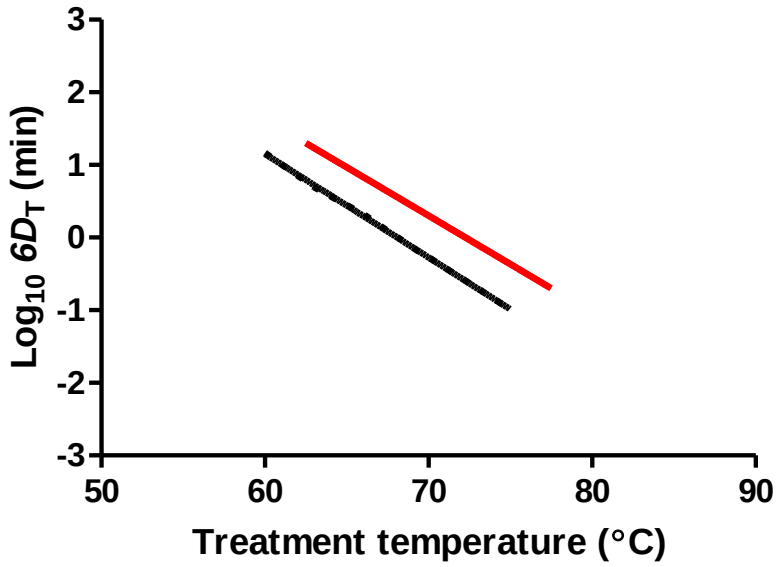


Figure 2.8: Theoretical thermal death time curves (TDT) to achieve 6 Log_{10} reductions of *Kocuria atrinae* (black line) and *Listeria monocytogenes* (red line). The latter based on the recommended treatments by FDA (FDA, 2011).

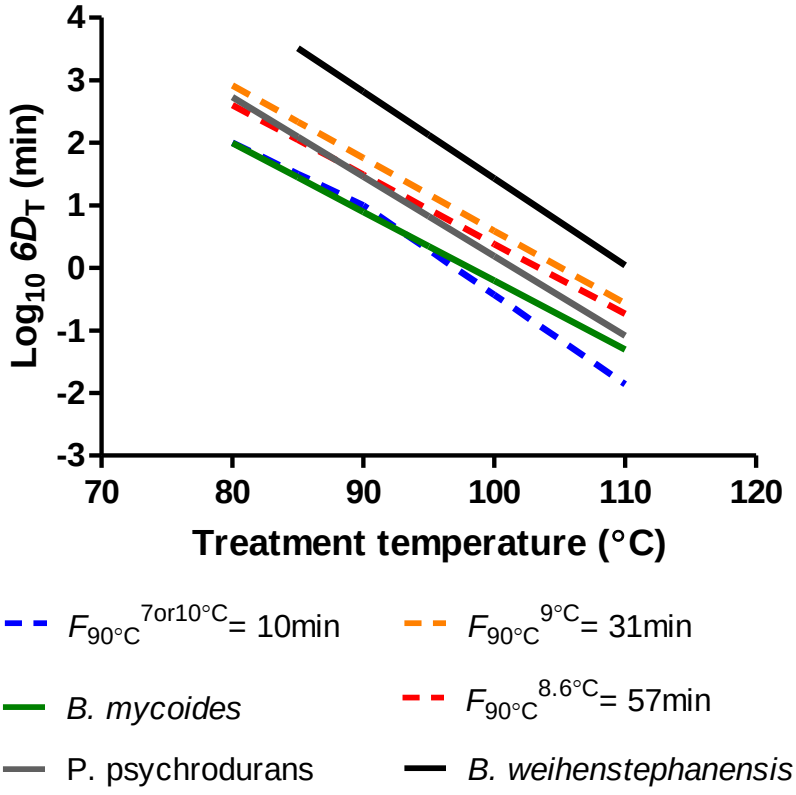


Figure 2.9: Theoretical TDT lines to achieve 6 Log_{10} reductions of the spore forming bacteria isolated from crab based on data presented in chapter 6 and heat treatments required to achieve similar reductions of *C. botulinum* non-proteolytic spores based on the recommended treatments by FDA ($F_{90°C}^{7or10°C}$ = 10 min; $F_{90°C}^{9°C}$ = 31 min; $F_{90°C}^{8.6°C}$ = 57 min) (FDA, 2011).

On the other side, Figure 2.9 shows the thermal treatments proposed for crab and crab based products considering *C. botulinum* non-proteolytic type E as the target microorganism. $F_{90°C}$= 10 min or equivalent, considering *z* values of 7°C for temperatures below 90°C, or 10°C for temperatures over 90°C. As it can be seen, this treatment would not reliably provide a reduction of 6 $\log_{10}$ cycles for *B. mycoides,* the most heat sensitive spore isolated from crab samples as it is shown in section 6.4.1. However, some researchers consider crab meat as an exception with regard to the inactivation of *Clostridium* spores due to its high concentration of lysozyme (Peterson *et al*., 1997). It is known that the presence of lysozyme improves the recovery of heat-treated *Clostridium* spores and therefore its heat resistance (Peck and Fernadez, 1995; Scott and Bernard, 1982). It has also been reported that this enzyme is present in fish and seafood up to levels of 200 µg/g (Lie *et al*., 1989; Lund and Notermans, 1992). For this reason, two more severe alternative treatments were proposed by the FDA for crab meat: $F_{90°C}^{9°C}$= 31 min (Blue crab, *Callinectes sapidus*) and $F_{90°C}^{8.6°C}$= 57 min (Dungeness crab, *Metacarcinus magister*) in order to achieve a satisfactory reduction of *Clostridium* spores (FDA, 2011; Peterson *et al*., 1997, 2002). These two alternative treatments are also represented in Figure 2.9. As the Figure shows, if the most severe recommended treatment, i.e. for Dungeness crab meat ($F_{90°C}^{8.6°C}$= 57 min), is to be applied to brown crab, an adequate reduction of two of the three spore formers isolated in this thesis (*B. mycoides* and *P. psychrodurans*) would be achieved. However, this treatment would not be sufficient to achieve a similar reduction in the population of *B. weihenstephanensis* spores, which would need treatment times 10-fold longer. Van Asselt and Zwietering (2006) reported similar differences when comparing the thermal resistance of various *Clostridium botulinum* and *Bacillus cereus* strains.

In 1998 *Bacillus weihenstephanensis* was proposed as a new species to accommodate some of the psychrotrophic strains of *Bacillus cereus* (Lechner *et al*., 1998). This spore-forming species is able to grow at temperatures of 5°C or less and the ability to produce emetic and diarrheal toxins, normally associated

with *B. cereus*, has also been reported (Stenfors and Granum, 2001; Stenfors *et al*., 2002; Thorsen *et al*., 2006, 2009). As a result, the isolation of this species in brown crab combined with its high heat resistance (higher than non-proteolytic species of *C. botulinum*) highlights their potential significance in terms of food safety. However, no disease associated with *Bacillus* spp. in brown crab products have been reported, probably due to products being packaged under vacuum, which can hinder the growth of *B. wiehentephanensis* (Samapundo *et al*., 2011). Overall, the results of this investigation showed that spores naturally present in brown crab meat could be more thermo-tolerant than the non-proteolytic strains of *C. botulinum*. Therefore, heat treatments designed for these spore-forming bacteria may not be sufficient to reduce populations to safe levels.

2.5 CONCLUSIONS

From the obtained results in this chapter it can be concluded that *Bacillus* spp., *Pseudomonas* spp. and *Staphylococcus* spp. are the main microbial groups present in raw white and brown crab meat. From the shelf-life study it can be concluded that the storage temperature has a greater effect on the lag phase than on the growth rate of the TVC_m during storage. A log-linear relationship was found between the storage temperature and the logarithm of shelf-life (hours) for both meat types (i.e. white and brown). *Bacillus* spp. and *Staphylococcus* spp. proved to be the bacterial groups present in a major proportion at the end of the shelf-life of the product. However, their proportions changed considerably depending on the storage temperature. Raw brown crab meat shows a greater heterogeneity in microbiota than white meat. In case of raw meat, *Carnobacterium divergens* and *Sewanella baltica* were the most frequently isolated microorganisms regardless of the meat type, while for cooked meat stored at 7-8°C, *B. mycoides*, *B. weihenstephanensis* and *P. psychrodurans* were the most frequently detected in all commercial meats and crabs cooked under controlled conditions at mild temperatures in the

laboratory. For vegetative cells, *Kocuria atrinae* showed the highest level of heat resistance among non-spore forming bacterial species isolated from brown crab meat, though importantly, the heat treatment recommended by the FDA to inactivate *L. monocytogenes* ($F_{70^oC}^{7.5^oC}$= 2 min) proved to be sufficient to inactivate it. In contrast, the most severe heat treatment recommended to inactivate non-proteolytic species of *C. botulinum* was enough to reduce 6 Log_{10} cycles of *B. mycoides* and *P. psychrodurans* but not *B. weihenstephanensis,* which would require a treatment time 10-fold longer. Overall, this section provides significant knowledge about the microbiota and the shelf-life of ready-to-eat brown crab (*Cancer pagurus*), a product of ample market but underestimated from a scientific point of view and highlights the importance of *Bacillus* spp. although additional studies would be required to determine the potential food safety risks associated with *B. weihenstephanensis* in brown crab based products.

Chapter 3

The use of ultrasound technology in the first cooking step of ready-to-eat whole brown crab (Cancer pagurus)

This chapter is as publish in Ultrasonics Sonochemistry, with some minor modifications to avoid duplication between chapters.

Condón-Abanto, S., Arroyo, C., Álvarez, I., Brunton, N., Whyte, P., & Lyng, J. G. (2018). An assessment of the application of ultrasound in the processing of ready-to-eat whole brown crab (*Cancer pagurus*). Ultrasonics Sonochemistry, 40, Part A, 497-504.

3.1 ABSTRACT/RESUMEN

ABSTRACT

This study assesses the potential of incorporating ultrasound as a processing aid in the production of whole cooked brown crab (*Cancer pagurus*). The FDA recommended heat treatment to reduce *Listeria monocytogenes* by 6 Log_{10} cycles in this product is a $F_{70ºC}^{7.5ºC}$ of 2 min. An equivalent *F* value was applied at 75°C in presence and absence of ultrasound in water alone or in water with 5% w/v NaCl added. Heat penetration, turbidity and conductivity of the cook water and also salt and moisture content of the crab meat (both white and brown meat) were determined. Ultrasound assisted cooking allowed a reduction of the cooking time by up to 15% while still maintaining an $F_{70ºC}^{7.5ºC}$ of 2 min. Ultrasound also enhanced the rate and total amount of compounds released from the crab, which suggests that crabs cooked in the presence of ultrasound would be expected to be cleaner. Ultrasound also proved to be effective in reducing the salt content but hardly affected the final moisture content of the crab meat.

RESUMEN

Este estudio se realizó para evaluar el potencial de la incorporación de la tecnología de los ultrasonidos como coadyuvante en la producción de buey de mar (*Cancer pagurus*) cocinado entero listo para el consumo. El tratamiento recomendado por la FDA para inactivar 6 ciclos logarítmicos de *Listeria monocytogenes* en este tipo de productos es un $F_{70ºC}^{7.5ºC}$ de 2 minutos. Se aplicó un *F* equivalente a 75°C en agua y agua con un 5% p/v de NaCl utilizando o no ultrasonidos durante el tratamiento. Se evaluó la penetración de calor y se midió la turbidez y conductividad eléctrica del agua de cocinado y el contenido de sal y humedad de ambos tipos de carne (blanca y marrón) de cangrejo tras los distintos tratamientos. El tratamiento con ultrasonidos permitió reducir hasta un 15% el tiempo total de tratamiento manteniendo un $F_{70ºC}^{7.5ºC}$ equivalente de 2 minutos. La aplicación de ultrasonidos durante el cocinado también mejoró la velocidad de salida de compuestos del cangrejo así como la cantidad total de estos compuestos, lo cual sugiere que los cangrejos cocinados en presencia de ultrasonidos deberían de estar más limpios. Los ultrasonidos fueron también efectivos para reducir el contenido de sal de la carne de cangrejo, pero prácticamente no afectaron a su contenido de humedad. En conclusión, los resultados obtenidos en este capítulo permitirían reducir el tiempo de cocción así como eliminar la etapa de lavado del proceso tradicional del cangrejo con las consiguientes consecuencias económicas y de calidad del producto.

3.2 INTRODUCTION

As mentioned before the production of ready-to-eat edible crab involve a series of general steps which have not evolved in line with current technological developments. Many producers still use traditional techniques and define their own cooking conditions in terms of time and temperature which leads to heterogeneity in the quality of marketable products (e.g. over or undercooking). The size of these companies is usually small and their investment in technology and process optimisation is generally low. However, novel processing technologies such as ultrasound have many benefits to offer them. For example, ultrasound technology is widely used in the food industry to enhance heat and mass transfer processes (Chandrapala, *et al.*, 2012; Chemat, *et al.*, 2011) which could have great relevance and be easily adopted to improve traditional immersion cooking processes used in the production of ready-to-eat crab.

High-intensity ultrasound involves intensities greater than 1 W/cm^2 and is performed at frequencies ranging from 18 to 100 kHz. Cavitation is considered the main mechanism by which this form of ultrasound enhances heat and mass transfer phenomena (Kim, *et al.*, 2004) though other effects such as acoustic streaming are also involved (Legay, *et al.*, 2011). The effects of ultrasound on heat transfer have been extensively studied since the 1990s in model systems such as water, metal tubes, metal balls, etc. (Huamao, *et al.*, 1997; Hyun, *et al.*, 2005; Oh, *et al.*, 2002) and its ability to enhance heat transfer in foods, mainly in processed meat products, during cooking processes has also been proven (Alarcon-Rojo, *et al.*, 2015; Vimini, *et al.*, 1983). The potential for ultrasound to assist different processes such as extraction (Khan, *et al.*, 2012; Luengo, *et al.*, 2014; Ma, *et al.*, 2009), cooking (Mason, *et al.*, 1996) and marinating (Cárcel, *et al.*, 2007; McDonnell, *et al.*, 2014; Turshan, *et al.*, 2013; Vinimi, *et al.*, 1983) have been assessed in vegetables tissues, meats and fish. However, its effects on the industrial heat processing of ready-to-eat crustaceans products have never been explored. Therefore, the aim of this

study was to assess the potential of ultrasound for the cooking of brown crab by accelerating heat and mass transfer processes. The increase of heat penetration should lead to a reduction in cooking times which in turn should enhance product quality whilst ensuring adequate levels of safety. In addition, the production of ready-to-eat brown crab involves a cleaning/cooling step which is needed to remove crab dirt and cook exudate deposits before packing. This step takes 3-4 h and constitutes a microbiological risk due to a possible re-contamination of the product, hence requiring a subsequent pasteurization step with the sole purpose of eliminating microbial contamination (Ghazala and Trenholm, 1996; Pagadala, *et al.*, 2012). The ability of ultrasound to enhance mass transfer could also be used to remove dirt and exudate from crab shells during cooking thus eliminating or reducing the severity of the subsequent pasteurization ultimately resulting in greater yields, less energy input and a milder heat- treated higher quality product.

Therefore the objective of this research was to evaluate the potential improvements induced by the application of ultrasound in the cooking process of ready-to-eat whole brown crab, with particular reference to the benefits of associated heat and mass transfer phenomena.

3.3 MATERIALS AND METHODS

3.3.1 Raw material and cooking conditions

All performed experiments were carried out with female crabs with weights ranging from 375 to 732g, landed in Ireland in the winter of 2014. Those were obtained from a local fishmonger and maintained alive at 4ºC in dry conditions for a maximum of 48 h. After storage, crabs were adequately euthanized (Roth and Øines, 2010) while maintaining the integrity of the carapace. Before cooking, each crab was characterised by measuring weight (grams) and dimensions (cm^2) (assuming that the crab shape was oval, the area of which was multiplied by two in order to account for both sides of the crab). After cooking the two types of crab meat, white and brown, were evaluated

separately. The white meat is the meat located in claws and legs and the brown meat inside the carapace. The distinction between the two types of meat was considered due to their different composition and market value.

Cooking experiments with and without ultrasound were performed immediately after euthanasia in an ultrasonic bath (Guyson mod. KS MK3 525, North Yorkshire, UK) with a tank capacity of 55L, a maximum ultrasound power of 900 W and a heating power of 2000 W. All trials were carried out by using the maximum volume of water (55L). Once the temperature of the water reached 75ºC, eight crabs were submerged and cooked for 45 min. Preliminary experiments (data not shown) showed that 45 min was a sufficient cooking time to apply an equivalent $F_{70ºC}^{7.5ºC}$ of 2 min, which corresponds to the FDA recommended heat treatment for ready-to-eat seafood products. This heat treatment ensures the inactivation of at least 6 Log_{10} cycles of *Listeria monocytogenes*, the target microorganism in pasteurised seafood products (FDA, 2011). For those experiments applying ultrasound, the maximum ultrasonic power of the tank was used, 900W (ultrasonic energy consumption). In order to standardize the cooking conditions as much as possible, the total weight of all batches ranged from 4.0 to 4.3kg. At least three independent replicates of each cooking conditions were performed on different working days.

3.3.2 Heat transfer study

To assess the effect of ultrasound on the heat transfer phenomena in crabs two different comparisons, based on mathematical models, were carried out.

3.3.2.1 Heat penetration curves

Heat penetration curves were obtained by placing a K type thermocouple (Alhborn, Holzikirchen, Germany) in the abdomen of the crab which corresponds to its cold spot (Figure 3.1) which had been previously identified in preliminary experiments (Figure 3.2).

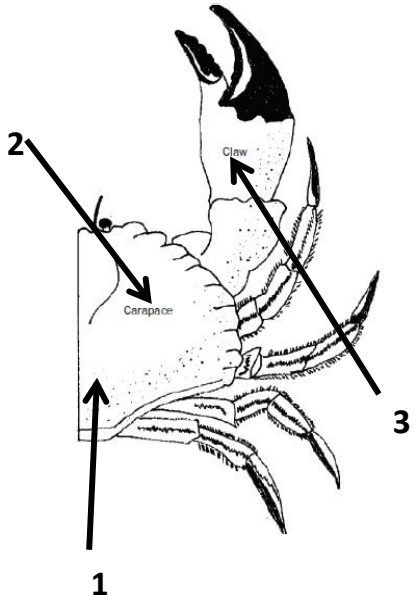


Figure 3.1: Scheme of the location of the three thermocouples during the heat penetration experiments: abdomen (1), mandibular (2) and claw (3). Image adapted from reference (BIM, 2017a).

The temperature was recorded using a data logger (Alhborn Type 2590-2) connected to a laptop with the data control software version 4.3 (32-bit). The heat penetration curves were subsequently fitted to the Ball & Olson Equation (Mafart, 1994) (Equation 3.1A and 3.1B):

$$\log \theta = \frac{1}{fh} \times \log j \qquad \text{(Equation 3.1A)}$$

$$\theta = \frac{T_{cook} - T_0}{T_{cook} - T_t} \qquad \text{(Equation 3.1B)}$$

Where *fh* is the maximum rate of heating up (dimensionless), *j* is the lag phase of the heat penetration curve (dimensionless), T_{cook} is the cooking temperature, i.e. 75 (°C), T_0 is the initial temperature in the crab's cold spot (°C) and T_t is the temperature reached in the crab's cold spot at specific times during the cooking process (°C).

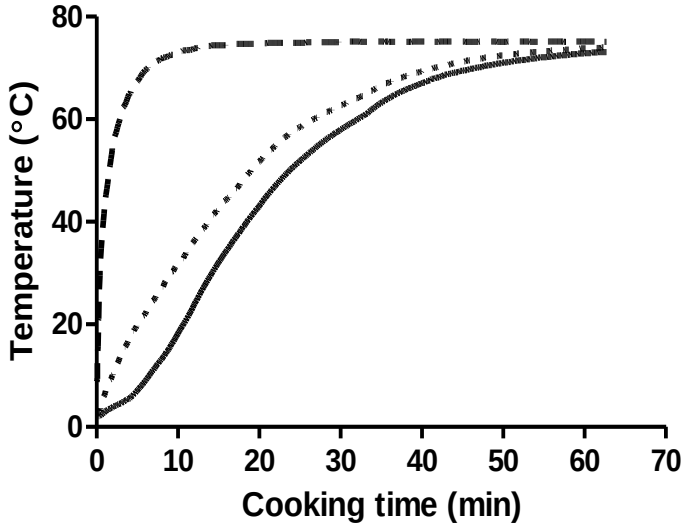


Figure 3.2: Time-temperature profiles over a cooking process at 75°C in the claw (dashed line), mandibula (dotted line) and abdomen (block line) of a 500 g crab in a conventional cooking process without ultrasound.

3.3.2.2 Lethality and F value

From the temperatures recorded during cooking, the equivalent lethality (L) and cumulative F equivalent values at each temperature were calculated using Equations 3.2 and 3.3, respectively.

$$L = 10^{\frac{T-Tref}{z}} \quad \text{(Equation 3.2)}$$

$$F = \sum_{x=0}^{x=i} L \times \Delta t \quad \text{(Equation 3.3)}$$

where T is the temperature (°C) reached in the crab's cold spot at specific times (x) during the cooking process, T_{ref} is the reference temperature considered for the target microorganism, *L. monocytogenes* (i.e. 70°C), z is the z value for the target microorganism, *L. monocytogenes* (i.e. 7.5°C), and Δt is the slot of time (min) during which the crab's cold spot is at the temperature T.

3.3.3 Microbiological examination of fresh crab meat

To assess the microbial reduction during the cooking process, crabs of a similar size (± 20g) were euthanized as described in Section 2.1 and stored at

4ºC for 72 h to allow the growth of the natural microbiota in the crab tissues (initial microbial load in crab meat was $\approx 10^2$ CFU/g). Following this, five crabs were cooked at 75ºC with and without ultrasound in the ultrasonic bath as described above. After 5, 10, 15, 30 and 45 min of cooking one crab was removed from the tank and submerged immediately in ice water in order to cool it down as quickly as possible. Following that, the white and brown meat from each crab was removed under aseptic conditions in a laminar flow cabinet (Faster, Mod. Bio 48. Ferrera, Italy). To assess the antimicrobial effect of sonication at temperatures below 30ºC (at this temperature the effect observed would be solely attributed to ultrasound and not to heat), the water from the bath was recirculated through a heat exchanger with a coolant to avoid temperature increases during treatment. Once the treatment conditions were stable (28 $\pm$ 2ºC), two crabs were submerged in the ultrasonic bath for 45 min and then their white and brown meat were extracted. Each treatment was performed three times on different days.

Total bacterial counts (TBC) were quantified by diluting 5 g of each type of meat from each crab in Maximum Recovery Diluent (MRD) (Oxoid, Hampshire, UK) and stomaching (400 circulator, Seward Stomacher, UK) for two min at 300 rpm. Then, 1 mL of the appropriate serial dilution was pour-plated in Tryptone Soya Agar (Oxoid), supplemented with 0.6% Yeast Extract (Oxoid), and incubated for 48 h at 30ºC. Longer incubation times did not increase the number of colonies observed on plates (data not shown). Microbiological assessment of raw crab meat was performed using the same procedure.

3.3.4 Mass transfer study

Conductivity and turbidity of the cook water were measured to assess the effect of ultrasound on mass transfer during the cooking of crabs (Method 1). In addition, the final salt and moisture content of the crab meat were also assessed after cooking (Method 2).

3.3.4.1 Method 1: Measurement of cook water turbidity and conductivity

Cook water turbidity was used as an indicator of the degree of exudate deposits removed during cooking. A volume of 10 mL of the cook water was taken at 5 min intervals during the 45 min cooking of each batch of eight crabs and the turbidity was measured using 1 cm of path length cuvettes in a spectrophotometer (UVmini-1240, Shimadzu). Measurements were performed at 515 nm, which was the wavelength at which the cook water showed the maximum absorbance (data not shown). Results were expressed in absorbance units at 515 nm. Cook water electrical conductivity was also used as an indicator of particulate loss, which is likely to be associated with the release of ionic compounds from the whole crab. Measurements were performed every 5 min during the 45 min cooking process of crab batches using a conductivity-meter (CyberScan mod. CON 400/410 & TDS 400). Each measurement was performed once the water had been allowed to cool down below 30ºC. Results were expressed in µS/cm.

3.3.4.2 Method 2: Measurement of salt and moisture content of crab meat

Salt content of white and brown crab meat was measured after cooking crabs in the presence or absence of ultrasound in water with and without 5% NaCl added (w/v), following an adaptation of the method described by Fox (1963). In brief, 2 g meat samples were placed in glass beakers (250 mL) to which 100 mL of a 0.1 N nitric acid solution (Fisher Scientific, Leicester, UK) was added. The mixture was then homogenized with an ultraturrax (DI 25 basic, IKA-WERKE, Germany) for 20 s at 10,000 rpm. After homogenization beakers were placed in a water bath (Davidson & Hardy LTD, Dublin, Ireland) at 65ºC for 15 min before cooling the samples to room temperature (≈20ºC). After cooling, samples were titrated against 0.1 N silver nitrate solution ($AgNO_3$) (Fisher Scientific) using a magnetic stirrer. During titration, silver concentrations were continually monitored using a coupled silver electrode with a reference

electrode (calomel) and the potential difference was measured in mV on a pH meter (Jenaway 3505, Bibby scientific Ltd., UK). The end of titration was determined when the pH-meter reached +225 mV. The salt content in brown and white crab meat was then calculated using Equation 3.4.

$$\% \text{ NaCl} = \frac{mL\ AgNO_3 \times 0.585}{weight\ of\ sample\ (g)} \qquad \text{(Equation 3.4)}$$

Crab meat moisture content was determined by oven drying following the AOAC (Association of Official Analytical Chemist) (1995) method. All analysis was carried out at least in triplicate.

3.3.5 Statistical analysis

T-tests (p=0.05) and ANOVA tests (p=0.05) followed by Tukey's test were used to define statistical differences among samples. GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used and differences were considered significant for P≤0.05. Error bars in the Figures correspond to the standard error of the mean.

3.4 RESULTS AND DISCUSSION

3.4.1 Heat transfer

To facilitate the study of the effect of ultrasound on heat penetration during the cooking of crabs, heat penetration curves were fitted to the Ball & Olson Equation due to its simplicity and goodness of fit (Table 3.1). The parameters *fh* and *j* were calculated from the heat penetration curves of crabs cooked in water at 75°C without (n = 27) and with ultrasound (n = 10, 900W). For comparison purposes, crabs were grouped by weight into 3 categories, i.e. small (<450g), medium (450-600g) and large (>600g). Table 3.1 also shows the R^2 and *RMSE* coefficients used as indicators of the goodness of fit of the model. In all cases the R^2 values were higher than 0.9, indicating a good goodness of fit and *RMSE* values ranged from 0.03 to 4.19. Regarding the *j* value, the ANOVA analysis revealed that there were no statistically significant

differences between crab weight categories regardless of whether ultrasound was applied or not (p>0.05).

The absence of differentes could be explained by variations on the morphology and integrity of the crab carapaces. For example, whilst every effort was made to maintain the integrity of the carapace during sample preparation, its integrity and morphology can vary depending on different factors such as the physiological status of the crab and the transportation (Edwards and Early, 2001; Tully, *et al.*, 2006; Woll, 2006). This would result in the uptake of cook water which could directly affect the lag-phase. When compared with the heat-only cooking process, the application of ultrasound reduced the *fh* value by 5.4%, 14.9% and 29.2% for the 'small', 'medium' and 'large' crabs, respectively. These results indicate that the use of ultrasound not only increases the heat penetration in the crab's cold spot but also reduces the effect of crab weight (and size by extension) on the heating ratio of the cold spot.

Table 3.1: Heat penetration parameters (*fh* and *j*, dimensionless) arising from the application of the Ball & Olson model to the heat penetration curves in the cold spot of brown crabs (*Cancer pagurus*) of different weights and sizes cooked with or without ultrasound in water at 75°C.

Weight (g)	Cooking type	Length (cm)	Width (cm)	*fh*	*j*	R^2	*RMSE*
394.5	Ultrasound-assisted	14.0	8.7	29.79	1.01	0.99	1.19
422.0	Ultrasound-assisted	14.3	8.9	28.85	1.45	0.99	0.63
432.0	Ultrasound-assisted	14.5	8.5	25.03	1.46	0.99	0.38
485.4	Ultrasound-assisted	15.1	9.5	36.07	1.52	0.99	0.58
624.9	Ultrasound-assisted	16.7	10.6	36.94	1.13	0.97	1.62
660.0	Ultrasound-assisted	16.0	9.5	32.51	1.12	0.99	0.82
693.3	Ultrasound-assisted	17.5	11.2	30.42	1.04	0.94	3.88
706.0	Ultrasound-assisted	17.0	10.5	28.42	1.0	0.99	1.24
822.0	Ultrasound-assisted	18.0	11.5	43.04	1.07	0.99	0.61
842.0	Ultrasound-assisted	19.3	12.5	37.39	1.59	0.99	0.46

Table 3.1 Continued

Weight (g)	Cooking type	Length (cm)	Width (cm)	*fh*	*j*	R^2	*RMSE*
300.0	Conventional	13.0	8.0	18.45	1	0.95	4.19
348.0	Conventional	14.0	9.0	21.44	1.05	0.97	1.54
375.5	Conventional	14.0	9.0	31.48	1.21	0.99	1.15
388.0	Conventional	14.0	8.5	20.68	1	0.95	3.47
398.0	Conventional	14.5	9.0	29.09	1.11	0.99	0.31
404.0	Conventional	14.5	8.5	26.45	1.02	0.99	0.55
411.4	Conventional	15.0	9.5	22.79	1.07	0.99	0.59
428.0	Conventional	14.0	9.5	17.12	1	0.98	1.09
432.0	Conventional	14.5	9.0	24.15	1.47	0.99	1.01
455.4	Conventional	14.5	9.0	33.99	1.4	0.99	0.91
462.8	Conventional	15.0	10.5	27.68	1.48	0.99	0.69
472.0	Conventional	16.0	9.0	27.55	1.27	0.99	0.03
478.0	Conventional	14.5	9.0	30.12	1.36	0.99	0.21
484.0	Conventional	15.0	9.0	34.14	1.23	0.99	0.39
500.0	Conventional	16.5	10.5	35.45	1.58	0.99	0.47
538.0	Conventional	17.0	10.5	37.88	1.13	0.99	0.19
553.6	Conventional	16.0	10.0	40.62	1.11	0.99	0.31
586.9	Conventional	16.5	10.0	35	1.06	0.99	1.62
614.6	Conventional	16.0	10.0	39.91	1.47	0.99	0.48
630.5	Conventional	16.5	11.0	43.4	1.09	0.99	0.24
640.7	Conventional	18.8	11.0	43.67	1.5	0.99	0.47
714.0	Conventional	18.0	11.5	41.35	1.4	0.99	0.69
732.7	Conventional	17.5	11.0	42.99	1.05	0.99	0.30
799.4	Conventional	17.5	11.0	40.91	1.06	0.99	1.91
816.8	Conventional	19.0	12.5	42.65	1.28	0.98	1.60
829.7	Conventional	18.0	11.0	40.97	1.29	0.99	0.64
869.7	Conventional	19.0	13.5	64.32	1	0.90	0.06

In addition, a linear relationship between *fh* values and crab weight was noted in each cooking process indicating that heat penetration in the crab cold spot is weight-dependent. Table 3.2 includes the first order Equations which correlate the increase in *fh* values with the increase of the weight of the crab in both cooking processes. Significantly different slopes were observed between Equations (P≤0.05), indicating that the crab weight affected heat penetration to a differing extent depending on the cooking process. When ultrasound was used to assist the cooking the slope was 2.6-fold smaller, meaning that the weight of the crab had a much smaller effect on increases in *fh* value. Hence, the larger the crab the greater the impact of ultrasound in enhancing the heating rate as indicated in Table 3.2.

Table 3.2: First order Equations correlating *fh* values (dimensionless) with the weight of brown crabs (g) during conventional and ultrasound-assisted cooking.

Cooking type	Equation	*RMSE*	*Bf*	*Af*
Conventional	$fh = 0.053 \times weight\ (g) + 4.9$	5.57	1.01	1.14
Ultrasound-assisted	$fh = 0.020 \times weight\ (g) + 20.4$	4.23	1.01	1.12

Much of the work published to date attributes the ultrasonic enhancement of heat transfer to the formation of cavitation bubbles (Wong and Chon, 1969), although other authors have also suggested that improvements in convection heat transfer may also be due to acoustic streaming (Gould, 1966; Hyun, *et al.*, 2005). Either way, it is generally accepted that ultrasonically induced heating is a result of energy dissipation from the accumulation of cavitation bubbles at the interface of the submerged body (Baffigi and Bartoli, 2012; Kiani, *et al.*, 2012). Additionally it is accepted that the number and density of cavitation bubbles can play an important role in the heat transfer caused by ultrasound and also that the cavitation of bubbles increases the micro-convection effect at the product surface (Kim, *et al.*, 2004).

In relation to the surface area, some researchers have reported a relationship between the weight and dimensions (width and length) of the

carapace of a crab (Klaoudatos, *et al.*, 2013). In the present study a linear relationship (represented by Equation 3.5) was found between the surface area (cm^2) of the carapace and the crab weight (g) (Figure 3.3).

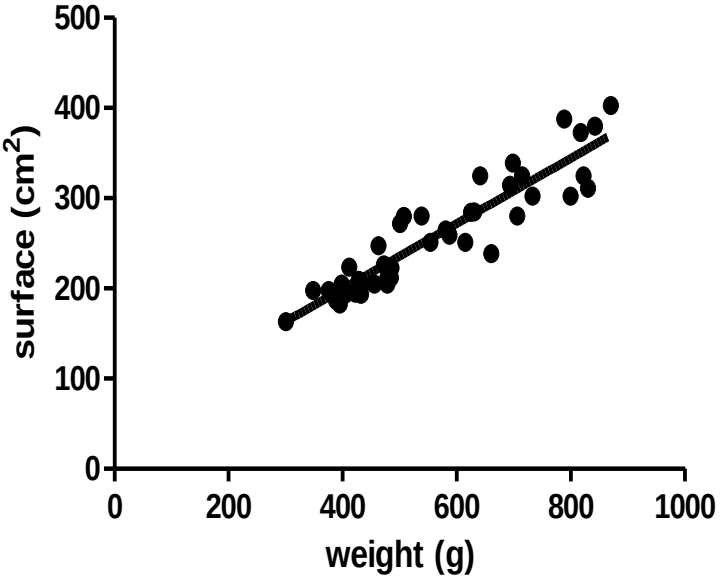


Figure 3.3: Relationship between crab's weight (from 300 to 870 g) and the total surface of the crab's carapace (cm^2).

$$Surface\ (cm^2) = 0.362 \times weight\ (g) + 54.83 \quad (R^2 = 0.85) \qquad \text{(Eq. 3.5)}$$

This relationship indicates that heavier crabs had higher carapace surface areas (Figure 3.3). Therefore the greater impact of the ultrasonic field on the reduction of *fh* values in heavier crabs could be attributed to their larger surface areas and as a consequence a greater amount of cavitation bubbles around their surface.

The effectiveness of a heat treatment in terms of microbial inactivation is given by the applied *F* value. As indicated before, the cooking processes applied in the current study were designed following FDA recommendations (FDA, 2011) to ensure a 6 Log_{10} reduction of *L. monocytogenes* in seafood products ($F_{70^oC}^{7.5^oC}$ = 2 min). A cooking process of 45 min in water at 75°C was previously demonstrated to achieve this minimum recommended *F* value in all

crabs irrespective of their weight (data not shown). For each cooking process, either with or without ultrasound, the actual equivalent $F_{70^oC}^{7.5^oC}$ value applied was calculated based on the corresponding heat penetration curves. Figures 3.4A, 3.4B and 3.4C show the $F_{70^oC}^{7.5^oC}$ values attained during the cooking of crab at 75°C without ultrasound (block line) and with ultrasound (dashed line) in small, medium and large crabs, respectively. Figure 3.4 also shows the threshold for the target $F_{70^oC}^{7.5^oC}$ of 2 min (horizontal dotted line).

The *F* value applied in the ultrasound-assisted cooking was 2.5-, 3.2- and 2.2-fold higher than the conventional heat-only cooking for the small, medium and large crabs, respectively. In other words, the cooking time was reduced by 15.5% (from 45 to 38 min), 16.9% (from 45 to 37.4 min) and 12.7% (from 45 to 39.3 min), respectively, while applying the same *F* value when ultrasound was used during cooking.

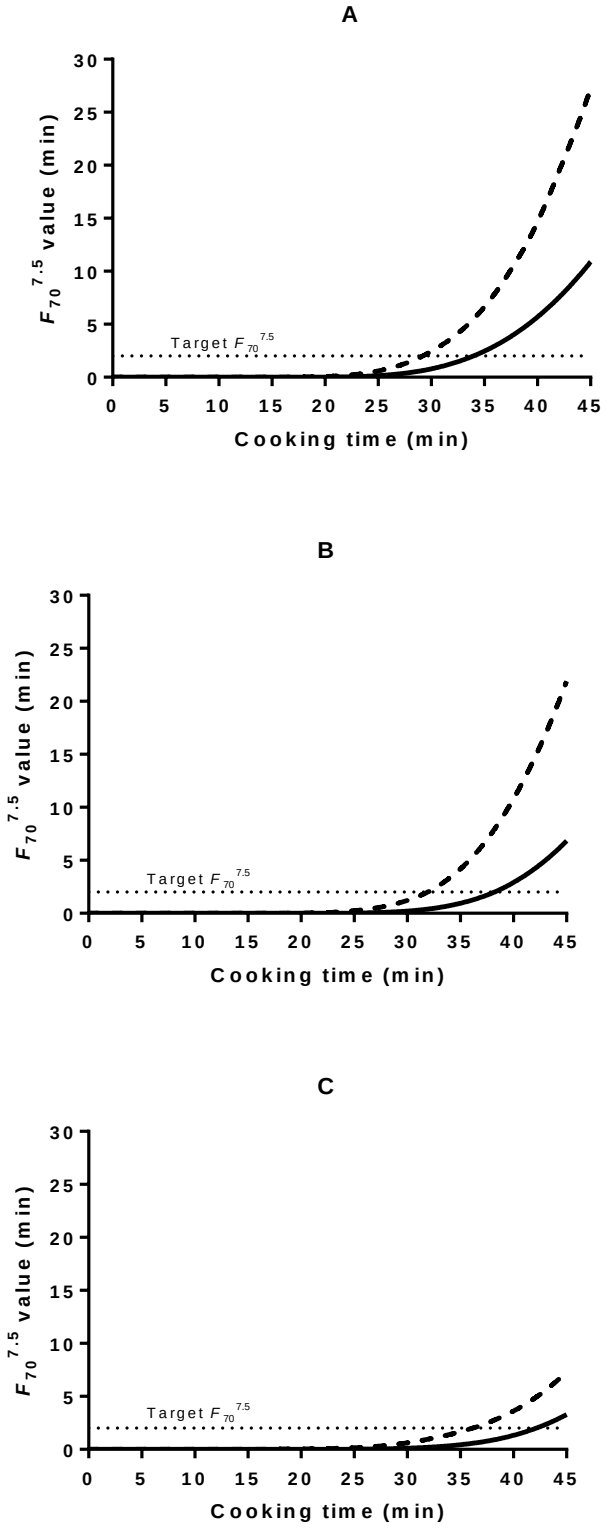


Figure 3.4: $F_{70^oC}^{7.5^oC}$ value (min) applied during the cooking process in the crab's cold spot (i.e. abdomen), with (dashed line) and without (block line) ultrasound for the (A) small, (B) medium and (C) large crabs. The horizontal dotted line represents the target $F_{70^oC}^{7.5^oC}$ of two minutes.

The efficacy of the two cooking processes was also evaluated by enumerating total bacterial levels in white (Figure 3.5A) and brown crab meat (Figure 3.5B). After 5 min of ultrasound-assisted cooking the microbial load in white meat was 0.6 Log_{10} cycles lower than in samples which had undergone regular cooking (2.4 *vs* 1.8 Log_{10} cycles), while brown meat needed a 10 min longer treatment to achieve similar reductions (2.3 *vs* 1.8 log_{10} cycles). These results indicate that the microbial reductions in crab are significantly higher in products exposed to an ultrasound assisted cooking. In addition, the effect of ultrasound alone was evaluated by measuring microbial loads in white and brown meat treated at temperatures below 30ºC for 45 min. In this case no differences were detected in the microbial loads regardless of the meat type (p>0.05).

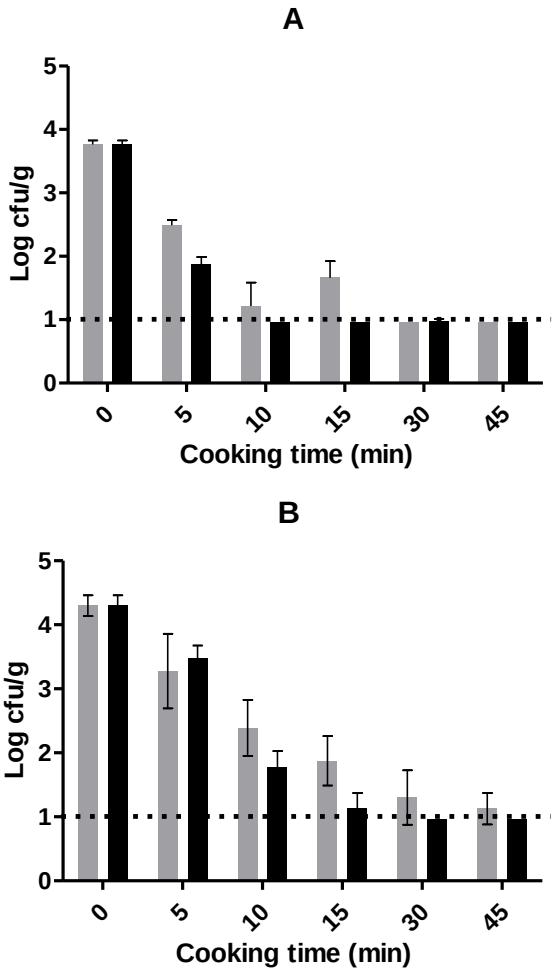


Figure 3.5: Microbial load over conventional (grey bars) and ultrasound assisted cooking processes (black bars) in (A) white meat and (B) brown meat. Dotted line shows the detection limit for the counts.

Some authors have suggested that the higher microbial inactivation levels often observed when ultrasound and heat are combined (i.e. thermo-sonication) is due to additive or synergistic effects between the two technologies (Arroyo, *et al*., 2012; Raso, *et al*., 1998). However, in the case of crab cooking, when ultrasound was applied at low temperatures the microbial loads did not decrease. Therefore, the greater microbial reduction observed could be attributed to the fact that ultrasound improved heat penetration in the crab rather than as a result of the effect of ultrasound itself. For example, as a consequence of more rapid heat penetration the cumulative *F* value increased at a higher rate which would result in greater reduction in microbial loads during ultrasonic cooking. Furthermore, ultrasound may have sub-lethally damaged bacterial cell envelopes thus reducing heat tolerance and therefore resulting in greater microbial reduction values compared to those samples that received the heat only treatment (Sala, *et al*., 1995).

3.4.2 Mass transfer

Ultrasound technology is widely used in the food industry to improve processes involving mass transfer phenomena such as cleaning, extraction, brining, pickling, marinating and curing (Chemat, *et al*., 2011; McDonnell, *et al*., 2014). In this section the effect of ultrasound on the mass transfer phenomena occurring during the cooking of crabs is quantified using two different methods.

3.4.2.1 Method 1: Turbidity and conductivity of cook water

Figure 3.6A illustrates the mean cook water turbidity value after cooking with and without ultrasound. It is very clear that the turbidity of the cook water increased more rapidly after 15 min when ultrasound was used to assist the process. After 40 min of cooking with ultrasound the turbidity of the cook water reached a maximum of 1.04 absorbance units which constitute a 113.7% increase compared to turbidity in conventional cook water. The conductivity of the cook water (Figure 6B) also increased in the presence of ultrasound after 10 min indicating a faster rate of ionic compound release from the crab. The

maximum increase in cook water conductivity was reached after 35 min of cooking, and was 55.7% higher than values observed in water used for the cooking without ultrasound. Regular commercial practice in the Irish crab industry involves a cleaning/cooling step with fresh water immediately after cooking. This step is critical in terms of microbial safety as recontamination can potentially occur. As a result, immediately after the cleaning/cooling step crabs are packed and pasteurized (Edwards and Early, 2001; Lalitha and Thampuran, 2012). If the turbidity of the cook water is considered as an indicator of the removal of dirt from the crab's surface, as Figure 3.7 suggests, the results obtained with ultrasound indicate that the use of this technology may have the potential for eliminating the cleaning step as this ultrasonically induced cleaning would be done concurrenly with the cooking.

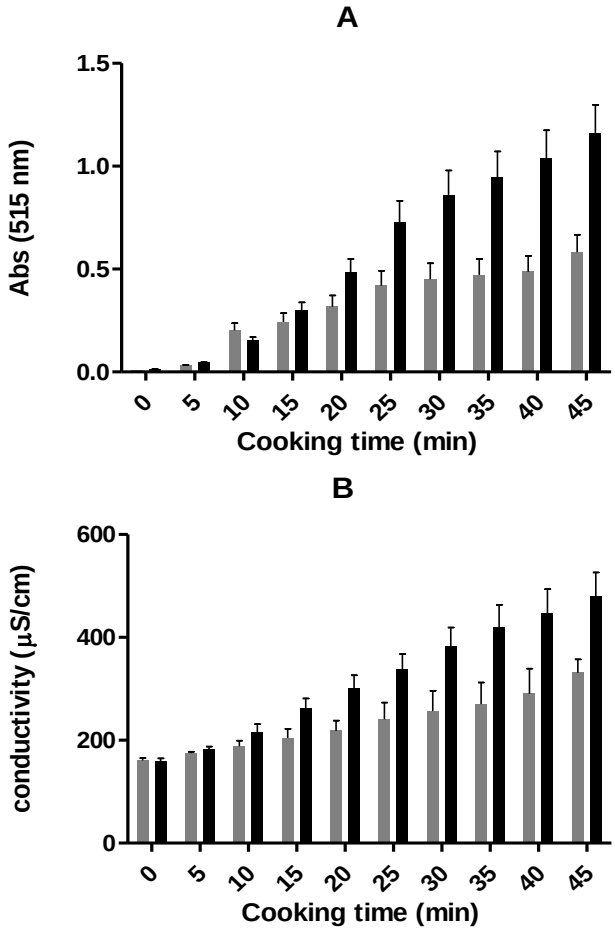


Figure 3.6: (A) Turbidity (OD_{515}) and (B) conductivity (µS/cm) values for the cook water during the cooking of brown crabs in water at 75°C with (black bars) and without (grey bars) ultrasound.

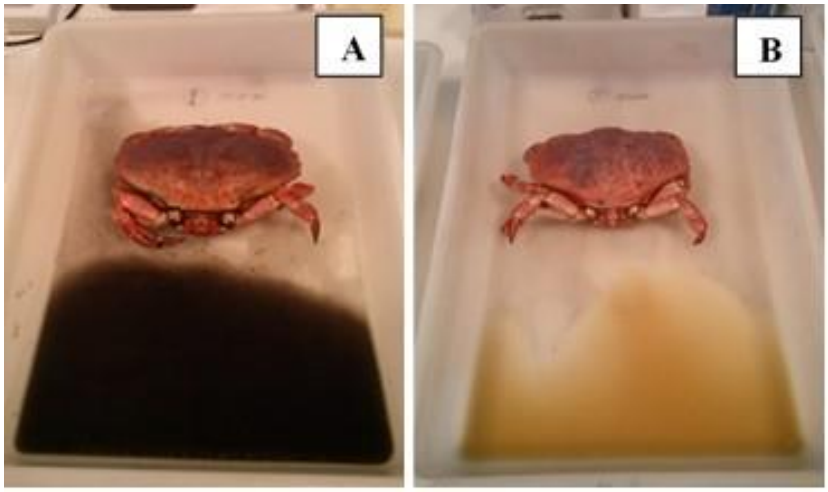


Figure 3.7: Picture of the crab's exudate after a conventional cooking (A) and ultrasound-assisted cooking (B).

3.4.2.2 Method 2: Salt content of the crab meat

The salt content in both white and brown crab meat was measured to assess the potential of ultrasound to transfer substances from the cook water into the crab meat. The initial salt content for raw crab was 1.43% in the white meat, which is located mainly in claws and legs, and 0.89% in the brown meat, which is located inside the carapace.

Salt content before and after cooking with and without ultrasound and in the presence or absence of 5% NaCl in the cook water is shown in Table 3.3. When the crabs were cooked in water without NaCl added the final salt content in white meat was reduced by 33.6% using regular cooking and by 46.1% for the ultrasound-assisted cooking. When 5% NaCl was added to the cook water, the salt content in white meat remained stable after regular cooking and was reduced by 21.1% following ultrasound assisted cooking. For brown meat, the salt content remained the same when crabs were cooked either with or without ultrasound in water without 5% NaCl added as opposed to white meat. However, when crabs were cooked in water with 5% NaCl added, the salt content in brown meat slightly increased from 0.89% (raw) up to 1.34% in the absence of ultrasound and remained unchanged with the application of ultrasound. Our results suggest that ultrasound did not facilitate the uptake of

salt during cooking in water with 5% NaCl added. This effect may be due to the physical barrier of the crab carapace which could act as a resonant box on which cavitation bubbles are produced on the internal surfaces. These bubbles could create micro-currents which could aid in the release of the salt from the meat to the cook water even against an osmotic gradient.

The effect on white meat when crabs were cooked in water without NaCl added suggests that ultrasound enhances the release of salt from the meat. Some authors observed a similar effect with enhanced extraction when vegetable tissues such as tomato peel were treated with ultrasound (Luengo, *et al.*, 2014; Riera, *et al.*, 2004). Other studies assessing the effectiveness of ultrasound for accelerating the marinating of meats reported contrasting findings to those in the current study (Cárcel, *et al.*, 2007; Mason, *et al.*, 1996; McDonnell, *et al.*, 2014) showed that the content of salt in slices of pork tenderloin increased when they were soaked in a saturated salt solution for 45 min with increasing ultrasound intensity. Also Siró *et al.*, (2009) showed a significant improvement in salt difussion in pork loins when ultrasound was applied. A similar effect was observed by Turhan *et al.*, (2013) who reported enhanced rates of marinating in anchovies when ultrasound was used.

Table 3.3: Salt content (%) and moisture content (%) in white and brown crab meat cooked with or without ultrasound in water and water with 5% NaCl. Values represent mean value ± standard error. For both salt and moisture content, ANOVA followed by Tukey's tests were performed for white and brown meat independently.

		White meat		Brown meat	
		Water	**Water with 5% NaCl**	**Water**	**Water with 5% NaCl**
Salt content (%)	**Raw**	1.43 (0.03)^{c}	1.43 (0.03)^{c}	0.89 (0.03)^{a}	0.89 (0.03)^{a}
	CC	1.06 (0.03)^{b}	1.33 (0.03)^{c}	0.96 (0.12)^{a}	1.34 (0.02)^{b}
	USC	0.84 (0.02)^{a}	1.17 (0.05)^{b}	0.98 (0.02)^{a}	0.81 (0.04)^{a}
Moisture content (%)	**Raw**	75.83 (3.85)^{b}	75.83 (3.85)^{b}	49.08 (0.54)^{a}	49.08 (0.54)^{a}
	CC	75.02 (1.65)^{b}	60.75 (0.92)^{a}	56.61 (1.44)^{b}	45.20 (0.20)^{a}
	USC	70.08 (0.89)^{ab}	69.98 (2.85)^{ab}	56.32 (1.70)^{b}	63.60 (1.51)^{c}

Raw: Raw meat, CC: Conventional cooking, USC: Ultrasound-assisted cooking. Different superscript letters indicate significant differences (P≤0.05).

3.4.2.3 Moisture content

Table 3.3 shows the moisture content of both white and brown meat before and after cooking with and without ultrasound in water with or without 5% NaCl added. Raw white meat had a moisture content of 75% while that of raw brown meat was 49%.

Regarding white meat, the moisture content remained unchanged (70-75.8%) when cooked in water without NaCl added regardless of the presence/absence of ultrasound. When NaCl was added the moisture content decreased in both cooking processes though this reduction was less evident in the presence of ultrasound.

In the case of brown meat, the moisture content increased at the same ratio, from 49% up to 56%, when crabs were cooked in water without NaCl added regardless of the cooking process. However, when cooking in water with NaCl added, the moisture content remained unchanged with the regular cooking process but increased by up to 63.6% in the presence of ultrasound.

These results revealed that overall ultrasound did not reduce the moisture content in any type of crab meat and in most cases even increased it. Other authors also observed similar effects when ultrasound assisted cooking or brining processes in meat (Jayasooriya, *et al.*, 2007; Smith, 2011; Vimini, *et al.*, 1983). Also in the case of marinated anchovies Turhan *et al.*, (2013) observed that the lower ultrasound intensity tested did not affect the final moisture content of the anchovy marinades although it decreased while ultrasonic intensity increased. Sánchez *et al.*, (1999) also reported that the use of ultrasound increased water losses during cheese brining. These authors attributed the reduction of the moisture to the cavitation phenomenon. This controversy could be attributed to the different matrixes and ultrasonic power used. In the case of the crab the carapace could act as a barrier, reducing the moisture losses. In addition, the low ultrasonic power used in this study (0.4

W/cm^2) could explain the small effect of ultrasonic cooking on the crab's moisture content.

The above literature has shown that ultrasound can either induce mass transfer in or mass transfer out of a matrix depending on the ultrasonic conditions and matrix characteristics. In our case, the cavitation of ultrasound that occurs in the interphase between the cook water and the carapace would create water jets which would clean the surface of the crab producing the increment of turbidity and conductivity. These implosions would limit the penetration of salt observed when 5% NaCl was added to the cook water and also would reduce the water movement from inside the crab to the cooking medium, as it has been described in other food matrices. It is clear that these mass transfer phenomena require more research and a deeper evaluation of each of them by studying the influence of different ultrasound parameters with adequate equipment. What it is clear from this study is that there are potential practical benefits for the crab cooking industry.

3.5 CONCLUSIONS

The main objective of this research was to evaluate the potential of ultrasound to enhance and optimize the cooking process of ready-to-eat brown crab (*Cancer pagurus*). From the results obtained it can be concluded that the application of ultrasound during the cooking process enhanced the heat transfer to the crab's cold spot (the abdomen) and also proved to be useful to reduce the effect of the crab weight on the heating rate. This effect would allow for (a) a reduction of the total cooking time while the same *F* value is applied or (b) an increase of 2.2-3.2 folds the total *F* value reached by applying the same treatment time. The application of ultrasound proved its efficiency to enhance the release of substances (dirt, cook loss deposits and ionic compounds) from the crab to the cook water which would also allow for the omission of the subsequent pasteurization routinely performed in the traditional procedure. Moreover, ultrasound prevented the uptake of NaCl in the brown meat when

cooked in water with 5% NaCl which was in contrast with the conventional cooking. Despite the evident advantages in economic and quality terms, more research needs to be done in this field to optimize the ultrasonic conditions for the processing of whole crab.

Chapter 4

Use of ultrasound technology combined with mild temperatures to reduce cadmium content of edible crab (Cancer pagurus).

This chapter is as publish in Ultrasonics Sonochemistry, with some minor modifications to avoid duplication between chapters.

Condón-Abanto, S., Raso, J., Arroyo, C., Lyng, J.G., Condón, S. and Álvarez, I. (2018). Evaluation of the potential of ultrasound technology combined with mild temperatures to reduce cadmium content of edible crab (*Cancer pagurus*). Ultrasonics Sonochemistry, 48, 550-554.

4.1 ABSTRACT/RESUMEN

ABSTRACT

The consumption of crustaceans is linked to certain health risks, particularly due to several highly toxic elements they contain, such as cadmium (Cd). Although the Cd content of one sole crab generally exceeds the total weekly recommended intake of cadmium as established by EFSA, efficient modern strategies to reduce Cd content in crabs have still not been developed. The objective of this research was to evaluate the potential use of ultrasound technology in combination with temperature (50-80ºC) with the purpose of releasing Cd from brown crab (*Cancer pagurus*), thereby reducing the Cd content in its meat. Female crabs were immersed in a water bath at 50, 65, and 80°C with and without the application of ultrasound. Cd concentration in the water was monitored over time. At the end of the process, Cd content in brown and white crab meat was also quantified. Treatment temperature did not have an influence on the release of Cd in absence of ultrasound, but proved to be an important variable when ultrasound assisted the process. Ultrasound increased Cd release rates 8.7-, 2.1- and 2.7-fold when applied at temperature of 50, 65 and 80°C, respectively. The maximum percentage of Cd extracted (22.8%) was observed at 50°C at an ultrasound input power of 200 W. These results have demonstrated for the first time that the application of ultrasound during the crab-cooking process could serve as an effective physical procedure for reducing the Cd content of crabs, thereby improving the product's safety for consumers.

RESUMEN

El consumo de crustáceos está ligado a ciertos riesgos para la salud, especialmente debido a ciertos elementos de alta toxicidad que contienen como el cadmio (Cd). Aunque el contenido de Cd de un solo buey de mar, generalmente excede el consumo semanal máximo recomendado por la EFSA, todavía no se ha desarrollado ninguna estrategia eficaz para reducir el contenido de este compuesto de los cangrejos. El objetivo de esta investigación evaluar el uso de la tecnología de ultrasonidos, en combinación con diferentes temperaturas (50-80°C), para reducir el contenido de Cd de la carne de buey de mar. Los cangrejos se sumergieron en agua a 50, 65 y 80°C en presencia o ausencia de ultrasonidos y se monitorizó la concentración de Cd en el agua de tratamiento a lo largo del tiempo. Además tras el procesado también se determinó la concentración de Cd en la carne blanca y marrón de los cangrejos tratados. La temperatura de tratamiento no influyó en la salida de Cd desde el cangrejo en aquellos tratamientos en los que no se aplicaron ultrasonidos. Sin embargo este parámetro de procesado fue una variable importante en el efecto de los ultrasonidos sobre la reducción de Cd. La aplicación de ultrasonidos aumentó las velocidades de salida del Cd 8,7. 2,1 y 2,7 veces en los tratamientos a 50, 65 y 80°C respectivamente. El máximo porcentaje de reducción de Cd (22,8%) se observó a la temperatura de 50°C utilizando una potencia ultrasónica de 200 W. Este trabajo demuestra que la aplicación de ultrasonidos durante el cocinado del buey de mar podría servir como un procedimiento efectivo para reducir la concentración de Cd del buey de mar, mejorando así su seguridad alimentaria.

4.2 INTRODUCTION

As mentioned before crustaceans are currently considered as a healthy choice in an equilibrated diet. However, the consumption of crustaceans is also associated with certain health risks due to high cholesterol levels in some edible parts, potential allergic reactions and contamination by toxic elements (Maulvault *et al*., 2012). Brown crab (*Cancer pagurus*) is one of the most appreciated crustaceans worldwide, especially in Southern European countries (Barrento *et al*., 2009a).

In the EU, the cadmium (Cd) content in crabs is regulated for muscle meat from appendages (legs and claws) i.e. white meat, being 0.5 mg/kg (wet weight) the maximum level allowed for compliance (Commission Regulation (EC) No 1881/2006; Commission Regulation (EU) No 420/2011). However, the cephalothorax, which comprises the digestive organs (hepatopancreas), i.e. brown meat, and known to contain high levels of Cd is excluded from the remit of the regulation. Several studies have warned of the risk in exceeding the tolerable weekly intake (TWI) of Cd established by EFSA (2009) (2.5 μg/kg body weight), especially in the case of consumers in countries where brown meat from crabs is popular, such as Portugal and Italy (Bolam *et al*., 2016; Maulvault *et al*., 2013; Noël *et al*., 2011; Wiech *et al*., 2017). The principal toxic effect of Cd is its toxicity to the kidney, although it has also been associated with lung damage and skeletal changes in occupationally exposed populations. Although Cd is relatively poorly absorbed into the body, once absorbed is slowly excreted, like other metals, and accumulates in the kidney causing renal damage. EFSA further indicated that, although adverse effects on kidney function are unlikely to occur for an individual exposed at the TWI level, exposure to cadmium at the population level should be reduced (EFSA, 2009). Consequently, the reduction of Cd in crab and crab-based products is highly desirable. Some investigations have studied the effect of different processes, such as cooking or freezing, on the Cd content of brown crab, but a negligible or inexistent effect was found (Maulvault *et al*., 20012; Wiech *et al*., 2017).

However, the use of new technologies such as ultrasound, which is know to enhance different mass transfer processes as pointed out before, with the purpose of reducing the Cd content in crabs has not been previously studied.

Therefore, the aim of this study was to evaluate the potential of the application of high-intensity ultrasound in the reduction of Cd content of brown crab, and also to evaluate if treatment temperature bears any influence on Cd removal.

4.3 MATERIAL & METHODS

4.3.1 Raw material and treatment conditions

All of the experiments in this research were carried out using female brown crabs with weights ranging from 600 to 800 g. Crabs from the North-West Atlantic were collected from a local supplier in Zaragoza, Spain: they had been previously slaughtered and immediately stored in frozen conditions at -20°C until use. Prior to processing, crabs were defrosted in a cold room at 4°C for 24 hours. After defrosting, crabs were processed in tap water, maintaining a ratio of 1 liter per 100 g of crab weight, using an Elma mod. TI-H10 ultrasonic bath (200 W, Elma Schmidbauer GmbH, Singen, Germany) with a tank capacity of 8.6 liters. All treatments were carried out at an ultrasonic frequency of 35 kHz and an amplitude of 100%, corresponding to an ultrasound input power of 200 W, at 50, 65 and 80°C (±2 °C), an output power of 117 W measured by calorimetry. Control treatments were carried out in the same bath and at the same temperatures without ultrasound.

Once the treatment conditions were stabilized, crabs were immersed in the ultrasonic bath for a maximum of 60 minutes. In the course of this treatment, 20 mL water samples were collected at preselected times for cadmium analysis. Crabs were removed from the bath after the treatment and stored for 24 hours in plastic bags at 4 °C. After storage, crabs were weighted, and white meat from claws and brown meat from the body (i.e. main shell cavity) were removed separately. Meats were subsequently dehydrated at 105°C in a convection drying oven (P-model Digitronic Selecta, Barcelona, Spain). After

drying, samples were weighed and then were milled and stored in hermetically sealed tubes until required for cadmium analysis. Each type of meat was analyzed in duplicate. A total of 72 samples of water (Before and after cooking process) and 60 samples of crab meat were analyzed.

4.3.2 Cadmium measurement

In order to quantify the amounts of cadmium present, water samples and dehydrated grounded samples of crab meat were analyzed by the University of Zaragoza Chemical Analysis Service, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). In brief, 0.2 g of dehydrated meat were diluted in 7 mL of HNO_3 (J.T.Baker^{TM}) and 3 mL of HCl (J.T.Baker^{TM}), and incubated at room temperature for 30 minutes in microwave tubes (MarsX-press Plus Wessel, CEM Corporation). After incubation, tubes were sealed and digested in a microwave system using a 15-minute heating ramp at 800 W and maintained at 200°C for 30 minutes. Digested samples were subsequently diluted using Milli Q water up to 50 mL. Water samples were directly injected. Instrumental settings for the ICP-MS are given in Table 4.1.

Table 4.1: DRC-e ICP-MS operating conditions

Nebulizer	**cross flow**
Spray chamber	Scott-type
Injector	Silica
Autolens	ON
RF Power	1.2 kW
Dual Detector	
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	1.2 L min^{-1}
Nebulizer gas flow	1 L min^{-1}
Sampling cone	Nickel
Skimmer cone	Nickel
Internal standard	Rh
Scan mode	Peak Hopping
Dwell time	50ms/AMU
Sample flow	1 L min^{-1}
Lens voltage	7.5 volts

4.3.3 Calculations and data analysis

The results obtained from the meat analysis were expressed as μg of Cd per gram of dry weight. For better comparison with published data, cadmium concentrations were converted to mg Cd per kg of wet weight, using Equation 4.1:

$$[Cd]_{ww} = [Cd]_{dw} \times dry\ weight/wet\ weight \qquad \text{(Eq. 4.1)}$$

where $[Cd]_{ww}$ represents the cadmium concentration in mg/kg (wet weight); $[Cd]_{dw}$ represents the cadmium concentration in mg/kg (dry weight); dry weight is the sample weight after drying (kg); and wet weight is the sample weight before drying (kg).

Cadmium content varies widely from one crab to another, as has been reported in the literature. Therefore, to improve the overall determination of the efficacy of ultrasound, the percentage of cadmium extracted (Eq. 4.3) was calculated for each individual crab and in relation with the different treatments, based on each crab initial cadmium content (Cd_0) by applying Equation 4.2:

$$Cd_0 = [Cd]_{water} \times V + [Cd]_{white} \times W + [Cd]_{brown} \times B \qquad \text{(Eq. 4.2)}$$

where Cd_0 represents the total amount of cadmium present in the crab before treatment expressed in μg; $[Cd]_{water}$ represents the cadmium concentration in the cooking water after treatment expressed in mg/L; V is the total volume of cooking water (L); $[Cd]_{white}$ is the cadmium concentration in white meat expressed in mg/kg (dried weight); W is the dry weight of white meat (kg); $[Cd]_{brown}$ is the cadmium concentration in brown meat expressed in mg/kg (dried weight); and B is the dry weight of brown meat (kg).

$$\%\ \text{Cd extracted} = \frac{\text{Cd}_{\text{water}}}{\text{Cd}_0} \times 100 \qquad \text{(Eq. 4.3)}$$

In Equation 4.3, Cd_{water} represents the total amount of Cd in the water after each treatment, in mg (equal to $[Cd]_{water} \times V$ in Eq. 4.2); and Cd_0 represents

the total amount of Cd present in the crab before treatment, in mg, as calculated from Eq. 4.2. All required information for calculus is shown in table 4.2.

As statistical analyses, one-way ANOVA and Tukey's post-test were performed with 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 are given as error bars in the Figures.

Table 4.2: Total samples weights (dry weight) and moisture content (%) of white and brown meats for each crab used in this investigation. The theoretical initial amount of Cd was calculated based on Equation 4.2.

	dw (g) White meat	Moisture white meat (%)	dw (g) Brown meat	Moisture Brown meat (%)	Theoretical initial Cd content (µg)
Raw	4.9	78.8	10.65	77.8	452.57
Raw	7.06	77.9	24.6	70.4	1228.63
Raw	11.07	74.8	23.17	61.2	65.71
80°C No US	6.26	84	20.69	76.2	1558.52
80°C No US	10.02	73.9	24.43	61.5	207.97
80°C No US	5.8	85.4	27.99	66.9	372.41
80°C US	4.36	88.1	15.55	65.8	564.60
80°C US	6.74	82.2	12.93	71.8	377.29
80°C US	10.43	74.7	18.11	60.7	524.56
65°C No US	3.6	83.4	2.23	90.3	80.91
65°C US	5.44	83.3	33.41	71.4	552.74
50°C No US	5.26	87.5	2.49	77.5	83.76
50°C No US	7.04	84.6	10.9	67.1	997.94
50°C US	8.25	59.5	8.94	83.4	361.61
50°C US	5.62	84.1	11.68	70.8	4.92

dw: dry weight. US, ultrasound assisted treatment; NOUS non-ultrasound assisted treatment.

4.4 RESULTS & DISCUSSION

The consumption of brown crab (*Cancer pagurus*) could represent a potential health risk if exceeding the tolerable weekly intake of Cd established by EFSA (2.5 µg/kg body weight) (EFSA, 2009). This applies especially to countries such as Portugal and Italy, where brown crab meat, known to contain high levels of Cd, is commonly consumed (Bolam *et al.*, 2016; Maulvault *et al.*,

2013; Noël *et al.*, 2011; Wiech *et al.*, 2017). Any strategy offering the possibility of reducing Cd content would therefore be of interest in terms of food safety. In this study, the application of ultrasound and temperature for this purpose has been tested for the first time. Table 4.3 shows the average values of Cd concentration measured in the white and brown meat of the crabs analyzed in this investigation, and the maximum concentration of Cd detected in the cooking water after the different processing conditions.

Table 4.3: Measured cadmium (Cd) concentrations in white and brown meats, both raw and after treatments at different temperatures with and without ultrasound (35 kHz, 100%, 200W, 60 minutes) and the maximum cadmium concentration detected in water after each treatment. Different letters indicate statistically significant differences within columns (P≤0.05).

Treatment		**[Cd] mg/kg (ww)**		**Max. [Cd] cooking water (ppm)** (mean ± SD)
Temperature	Ultrasound	brown meat (mean ± SD)	White meat (mean ± SD)	
Raw crab	-	8.37 ± 5.56[a]	0.24 ± 0.20[a]	-
80 °C	Yes	9.32 ± 2.04[a]	0.14 ± 0.06[a]	6.46±0.42[a]
80 °C	No	10.65 ± 6.64[a]	0.37 ±0.32[a]	5.17±0.04[b]
65 °C	Yes	11.26 ± 5.31[a]	0.89 ± 0.96[b]	3.40±0.12[c]
65 °C	No	5.60 ± 3.25[a]	0.17 ±0.49[a]	0.21±0.02[d]
50 °C	Yes	6.84 ± 3.05[a]	1.05 ± 0.69[b]	14.58±0.30[e]
50 °C	No	17.20 ± 11.48[a]	0.40 ± 0.29[a]	2.40±0.02[f]

ww, wet weight

Cd content ranged from 5.6 to 17.20 mg/kg (ww) and from 1.10 to 14.61 mg/kg (ww) for crab brown meat, cooked and raw respectively, which is in accordance with the range reported in the literature. Noël *et al.*, (2011) reported Cd levels from 0.78 to 27.6 mg/kg (ww) and from 6.38 to 61.8 mg/kg (ww) in the brown meat of common crabs (*Cancer pagurus*) captured in open or coastal areas of the Atlantic North East respectively, collected in Ireland (IE) and United Kingdom (UK). Bolam *et al.*, (2016) analyzed Cd levels in the brown meat of *Cancer pagurus* sampled throughout the UK, and observed concentrations between 0.11 and 26 mg/kg (ww). Maulvault *et al.*, (2012) reported Cd concentrations between 5.0 and 8.4 mg/kg (ww) in the brown meat of crabs caught on the Scottish coast. Values for brown meat obtained in our study are also in accordance with those reported for crabs from Southern Norway, which showed concentrations from 5.7 to 12 mg/kg (ww) after

different procedures. The same study, however, reported significantly higher Cd levels of up to 44 mg/kg (ww) in crabs from Northern Norway (Wiech *et al.*, 2017).

Values presented in Table 4.3 also shows that, when results are expressed as the average value of all studies conducted using different treatment conditions, no statistically significant differences in terms of Cd content in brown meat can be observed between processed and unprocessed crabs. These findings are in agreement with those reported by Maulvault *et al.*, (2012) and Wiech *et al.*, (2017) regarding the effect of different cooking and freezing temperatures on the Cd content of crabs.

In the case of white meat (Table 4.3), Cd concentrations ranged from 0.14 to 1.05 mg/kg (ww). These values were significantly lower than those detected in brown meat, thus confirming results reported by other authors (Barrento *et al.*, 2009a; Maulvault *et al.*, 2012, 2013; Noël *et al.*, 2011; Wiech *et al.*, 2017). Similar to what was observed for brown meat, no statistically significant differences were observed in terms of Cd content when comparing untreated and only-temperature-treated crabs. However, significantly higher concentrations of Cd were detected in white meats treated by ultrasound at 50°C and 65°C. Other authors have also reported that certain treatments such as freezing, thawing or cooking may affect the concentration of Cd in the white meat of brown crab (Wiech *et al.*, 2017), as well as in other crustacean species such as crayfish (Jorhem *et al.*, 1994).

Table 4.3 also shows the concentration of Cd measured in the process water after each treatment. It can be observed that the Cd concentration in the process water increased under all experimental conditions investigated. Other authors have also reported increases of Cd concentration in the cooking water in similar experiments performed with bivalves (Houlbrèque *et al.*, 2011; Metian *et al.*, 2009), although Jorhem *et al.*, (1994) did not observe significant changes in the concentration of heavy metals in water after cooking crayfish.

As a wide variability in Cd content across all crabs used in this investigation was observed, the variability between individuals could mask the effect of the treatments if the results were analyzed using the average value of all individuals. Therefore, data obtained on the effect of ultrasound at different temperatures on Cd release were analyzed for each sample individually, and results were expressed as the average of the % of Cd release. As an example, Figure 4.1 illustrates the release of Cd into water, with or without ultrasound at different temperatures. In all cases, Cd release followed a linear kinetics. Equations describing the release of cadmium across time are shown along with determination coefficients and root mean square errors in Table 4.4.

When the slopes of the regression lines, which correspond with the Cd release rate (% Cd released per minute) were compared, no significant differences existed among treatment temperatures in treatments without ultrasound. However, when ultrasound was used to assist the treatments, significantly higher Cd release rates were observed at all temperatures (Figure 4.1 and Table 4.3). Data also showed that the increase exerted by ultrasound on the Cd release rates depended on water temperature. Thus, ultrasound increased the Cd release rate 8.7-fold at 50°C, while at 65°C and 80°C the increments were 2.1- and 2.7-fold, respectively. This greater effect of ultrasound at lower temperatures could be attributed to the influence of temperature on the ultrasound cavitation phenomenon. Indeed, the effects of high-power ultrasound are generally attributed to acoustic cavitation (Feng and Tang, 2010). Some authors have also observed a correlation between the energy transferred into the treatment media due to cavitation and the magnitude of ultrasonic effects on extraction processes and microbial inactivation (Luengo *et al.*, 2014; Mañas *et al.*, 2000). Additionally, the amount of energy transferred by cavitation is reduced when the vapour tension of the liquid increases (Knorr *et al.*, 2004). Therefore, increases in treatment temperature would imply lower ultrasonic effects. This phenomenon could

explain why the greatest effect of ultrasound on the Cd release rate was detected at 50°C, rather than at higher temperatures.

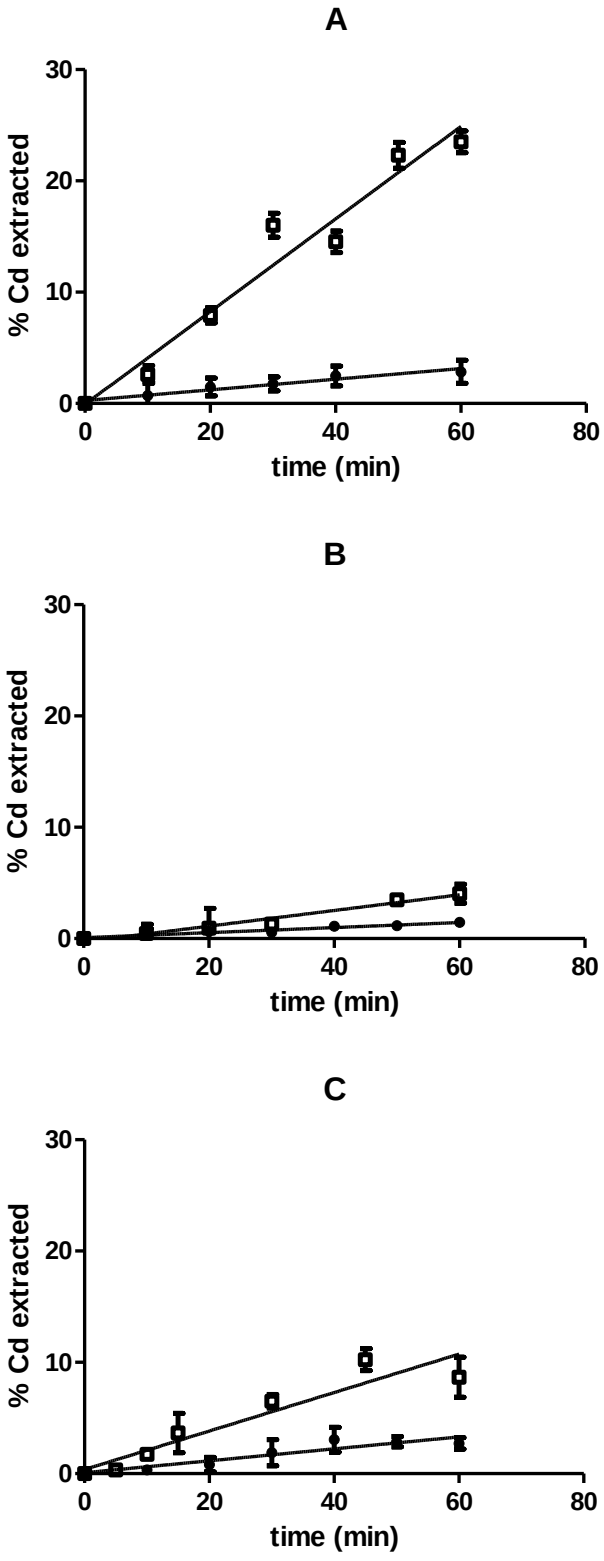


Figure 4.1: Release rate of Cd (% of Cd released per min) in crabs treated at 50°C (A), 65°C (B) and 80°C (C) with (□) and without (●) ultrasound.

The main reservoir of Cd in brown crab is located in the hepatopancreas, where this element is ligated to metallothionein (MT) by metal SH links (Barrento *et al.*, 2009c; Pedersen *et al.*, 1994). Although MTs are considered thermostable proteins, the metal links are thermosensitive (Bragigand and Berthet, 2003). This would explain the negligible, *viz.* inexistent differences between the Cd release rates at all three temperatures when ultrasound was not applied.Once the minimum temperature required to break the metal links is reached, the release rates would either remain stable or become only slightly influenced by convection phenomena at higher temperatures. On the other hand, ultrasound technology has proved to be effective in enhancing a series of mass transfer processes for various food matrices due to different mechanisms such as sonocapillary, sonoporation, shear forces, detexturation or a combination of them (Chandrapala *et al.*, 2012; Chemat *et al.*, 2011, 2017; Khadhraoui *et al.*, 2018). In chapter 3 is observed the potential of ultrasound for enhancing mass transfer processes during the cooking of brown crab and is hypothesized the possibility that the crab carapace could act as a resonant box which could induce cavitation inside the crab. This could explain the enhancement of the Cd release rate observed in this research in conjunction with the application of ultrasound technology.

Table 4.4: First order equations which correlate the percentage of cadmium extracted with the treatment time during the different processes applied.

Treatment		Equation	R^2	*RMSE*
Temperature	**Ultrasound**			
80°C	**Yes**	% Cd released = 0.1728*t + 0.375	0.86	0.519
80°C	**No**	% Cd released = 0.0649*t - 0.434	0.91	0.163
65°C	**Yes**	% Cd released = 0.0679*t - 0.255	0.94	0.159
65°C	**No**	% Cd released = 0.0321*t - 0.076	0.94	0.046
50°C	**Yes**	% Cd released = 0.4162*t - 0.073	0.95	0.755
50°C	**No**	% Cd released = 0.0479*t + 0.267	0.95	0.103

R^2, determination coefficient; RMSE, root mean square error

Figure 4.2 shows the percentage of Cd reduction in crab after each treatment depending on the initial amount of cadmium calculated for each crab. When crabs were not treated with ultrasound, the percentage of extracted Cd

ranged between 2 and 3%, and no statistically significant differences were observed across the different temperatures applied. However, in ultrasound-assisted treatments, the observed Cd content decreases were 22.8%, 4.4% and 7.6% for 50°C, 65°C and 80°C, respectively.

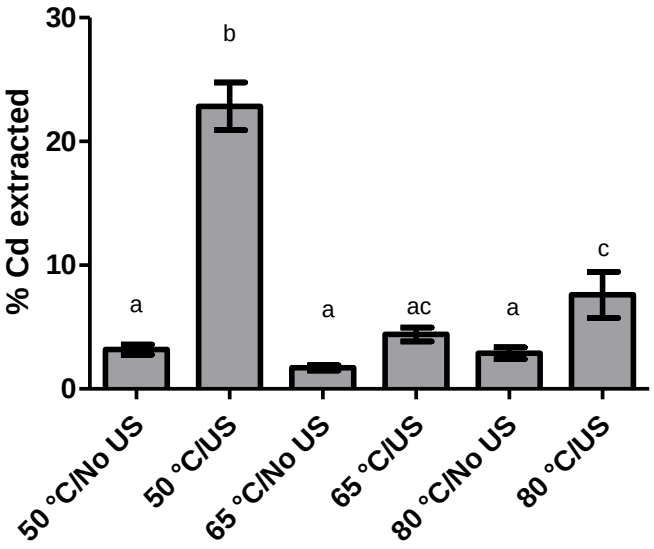


Figure 4.2: Percentage of Cd removed in crabs treated at different temperatures with or without ultrasound. Bars with different letters indicate statistically significant differences (P≤0.05).

As described by the kinetics, the greatest effect of ultrasound on Cd removal was observed at 50 °C. These results show that part of the Cd was released from the crab during the cooking process, even in the absence of ultrasound, although other researchers have reported contradictory results in this respect (Bolam *et al.*, 2016; Maulvault *et al.*, 2011, 2012; Wiech *et al.*, 2017). Such variability in the reported results could be attributed to the low quantity of Cd removed during the process (i.e. up to a maximum of 3% in the present study), and also to the high variability of Cd content in individual crabs (Wiech *et al.*, 2017). To the best of our knowledge this is the first research study in which the potential of ultrasound to reduce the Cd content of brown crab has been evaluated. The obtained results have demonstrated that in all cases the application of ultrasound enhances the amount of Cd released from the crab, allowing a reduction of up to 22.8% of the initial Cd present in the

crab. On the other hand, the results obtained also showed that the ultrasonic effect depends on treatment temperature. Further research would therefore be necessary in order to optimize process conditions with the purpose of defining an adequate procedure capable of improving crab meat chemical safety. Based on these results, it can be proposed that the production of ready-to-eat crabs and crab-based products is the area in which ultrasound technology could be most easily implemented to reduce the Cd content of crab meats. Attaining an overall reduction of the risk associated with Cd intake for consumers who eat crab meat on a regular basis.

4.5 CONCLUSIONS

The main objective of the present work was to evaluate the potential of ultrasound technology for reducing the cadmium content of brown crab (*Cancer pagurus*). The results showed that without ultrasound the Cd release rate was independent of the treatment temperature within the studied range (50-80°C). Without ultrasound, the percentage of Cd removed was less than 3% and no differences were detected among the different temperatures tested. Conversely, the application of ultrasound (35 kHz and 200 W input power) increased the release of Cd from the crab into the water at all temperatures tested. The effect of ultrasound on Cd removal was dependant on temperature. The maximum Cd release rate (0.41% Cd extracted/min) and the maximum percentage of Cd extracted (22.8%) were observed at 50°C. Overall, the results obtained highlight the potential for ultrasound technology to reduce the Cd content in brown crab. Further research is needed though to optimize treatment conditions for maximum Cd reduction.

Chapter 5

Optimization of the second pasteurization step of ready-to-eat whole edible crab (Cancer pagurus)

This chapter is as submitted to Food and Bioprocess technology, with some minor modifications to avoid duplication between chapters.

Condón-Abanto, S., Raso, J., Arroyo, C., Lyng, J.G. and Álvarez, I. Quality-based thermokinetic optimization of ready-to-eat whole edible crab (*Cancer pagurus*) pasteurization treatments. Submitted to Food and bioprocess technology. (First review 4/7/2018)

5.1 ABSTRACT/RESUMEN

ABSTRACT

Traditional processing practices used in the manufacture of ready-to-eat edible crab products include a double heat treatment involving an initial cooking step (first heat treatment) followed by washing and packaging and finally a second heat treatments for pasteurization purposes. The latter pasteurization step results in the most severe impact on product quality. The main objective of this research was to optimise this pasteurization step using quality index degradation kinetic approach. Preliminary work involved the characterization of temperature rise in the crab cold-spot during pasteurization. Equivalent treatments ($F_{90^oC}^{10^oC}$=10 min) were defined in order to assess the impact of pasteurisation temperature on different crab quality indexes in both crab meat types (i.e. white and brown meat). Colour degradation of crab white meat was defined as the critical quality parameter to be monitored during thermal pasteurisation. The effect of time and temperature on kinetics of white meat colour change (ΔE^*) were characterized and fitted to an exponential Equation. Following this, an industry focus group was used to define white meat colour change vs product quality and defined "good" ($\Delta E^* \leq 7$), "acceptable" ($7 < \Delta E^* < 9$) and "unacceptable" ($\Delta E^* \geq 9$) quality. Finally, using the developed Equations, optimal pasteurization conditions were defined and validated. To produce "good" quality crab optimal temperatures ranged between 96 and 100°C while temperatures between 104 and 108°C produced "acceptable" quality in crabs of 400 and 800g, respectively. Overall, the results show that the Equations obtained could be used in a Decision Support System (DSS) to define heat pasteurization conditions to optimise the quality of ready-to-eat edible crab.

RESUMEN

El procesado tradicional del buey de mar listo para el consumo generalmente implica un doble tratamiento térmico. Primero se aplica un cocinado inicial (primer tratamiento térmico) tras el cual el cangrejo se limpia y envasa para finalmente aplicar un segundo tratamiento térmico con el propósito de pasteurizar el producto. Esta pasteurización provoca un impacto considerable sobre las propiedades organolépticas del producto finalizado. El objetivo de este trabajo fue optimizar las condiciones de pasteurización del buey de mar mediante el estudio de su cinética de cambio de calidad durante el procesado térmico. En una primera fase se evaluó el perfil térmico del buey de mar durante la pasteurización. A continuación se definieron distintos tratamientos equivalentes ($F_{90ºC}^{10ºC}$=10 minutos) para evaluar el efecto de la temperatura de pasteurización sobre diferentes parámetros de calidad en ambos tipos de carne cangrejo (es decir: carne blanca y carne marrón). La degradación del color de la carne blanca fue identificada como el parámetro de calidad más adecuado para realizar la optimización del proceso. Por ello se caracterizó el efecto del tiempo y la temperatura de pasteurización en la cinética de cambio de color (ΔE^*) de la carne blanca, ajustándola a una ecuación exponencial. A continuación se utilizó un *focus group* compuesto por productores de cangrejo para definir la calidad en función del cambio de color de la carne blanca. Así se definió la calidad "buena" ($\Delta E^* \leq 7$), "aceptable" ($7 < \Delta E^* < 9$) e "inaceptable" ($\Delta E^* \geq 9$) de los cangrejos. Finalmente se desarrollaron una serie de ecuaciones con las que se definieron y validaron diferentes condiciones de pasteurización. Para producir cangrejos con una buena calidad se definieron unas temperaturas óptimas de pasteurización de 96 y 100°C mientras que temperaturas de 104 y 108°C produjeron una calidad aceptable para cangrejos de 400 y 800 g respectivamente. Las ecuaciones desarrolladas en esta investigación podrían utilizarse como sistema de soporte de decisiones para definir las condiciones de pasteurización optimizando la calidad del buey de mar listo para comer.

5.2 INTRODUCTION

Thermal processing is the most commonly used food industry technology to produce microbiologically safe and shelf-stable foodstuffs. However, when it comes to solid foods, heat treatments generally require long times, which in turn may promote undesirable quality changes such as the development of off-odours, off-flavours, nutrient degradation and colour changes (Ling *et al.*, 2015; Liaotrakoon *et al.*, 2013; Zabbia *et al.*, 2011). The selection of effective heat processing conditions, that produce adequate microbial inactivation while also minimising undesirable heat-related changes, is important for the food industry. A kinetic model development approach is a possible solution towards solving this challenge. Such an approach can provide valuable information to predict and control product quality during thermal processing (Haefner, 2005). The effect of heat treatments on the inactivation kinetics of microorganisms and enzymes have been extensively reported in the literature and different modelling Equations have been proposed (FDA, 2000; IFT, 2003). These models are widely accepted and used in the food industry to define the minimum required processing conditions to produce safe products. Some other studies have reported the effect of heat processing on the degradation kinetics of different quality attributes such as colour, nutrients and texture (Fante and Noreña, 2012; Hadjal *et al.*, 2013; Jaiswal *et al.*, 2012; Yu *et al.*, 2011). However, these models have to be developed for each individual food product due to the complexity of the reactions involved. Additionally, most of these studies have been performed in plant-based products. Relatively a few of those have been performed on fish or seafood products (Kong *et al.*, 2007; Ovissipour *et al.*, 2013).

As indicated previously, in the production of ready-to-eat whole crab and crab based products a second in-pack pasteurization, to ensure the microbiological safety, is generally applied. This second in-pack pasteurization is designed to inactivate the bacterial spores that may be present in these products. This step's target is a 6 Log_{10} cycle reduction of *Clostridium botulinum*

non-proteolytic type E (FDA, 2011; Huss, 1997; Linton *et al.*, 2003). However, the intense heat treatment required to obtain this objective, can result in undesirable changes in the final product if the heat process is not adequately designed and controlled.

The impact of this second pasteurization step on the quality characteristics of edible crab has not been previously studied. Therefore the main objectives of this research were: (1) to characterise the thermal profiles of edible crab in a pilot plant scale retort; (2) to evaluate the main quality attributes of edible crab affected by the thermal treatment; (3) to define the heat degradation kinetics of the most affected quality attribute under controlled conditions; and (4) to develop suitable Equations to allow the definition of the optimal thermal pasteurization conditions for ready-to-eat whole edible crab.

5.3 MATERIAL AND METHODS

5.3.1 Raw materials and crab preparation

All experiments were carried out using female North Atlantic crabs (400-900 g), obtained from a local supplier in Zaragoza (Spain). Crabs were sacrificed by the supplier on collection and immediately stored in frozen conditions at -20°C until required for analysis (maximum 30 days). Prior to use, crabs were defrosted in a cold room at 4°C for 24 hours and then weighed. Once defrosted, crabs were cooked in a thermostatic bath (CC-E, Huber, Germany) containing water with 2% w/v salt, at 95-98°C for 20 minutes as recommended by the Irish crab industry (Edwards and Early, 2001). After cooking, crabs were cooled in ice-water until a temperature below 20°C was achieved in the cold-spot which was located in the crab's abdomen as we demonstrated in a previous study (Condon-Abanto *et al.*, 2018). Temperature was measured using a K type thermocouple (Ahlborn, Holzkirchen, Germany) connected to a data logger (Ahlborn, mod. Almemo® 710). After cooling, crabs were weighed and vacuum packed (Model EV-13, Tecnotrip, Tarrasa, Barcelona, Spain) prior to pasteurization.

5.3.2 Quality measurements

5.3.2.1 Moisture content and water holding capacity

Moisture content was determined by oven drying (P-model Digitronic Selecta, Barcelona, Spain) following the AOAC (2000) method and results were expressed on a wet weight percentage basis. For the water holding capacity (WHC) determinations, samples (3 g) were were wrapped in a plastic mesh and placed in a 50 mL centrifuge tube half-filled with 6 mm diameter glass beads. Samples were centrifuged (Megafuge 1.0 R, Kendro, Germany) for 10 min at 3000 × *g* at room temperature (Martínez *et al.*, 2017). WHC was expressed as the percentage of post cook wet, weight moisture using Equation 5.1.

$$WHC = \frac{(sample\ weight\ (g) \times moisture(\%)) - lost\ water\ after\ centrifugation\ (g)}{(sample\ weight\ (g) \times moisture(\%))} \times 100 \qquad \text{(Eq. 5.1)}$$

5.3.3 Heat treatment characterization

After the crabs were vacuum-packed, the temperature rise in different locations within the crab anatomy was evaluated. Similar to the crabs cooked by immersion in water (Chapter 3), the crab cold-spot was located in the abdomen (Figure 5.1). Once the crab's cold-spot was identified, a series of time temperature curves were obtained at heat processing temperatures ranging from 85°C to 120°C in a 90 litre capacity pilot plant scale superheated water shower retort MicroMar-mini (Marrodan, Lodosa, Navarra, Spain). Temperatures were monitored using a K type thermocouple (Ahlborn, Holzikirchen, Germany) placed in the crab cold-spot at time intervals of 1 minute using a data logger (Ahlborn, mod. Almemo® 710) connected to a laptop with the data control software version 5.18.2.34 (64-bit). The temperature evolution with time was subsequently fitted to the Ball & Olson Equation (Mafart, 1994) as explained in Chapter 3 using Equations 3.1A and 3.1B. The estimated parameters as well as R^2 (coefficient of determination) and *RMSE* (Root Mean Square Error) are shown in Table 5.1.

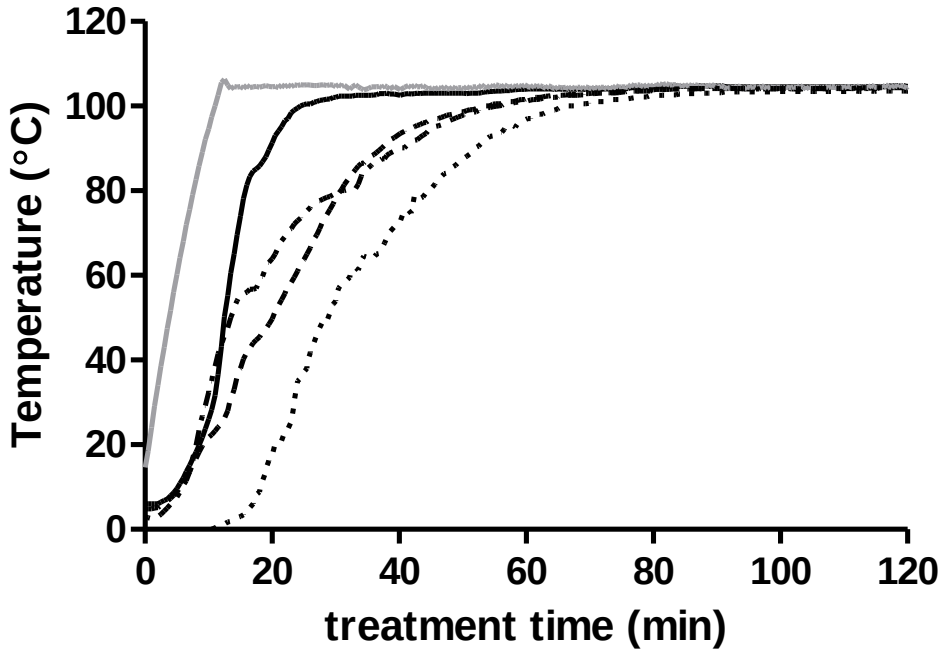


Figure 5.1: Time/temperature profiles during thermal pasteurization in a shower retort at 105°C in the crab's claw (block line), mandibula (dotted lines) and abdomen (dotted line) of a 790 g crab. Grey line shows the thermal profile of the autoclave.

Based on these Equations, equivalent pasteurization treatments at different temperatures were calculated based on the crabs weight to reach a 6 Log_{10} reduction of *C. botulinum* non-proteolytic type E. This is based on the recommendations by the U.S. Food and Drug Administration (FDA, 2011). As explained in Chapter 3 equivalent treatments were calculated using the equivalent lethality (L) and cumulative F equivalent values at each of the temperatures recorded during pasteurization using Equations 3.2 and 3.3 respectively. After pasteurization, brown and white crab meats were removed and separated for quality measurements.

Table 5.1: Heat penetration parameters (*fh* and *j*, dimensionless) arising from the fitting of the Ball & Olson Equation to the heat penetration curves in the cold-spot of various edible crabs (*Cancer pagurus*), of different weights and sizes, during thermal pasteurization in a shower retort at different temperatures.

Weight (g)	long diameter (cm)	short diameter (cm)	*fh*	*j*	R^2	*RMSE*
406	15.5	10	38.3	1.20	0.99	0.34
406	15.5	10	24.8	1.90	0.99	0.73
406	15.5	10	34.6	1.10	0.99	1.12
510	15	10	51.5	1.20	0.99	0.87
537	15.5	10.5	40.2	1.80	0.99	0.88
537	15.5	10.5	48.9	1.90	0.99	1.73
537	15.5	10.5	51.7	1.60	0.97	4.79
537	15.5	10.5	55.0	1.90	0.98	3.33
590	16	11	58.3	1.10	0.97	2.33
590	16	11	60.6	1.60	0.99	1.97
590	16	11	58.6	1.40	0.99	1.77
590	16	11	39.6	1.70	0.99	1.23
600	16	11	56.9	1.20	0.99	1.43
600	16	11	63.7	1.20	0.98	1.38
600	16	11	58.4	1.30	0.99	1.61
600	16	11	34.1	1.80	0.99	1.60
692	18	11.5	53.2	1.60	0.99	0.54
692	18	11.5	52.5	1.90	0.99	1.11
692	18	11.5	61.0	1.30	0.99	1.37
692	18	11.5	41.9	1.80	0.99	1.59
703	17	10.5	62.4	2.33	0.99	1.74
751	17	11.5	48.1	1.76	0.98	3.73
752	18	12	59.0	1.80	0.98	2.51
752	18	12	40.7	1.90	0.98	1.82
752	18	12	51.9	1.20	0.99	0.84
753	18	11.5	62.0	1.10	0.99	0.83
753	18	11.5	62.9	1.40	0.99	0.41
753	18	11.5	43.4	1.60	0.99	1.9
800	19	11.5	43.4	2.36	0.99	1.67
805	17.5	11.5	61.2	1.51	0.99	1.66
909	19	12	79.7	1.48	0.99	2.30

5.3.4 Colour measurement

A tristimulus colorimeter (Spectrophotometer CM-2002, Konica Minolta, Japan) was used to determine colour parameters of brown and white crab meat after pasteurization. Results were expressed using the L*, a*, b* CIE colour space. Other colour parameters such as Chroma (C*), Hue angle (H*), total colour change (ΔE*), Whiteness index (WI*) and Browning index (BI*) were estimated from L*, a* and b* using the following Equations (Pathare *et al.*, 2013):

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2} \quad \text{(Eq. 5.2)}$$

$$H^* = tan^{-1}(b^*/a^*) \quad \text{(Eq. 5.3)}$$

$$\Delta E^* = [(L_0 - L^*)^2 + (a_0 - a^*)^2 + (b_0 - b^*)^2]^{1/2} \quad \text{(Eq. 5.4)}$$

where L_0, a_0 and b_0 represents the initial values of colour for white and brown meats prior to pasteurization and L*, a* and b* are those values following retort pasteurization.

$$WI^* = [(100 - L^*)^2 + (a^*)^2 + (b^*)^2]^{1/2} \quad \text{(Eq. 5.5)}$$

$$BI^* = 100 \times \frac{(X - 0.31)}{0.17} \quad \text{(Eq. 5.6)}$$

Where, $X = \frac{a^* + 1.75(L^*)}{5.645(L^*) + a^* - 3.012(b^*)}$

From these colour parameters, Chroma (C*) indicates the colour saturation, Hue angle (H*) indicates the colour tone, ΔE* quantifies the total colour change taking as reference the colour after cooking (step 1) and before thermal pasteurization (cook step 2), whiteness index (WI*) indicates the degree of whiteness and Browning index (BI*) indicates the purity of the brown colour.

5.3.4.1 Colour change kinetics

The study of the effect of temperature on colour change kinetics was performed in white meat only. This study was performed under controlled conditions as follows: After cooking step 1 (as described in section 2.1), white meat from crab claws and legs was removed. A glass tube with a screw cap lid was filled with approximately 5 g of white meat. The tube was the sealed using a septum and screw cap. A hole in the tube cap allowed the insertion of a syringe to relieve the internal pressure in the tube by removing air/vapour during the beginning of the heat treatment (a schematic description of the tubes is shown in Figure 5.2). The colour changes in the white meat during the heat treatment were measured through the tubes using a tristimulus colorimeter (Chroma Meter CR-400). Preliminary trials showed that the glass tube did not interfere with the colour measurements (data not shown). After the initial colour measurements, tubes were submerged in an oil bath (CC-E, Huber, Germany) at 95°C, 105°C and 115°C. Preliminary experiments showed that the tube needed less than one minute to reach the target temperature (data not shown). A single tube was removed at preselected times and immediately cooled down by submerging it in ice-water. After cooling it down to room temperature ($T \leq 20$ °C) the white meat colour was measured again and all colour parameters calculated. All experiments were performed in triplicate and ten colour measurements were averaged for each replicate (i.e. tube).

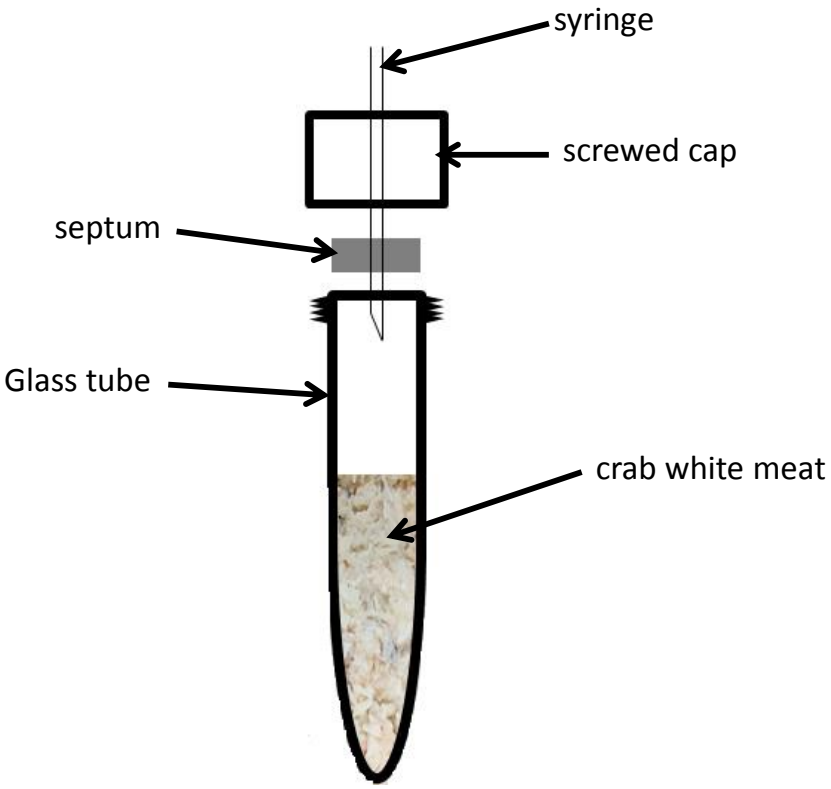


Figure 5.2: Diagram of the tubes used to study of the colour degradation kinetics of crab white meat.

The results obtained were fitted to zero order, fist order and pseudo-first order association (Eq. 10 A, B and C) kinetic equations, as recent reviews suggest for describing the kinetics of colour change in different food matrices (Ling *et al.*, 2015; Pathare *et al.*, 2013). Due to the complexity of food products, different kinetics have been used to describe for modelling of quality attributes in foods. Depending of the process, food or attribute, kinetics varies from zero-order formation to complex kinetics involving parallel and consecutive reactions, for instance the Maillard reaction, or degradation of some enzymes during heating (van Boekel and Tijskens, 2004). The order of kinetic was selected based on R^2 as suggested by Mondal & Dash (2017). The highest correlation (R^2) was obtained for the pseudo-first order association kinetic equation for the total colour change index (ΔE*), so for subsequent calculations this parameter was the only one considered.

$$Y = Y_0 - k \times t \quad \text{(Zero-order kinetic)} \quad \text{(Eq. 5.7A)}$$

$$Y = Y_0 - e^{-k \times t} \quad \text{(First-order kinetic)} \quad \text{(Eq. 5.7B)}$$

$$Y = Y_0 + (Y_{max} - Y_0) \times (1 - e^{-k \times t}) \quad \text{(Pseudo-first order kinetic)} \quad \text{(Eq. 5.7C)}$$

Where Y_0 represents the initial colour, Y_{max} is the value in which the colour becomes stable for a time (t) value equal to ∞, and k is the rate constant (min^{-1}). Then the effect of the treatment temperature on k and Y_{max} was studied by plotting the decimal logarithm of these parameters versus the treatment temperature.

Finally, to validate the developed Equations, 16 crabs were heat-treated at temperatures ranging from 90 to 120 °C, and the temperature of the crab's cold spot was monitored. After treatments, quality parameters were analysed.

5.3.4.2 Data processing and statistical analyses

Data were processed using GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Different Equations were used to fit the data using the same software. The fitting showing the highest correlation coefficient (R^2) was considered for further calculations (Mondal & Dash, 2017). Microsoft Excel 10 was used to develop the final Equations. For the statistical analyses, t-tests (p=0.05) and ANOVA tests (p=0.05) followed by Tukey's test were performed using GraphPad PRISM 5.0 software to define statistical differences among samples and differences were considered significant where P≤0.05. Error bars in the Figures correspond to the standard error of the mean for at least three independent replicates.

5.4 RESULTS AND DISCUSSION

In addition to microbial inactivation, heat treatments also induce a series of chemical reactions which modify attributes of foodstuffs. These reactions can be positive including desirable flavour, colour or texture development, but they

can also include reactions which are regarded as negative such as undesirable colour, odour, and nutrient degradations. Ultimately, these reactions will influence consumer acceptance and, for this reason, an understanding of their development kinetics is essential in order to design thermal processes that better align with consumer satisfaction (Ling *et al.*, 2015). In the case of edible crab, severe heat treatments are generally required for bacterial spore inactivation. Therefore, the main objective of this research was to optimize the pasteurization treatment conditions (i.e. second thermal treatment) for edible crab after packaging based on the kinetics changes of some crab quality parameters and define different treatment conditions based on the thermal profile of crabs, in order to ensure one of the recommended heat treatments for this type of ready-to-eat products (F90ºC for 10 minutes) (FDA, 2011).

5.4.1 Effect of pasteurization temperature on quality characteristics of edible crab

The first step was the development of an Equation (derived from Equations 3.1A and 3.2B) with the objective of characterizing the temperature rise in the crab cold-spot i.e. the abdomen during pasteurization. Crab weight was also factored into this Equation due to the important effect of this parameter on the temperature increase rate. Figures 5.3A and 5.3B show the effect of the crabs weight on the Equation parameters *fh* and *j*. It is observed that crab weight had a negligible effect on the *j* values. Therefore, to compose the final Equation this parameter was fixed at a constant value of 1.56 (±0.35) units. By contrast, a linear relationship was found between *fh* value and crab weight. Essentially this means that the temperature rise in the crab cold-spot is weight-dependent and both parameters are correlated in Equation 5.8. These observations are in agreement with those presented in chapter 3 where the heat penetration of the crab cold-spot was monitored during the initial cooking process.

$$fh = 0.04616 \times Weight + 22.04 \qquad \text{(Eq. 5.8)}$$

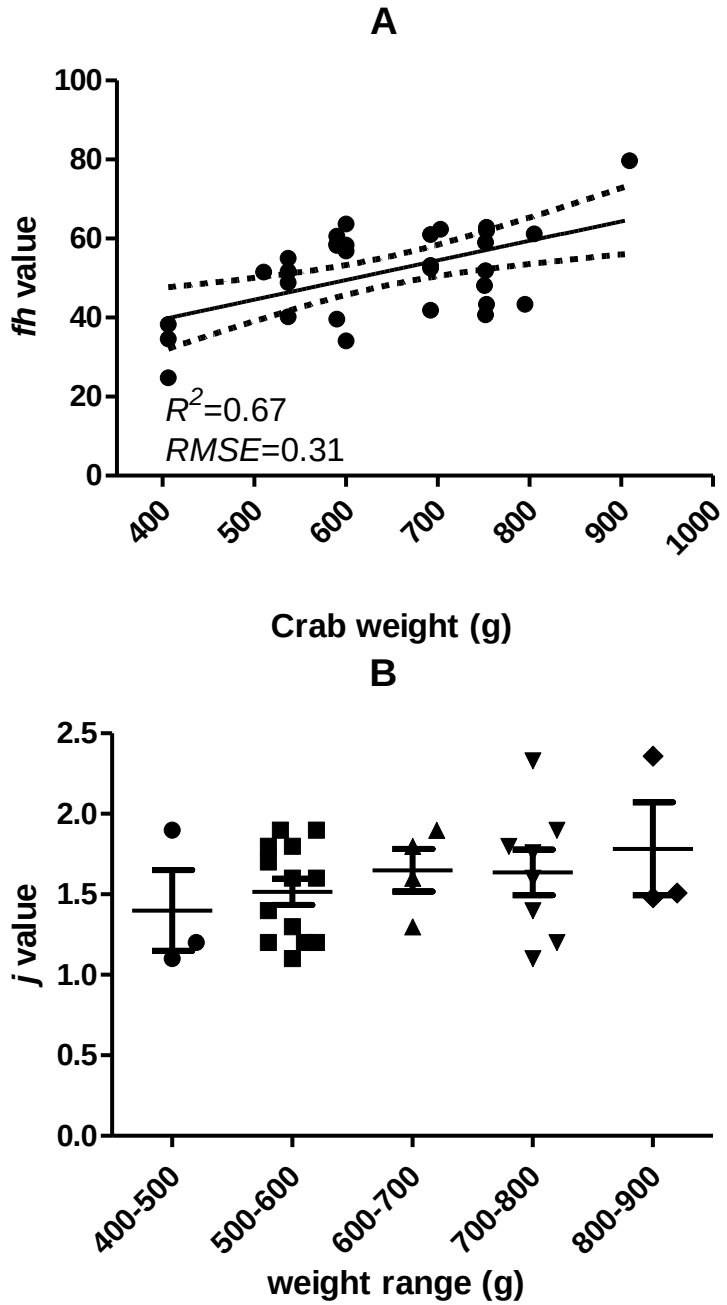


Figure 5.3: Effect of crab weight on the heat penetration parameters *fh* (A) and *j* (B), estimated from the fitting of Ball & Olson Equation to the thermal profiles obtained in the crab cold-spot. Figure 5.3A also shows the regression line (block line) as well as the 95% confidence intervals (dotted lines).

The combination of Equations 3.1A, 3.1B and 5.8, produced Equation 5.9 that predicts the evolution of the temperature in the crab cold-spot during retort pasteurization, as influenced by the initial temperature of the crab, pasteurization temperature and crab weight.

$$T_t = T_c - \frac{T_c - T_0}{10^{\left(\left(\frac{1}{(0.046 \times W + 22.04)} \times t\right) - \text{Log } 1.56\right)}} \qquad \text{(Eq. 5.9)}$$

Where T_t is the temperature (°C) at a certain time t (min), T_c is the pasteurization temperature, T_0 is the initial temperature in the crab 'cold-spot' and W is the crab weight (grams).

Based on Equation 5.9 and using Equations 3.2 and 3.3, a series of equivalent pasteurization treatments at temperatures ranging from 90 to 120ºC were defined in order to apply the heat treatment recommended by the FDA for crustaceans (i.e. $F_{90^oC}^{10^oC}$=10 min), in order to produce a 6 Log_{10} cycle reduction in the population of *Clostridium botulinum* non-proteolytic type E.

Table 5.2 summarizes the range of pasteurization conditions in terms of time and temperature as a function of the crab weight ranges. Treatment temperatures did not influence the moisture content which ranged between 58.93 and 65.33% for brown meat and between 77.3 and 79.23% for white meat. By contrast, small differences in the WHC values were detected in both brown and white meat. This parameter varied from 59.75 to 69.77% and from 61.65 to 87.35% for white and brown meat, respectively. Observed moisture values are in the range of previously published data which focused on the effect of crab cooking methods. These results also show that moisture content of white meat is higher than brown (Anacleto *et al.*, 2011; Cóndon-Abanto *et al.*, 2018; Martínez *et al.*, 2017; Maulvault *et al.*, 2012). In the case of WHC, the values obtained in the present study were higher than those reported by Martínez *et al.*, (2017) for *Callinectes sapidus*. From a quality perspective, the objective of cooking crab is to coagulate meat proteins in order to produce specific organoleptic attributes. The cooking process also induces shrinkage of meat fibres and aggregation/denaturation of myofibrillar proteins which eventually lead to water loss (Hong *et al.*, 1993; Kong *et al.*, 2008). Therefore changes in moisture and WHC of the crab meat occur during the first cooking step, rather than during the second heat treatment, the objective of the latter being pasteurization.

Table 5. 2: Average values of moisture, water holding capacity and colour of white and brown meat after retort pasteurization ($F_{90^oC}^{10^oC}$=10 min) at different temperatures.

			Brown meat				White meat			
Temperature (°C)	Crab weight (g)	switch-off time (min)	Moisture (%)	Water Holding Capacity (%)	BI*	ΔE*	Moisture (%)	Water Holding Capacity (%)	BI*	ΔE*
No retort	-	-	61.9 (3.4)^{a}	63.5 (1.2)^{a}	96.4 (4.9)^{a}	0	78.4 (1.6)^{a}	61.0 (3.4)^{ab}	10.1 (1.9)^{a}	0
90	700-800	104	58.9 (1.6)^{a}	87.3 (1.6)^{b}	91.4 (8.9)^{ab}	6.6 (3.2)^{ab}	77.3 (3.2)^{a}	66.2 (0.7)^{ab}	14.7 (3.4)^{b}	3.8 (1.8)^{a}
100	700-800	78	61.8 (2.6)^{a}	85.9 (6.7)^{b}	98.6 (4.2)^{a}	5.0 (0.9)^{a}	79.2 (1.4)^{a}	59.7 (2.6)^{a}	22.9 (1.9)^{c}	10.6 (1.1)^{b}
110	700-800	61	60.0 (0.7)^{a}	74.1 (5.1)^{ab}	110.7 (6.2)^{a}	5.4 (2.1)^{ab}	77.3 (0.7)^{a}	69.7 (1.9)^{b}	31.3 (1.5)^{d}	13.1 (0.9)^{b}
120	650-800	47	65.3 (4.4)^{a}	61.6 (4.9)^{a}	70.3 (4.1)^{b}	8.8 (1.1)^{b}	77.8 (0.6)^{a}	68.5 (2.1)^{b}	39.3 (5.1)^{e}	18.5 (1.0)^{c}

BI*, Browning index; ΔE*, colour change parameter (dimensionless). The number in brackets represents standard deviations of three different crabs. Superscript letters shows statistically significant differences (P≤0.05) within columns.

In the case of meat colour, two parameters ΔE* and BI* were calculated (Eq. 5.4 and 5.6, respectively) to assess the effect of pasteurization temperature on crab meat colour (Table 5.2). BI* parameter ranged from 70.31 and 110.7 units and ΔE* parameter ranged from 5.05 and 8.85 units for the brown meat. For comparison purposes, the BI* value of brown meat before pasteurization was 96.49 and parameters L*, a* and b* ranged between 43.3-45.4, 15.9-17.1 and 20.6-24.2, respectively. These values are in the range of those reported by Anacleto *et al.*, (2011) for single cooked edible crab. However, no values which compare the results obtained for double heat processing have been found in literature. The slight differences observed in the colour of brown meat following the different pasteurization treatments might be attributed to the intrinsic variability between crabs instead of the pasteurization temperature itself. It is well known that brown meat content is very variable, even within crabs of the same batch. It is also highly affected by seasonality (Woll, 2006). These factors probably impacted on the findings from the present study which did not show any trend between pasteurization temperature and colour parameters of brown meat.

Contrary to what was observed in brown meat, pasteurization significantly affected the colour parameters of white meat. BI* ranged between 14.74 and 39.39 units and the total colour change (ΔE*) ranged between 3.88 and 18.52. For the non-pasteurized crab, the BI* calculated was 8.05 and values L*, a* and b* ranged from 66.7 to 73.3, 0.37 to -3.3, and 4.1 to 10, respectively. The L* values observed in this investigation are in agreement with those reported by Anacleto *et al.*, (2011), while the observed a* and b* values were lower. Our results are also in accordance with those reported by Requena *et al.*, (1999) who observed that more intense thermal treatments led to a darker colour in canned blue crab. From the results presented in Table 5.1, it can be concluded that white meat colour is the quality parameter most affected, among the parameters which were studied in this research, by the pasteurization conditions. Therefore, this is one of the essential parameters to

be controlled during the heat treatment optimization of ready-to-eat whole edible crab once its microbial safety has been assured with the recommended heat treatments.

5.4.2 Colour change kinetics of edible crab white meat

Once it was identified that the colour of white meat is a critical parameter which determines the quality of edible crab after pasteurization, the effect of temperature on meat colour vs time was studied under the controlled conditions described in Section 5.2.4. Table 5.3 shows the colour parameters L*, a*, b* measured in white meat during processing at different temperatures. The table also shows calculated colour parameters (i.e. C*, H*, BI*, ΔE* and WI*). Of the calculated colour parameters, ΔE*was selected to describe the colour change kinetics of white meat since it was the only colour parameter that varied with both time and temperature.

Equation 5.7C was used to describe the effect of heating time on ΔE* at different temperatures. Figure 5.4 illustrates the fitting of Eq. 5.7C to the data describing the evolution of ΔE* with time at 95, 105 and 115°C. R^2 values were 0.88, 0.92 and 0.93 at 95, 105 and 115°C, respectively indicating good correlations. As shown in the Figure, ΔE* increased rapidly in the early stages of the heat treatments, but then began to plateau. Figures 5.5A and 5.5B show the relationships between the logarithm of the parameters k and $Y_{max,}$ respectively vs. treatment temperature. The following linear Equation describes the effect of the temperature on the logarithm of these parameters:

$$Log\ k = 0.0248 \times T - 4.049 \qquad (R^2 = 0.91) \qquad \text{(Eq. 5.10)}$$

$$Log\ Y_{max} = 0.00946 \times T - 2.273 \qquad (R^2 = 0.82) \qquad \text{(Eq. 5.11)}$$

where T represents the treatment temperature in °C.

The variation of ΔE* with temperature (k values) increased 1.8 and 3.0 fold (0.0207, 0.0377 and 0.0615 min^{-1} for 95, 105 and 115 °C, respectively), when increasing the temperature from 95 to 115ºC. From this relationship, a z value

of 40.37°C was calculated for the colour change rate of crab's white meat (Figure 5.5A). The maximum ΔE* values reached (represented by Y_{max} parameter) were 8.08, 10.55 and 12.79 ΔE* units at 95, 105 and 115°C, respectively (Figure 5.5B).

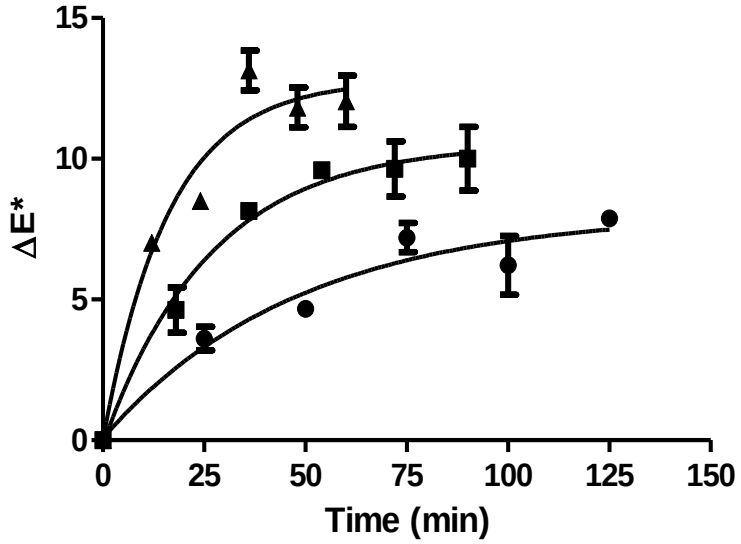


Figure 5.4: Effect of the processing time on the evolution of ΔE* of crab white meat at 95°C (●), 105°C (■) and 115°C (▲). Figure also shows the data fitting lines to Equation 5.10C. Error bars represent the standard deviation of three replicates.

Considering the Log-linear relationships between *k*, Y_{max} and temperature (*T*) and substituting these relationships (as represented by Eq. 5.10 and 5.11) into Eq. 5.7C, Eq. 5.12 would predict the total colour change in the crab's white meat during the thermal processing:

$$\Delta E^* = \left(10^{(0.009464\times T-2.273)}\right)\times\left(1-e^{\left(\left(-\left(10^{(0.0248\times T-4.049)}\right)\right)\times t\right)}\right) \qquad \text{(Eq. 5.12)}$$

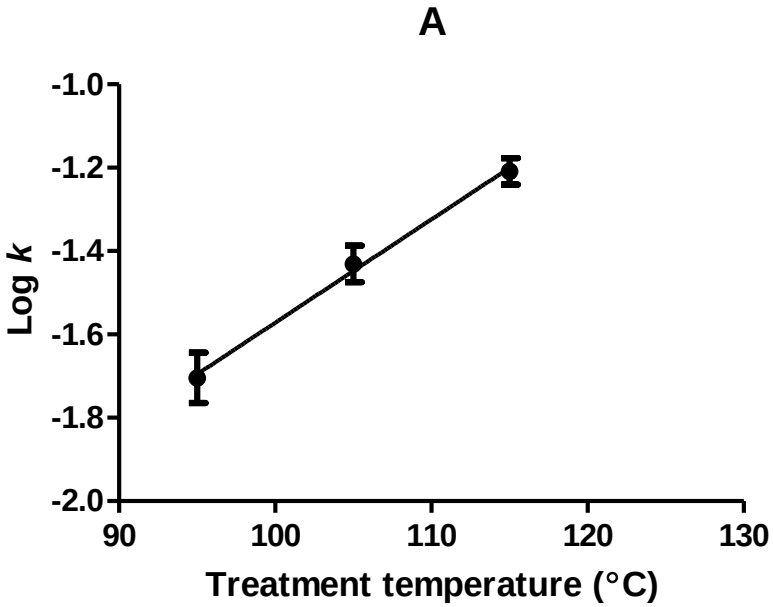
Where *T* and *t* are the temperature and time of the thermal treatments, respectively.

Colour of processed crab meat has been classified in five general categories: blue, relates to crab blood and/or thermal processing; brown is associated with the Maillard reaction; black, relates to metal reactions during canning; yellow is due to lipid oxidation and red is associated with pigments present in white meat (Requena *et al.*, 1999).

Table 5. 3: Colour parameters of crab's white meat at different times during heat processes at different temperatures.

Temp. (°C)	time (min)	L*	a*	b*	C*	H*	BI*	WI*	ΔE*
95	0	67. 46 (1.2)	-0.25 (0.5)	6.19 (0.3)	6.22 (0.3)	92.50 (4.5)	9.12 (1.0)	66.87 (1.1)	0
	25	63.8 (1.1)	-0.59 (0.3)	7.38 (0.4)	7.41 (0.4)	92.60 (2.5)	11.33 (1.0)	63.03 (1.1)	4.33 (1.6)
	50	63.06 (2.1)	-0.26 (0.7)	7.59 (0.6)	7.63 (0.6)	92.28 (5.2)	12.29 (2.2)	62.26 (2.2)	4.73 (1.8)
	75	59.96 (2.8)	0.39 (0.6)	8.72 (1.1)	8.74 (1.1)	87.64 (3.5)	15.94 (3.0)	59.00 (2.7)	7.15 (3.0)
	100	61.04 (1.4)	-0.71 (0.5)	8.78 (0.6)	8.82 (0.6)	94.62 (3.4)	14.28 (1.0)	60.04 (1.3	6.14 (2.0)
	125	60.68 (1.5)	-0.53 (0.4)	9.03 (0.8)	9.06 (0.8)	93.33 (2.6)	15.11 (1.7)	59.65 (1.6)	5.64 (1.8)
105	0	67.46 (1.2)	-0.25 (0.5)	6.19 (0.3)	6.22 (0.3)	92.5 (4.5)	9.12 (1.0)	66.86 (1.1)	0
	18	62.9 1(1.6)	-1.17 (0.9)	8.09 (0.6)	8.22 (0.5)	98.45 (0.4)	12.07 (2.0)	61.99 (1.5)	4.61 (1.6)
	36	58.98 (2.9)	-1.05 (1.4)	7.94 (0.9)	8.16 (0.8)	98.24 (9.0)	13.00 (4.0)	58.28 (3.0)	8.10 (2.1)
	54	56.66 (2.5)	-0.21 (0.6)	9.81 (0.8)	9.83 (0.8)	91.06 (3.4)	18.34 (1.8)	55.55 (2.3)	9.52 (2.4)
	72	56.30 (1.6)	0.14 (0.5)	9.87 (1.0)	9.89 (1.0)	89.28 (2.8)	19.11 (2.8)	55.19 (1.6)	9.46 (2.3)
	90	56.21 (1.8)	-0.05 (0.2)	9.65 (0.6)	9.65 (0.6)	90.36 (1.4)	18.38 (1.9)	55.11 (1.8)	9.84 (2.3)
115	0	67. 46 (1.2)	-0.25 (0.5)	6.19 (0.3)	6.22 (0.3)	92.50 (4.5)	9.12 (1.0)	66.87 (1.1)	0
	12	59.44 (0.9)	-1.18 (0.5)	8.81 (1.0)	8.90 (1.0)	97.49 (2.6)	14.19 (1.6)	58.48 (0.9)	7.04 (0.8)
	24	57.50 (1.6)	0.30 (1.0)	8.88 (0.8)	8.93 (0.8)	88.55 (6.0)	16.77 (2.6)	56.59 (1.5)	8.39 (2.1)
	36	53.86 (2.1)	1.32 (1.4)	10.70 (1.5)	10.85 (1.6)	83.79 (6.4)	23.75 (6.2)	52.55 (2.3)	12.82 (2.6)
	48	52.34 (2.4)	1.16 (0.9)	10.34 (1.5)	10.43 (1.6)	84.11(4.0)	23.49 (5.6)	51.14 (2.5)	11.71 (2.9)
	60	51.49 (2.3)	1.49 (0.3)	10.10 (0.7)	10.22 (0.7)	81.64 (1.8)	23.6 (2.3)	50.49 (2.3)	12.17 (2.2)

L*, lightness; a*, green-red component; b*, blue-yellow component; C*, Chroma; H*, hue angle; BI*, Browning index; WI*, whiteness index; ΔE*, colour change parameter. Number in brackets represents the standard deviations of three different replicates.



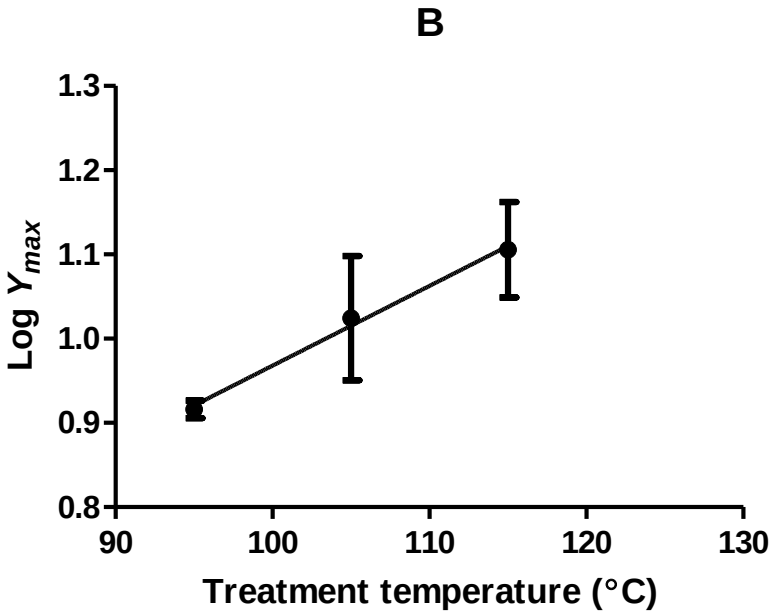


Figure 5.5: Effect of the treatment temperature on the colour change rate (*k*) (A) and maximum colour change developed (Y_{max}) (B) on crab white meat. Error bars show the standard deviation of three replicates.

Figure 5.6 shows that the ΔE* is highly correlated with Browning index (BI*). This correlation seems to indicate that the main factor involved in the crab meat colour change during thermal pasteurization is related with the Maillard reaction, since this is the main reaction responsible of the development of heat induced brown colours in crab meat (Requena *et al.*, 1999). However, while the Maillard reaction has been associated mainly to zero or first-order kinetics, colour changes on crab white meat followed a pseudo-first order kinetics. Therefore other process such as those related with the degradation of other natural pigments present in crab's meat, such as carotenoids (mainly astaxanthin) (Maoka, 2011) are the main reactions responsible of the red colour which develops in most crustaceans during cooking, might also affect the colour develpement of brown crab's white meat colour. Though, the main reservoir of these pigments is the shell some of those are also present in the meat or could be transferred from the shell to the meat during processing. Therefore, these pigments could be also involved on the colour changes of white meat during thermal processing (Ling *et al.*, 2015; Requena *et al.*, 1999).

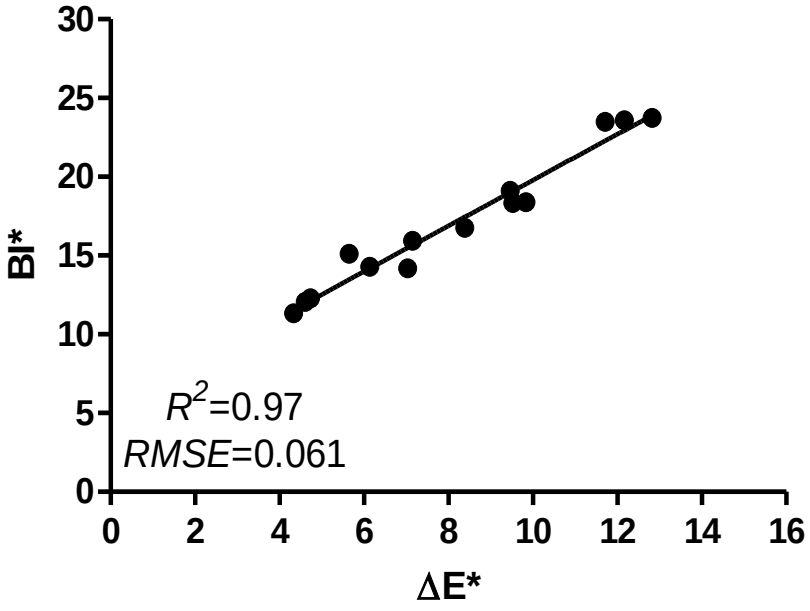


Figure 5.6: Correlation between Browning index (BI*) and total colour change (ΔE*) after different thermal treatments.

5.4.3 Heat process optimization and validation

In the final stage of this study, the optimization of the pasteurization of ready-to-eat whole edible crab was performed based on microbial recommendations of the U.S. Food and Drug Administration (FDA, 2011) (i.e. a target 6 decimal logarithmic reductions of *Clostridium botulinum* non-proteolytic type E). For this optimization, Equation 3.2 and 3.3 that describe the lethality of heat treatments, Equation 5.9 that describes the heat penetration and Equation 5.12 that describes the colour change kinetics were used in conjunction with the data obtained from a survey conducted among edible crab producers in Ireland, to define the quality associated with colour developed during thermal pasteurization. Figure 5.7 shows the results obtained from the survey performed to the Irish crab producers. As the figure shows, higher values of ΔE* parameter are associated with lower quality crab meat. The samples cooked/pasteurised during this investigation (in Sections 5.3.1 and 5.3.2) were classified, by the producers, as "good quality" (ΔE* ≤ 7), "acceptable quality" (7< ΔE* <9) and "non-acceptable quality" (ΔE* ≥ 9) based on their colour. Previous work on other products has suggested that ΔE* values between 1.5 and 3 units represent appreciable differences between food samples (Adekunte *et al.*, 2010; Pathare *et al.*, 2013).

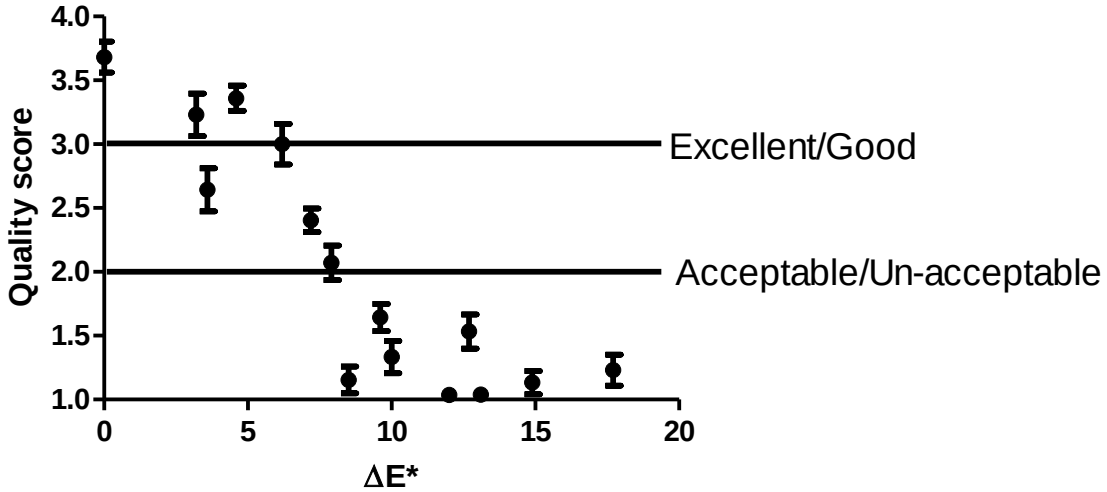


Figure 5.7: Quality score associated with crab white meat colour change based on the Irish crab producers perception. Number 4 indicates the maximum quality and 1 the lowest.

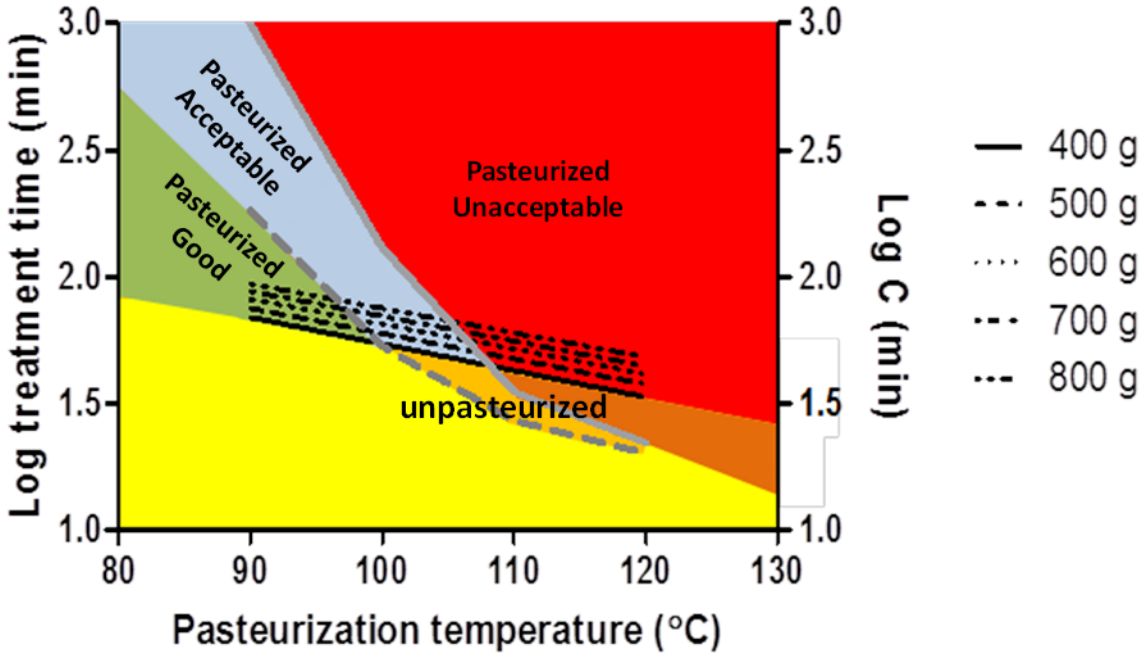


Figure 5.8: Theoretical optimisation graph for the pasteurization process of ready-to-eat whole edible crab based on Equations 5.12 and 5.13. Grey lines represent the quality boundaries between "good" and "acceptable" (dashed line) and between "acceptable" and "non-acceptable" (block line). Black lines represents the minimum processing conditions (time/temperature) required for crabs of different weights (from bottom to top 400, 500, 600, 700 and 800 grams) to achieve an adequate F value based on the inactivation of *C. botulinum* non-proteolytic type E ($F_{90^oC}^{10^oC}$=10 min).

Boundary lines (grey lines, dashed and solid) which define the time/temperature combinations based on the crab's quality are shown in Figure 5.8. The area below the dashed line represents the time/temperature combinations which would maintain the ΔE* below or equal to 7 (i.e. "good quality"). The area between the dashed line and the solid line corresponds to the time/temperature combinations which would produce crab with "acceptable quality" (ΔE* >7 and <9); and the area above the solid line is related to the time/temperature combinations which would produce crab of "non-acceptable quality" (ΔE*≥9). Figure 5.8 also shows a series of black lines which define the required time/temperature combinations, based on the heat penetration Equation developed in Section 5.3.1, to reach 6 Log_{10} reduction of *C. botulinum* non- proteolytic type E. Different lines correspond to the different crab weights. Based on Figure 5.8, the shorter pasteurization treatments recommended to maintain "good quality" are 100ºC for 55 minutes and 96°C for 83 minutes for crabs of 400 and 800 grams, respectively (Green area), and "acceptable

quality" at 108°C for 45 minutes and 104°C for 70 minutes for crabs of 400 and 800 grams, respectively (Blue area). The red area represents the pasteurization conditions which ensure microbiological safety, based on the recommended *F* value, but produce undesirable colour changes. The areas which are orange and yellow show the treatments conditions in which a product which is not microbiologically safe is produced.

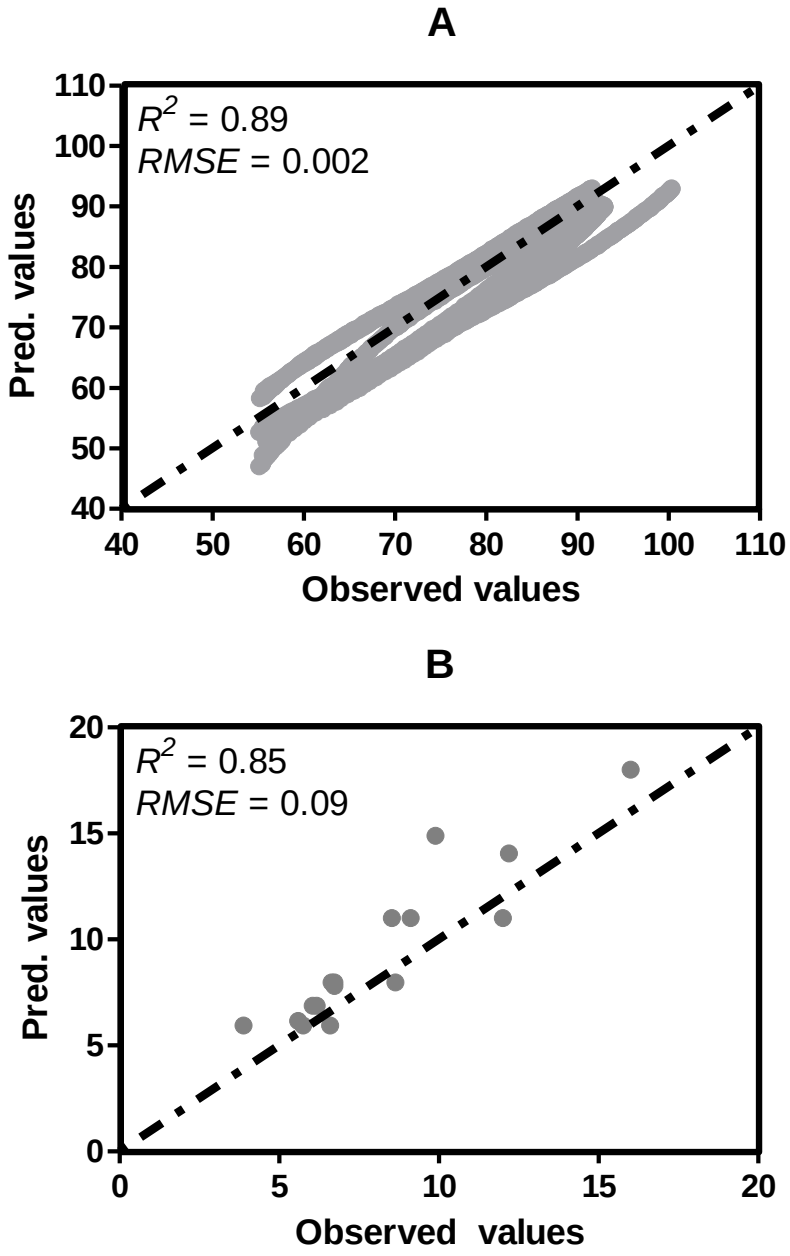


Figure 5.9: Observed versus predicted values for the thermal profiles (A) and white meat colour degradations (B) of crabs of different weights that were pasteurised under the conditions defined in Figure 5.5.

Finally, to evaluate the robustness of the predictions obtained from the Equations which were developed, a series of treatments, at different temperatures ranging from 90 to 120°C, were defined based on Figure 5.8. To validate the predictions of the heat penetration Equation (Equation 5.9), the crab's cold-spot temperatures were monitored during the treatment. These measured temperatures from crabs of different weights were plotted against the predicted temperatures from Equation 5.9. As Figure 5.9A shows, very good correlations were observed between real and predicted values. Since the treatments were designed to apply an equivalent *F* value of $F_{90°C}^{10°C}$= 10 min, the total *F* values from measured temperatures were also calculated using Equations 3.2 and 3.3.

In all cases, the total F value achieved was higher than 10 min, confirming the robustness of the Equation developed. After the pasteurization treatments, all quality parameters were also measured. As expected, no differences were observed in moisture and WHC among the crabs pasteurized at different temperatures of the white and brow meat and no differences in meat colour values were observed in the brown meat. However, significant differences were observed in white meat colour depending on the pasteurization conditions as previously demonstrated. To evaluate the robustness of the predictions provided by Equation 5.12, the observed ΔE* parameters were plotted against of the ΔE* parameters calculated based on the theoretical heat penetration of crab claws (Figure 5.9B). As observed, Figure 5.9B shows a good correlation between the observed and predicted ΔE* values confirming the applicability of the results obtained.

5.5 CONCLUSIONS

The main objective of the present work was to evaluate the effect of the pasteurization treatments on different quality attributes of edible crab and to identify optimal thermal treatments using mathematical predictions. From the results obtained, the crab cold-spot was located in its abdomen during retort

pasteurization. The time necessary to achieve a target F value was dependent on the crab weight. The pasteurization temperature did not affect moisture, water holding capacity or the colour of crab brown meat. Colour change of white meat was the most affected quality attribute during the thermal pasteurization and was consequently used as the indicator for heat process optimization. The colour change kinetics revealed that the degradation of the colour of white meat followed a pseudo-first order kinetics during thermal treatment. Both the maximum colour change achieved and the colour change rate, increased exponentially with treatment temperature. The Equations developed in this investigation proved to be useful for designing the thermal pasteurization treatments for ready-to-eat whole edible crab. These Equations indicated that the maximum temperatures to pasteurize whole edible crab were: between 96 and 100°C and between 104 and 108°C (depending on the crab weights) in order to obtain "good" or "acceptable" quality, respectively. Results obtained in the present work may be transfer to the crab industry for defining the pasteurization conditions with less impact on product quality.

Chapter 6

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

This chapter is as published in International Journas of Food Microbiology, with some minor modifications to avoid duplication between chapters.

Condón-Abanto, S., Arroyo, C., Álvarez, I., Condón, S., & Lyng, J. G. (2016). Application of ultrasound in combination with heat and pressure for the inactivation of spore forming bacteria isolated from edible crab (*Cancer pagurus*). International Journal of Food Microbiology, 223, 9-16.

6.1 ABSTRACT/RESUMEN

ABSTRACT

This research was performed to characterize the resistance of three different bacterial spores species isolated form 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 (100 kPa) 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 Log_{10} 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.

RESUMEN

Esta investigación se realizó para determinar la resistencia al calor de tres bacterias esporuladas, aisladas del buey de mar (*Cancer pagarus*) pasteurizado, y evaluar el potencial de los tratamientos de mano-sonicación (MS) y mano-termo- sonicación (MTS) para su inactivación. Las bacterias formadoras de esporos utilizadas en esta investigación fueron *Bacillus mycoides*, *Bacillus weihenstephanensis* y *Psychrobacillus psychrodurans*. La termorresistencia de las tres especies se dermino entre 80 y 110°C y su resistencia a los tratamientos de ultrasonidos bajo presión (100 kPa) entre 35 y 95°C. Las curvas de inactivación se describieron con las ecuaciones de Gereraerd (caída exponencial+hombro) y de Bigelow & Easty utilizando la herramienta de Excel Ginafit. En aquellas especies esporuladas en las que se detectaron hombros en las curvas de inactivación por calor, también se detectaron hombros en las curvas de inactivación de los tratamientos de MS/MTS, aunque la aplicación de un campo ultrasónico redujo la duración de los mismos. *B. weihenstephanensis* fue la bacteria esporulada más termorresistente requiriendo 1.4 minutos de tratamiento a 107.5°C para reducir 4 ciclos logarítmicos su población (z_T = 7.1°C) mientras que *B. mycoides* fue el más sensible al tratamiento térmico requiriendo 1.6 minutos de tratamiento a 95°C (z_T = 9.1°C) para producir una reducción similar de la población. *B. mycoides* mostró la mayor resistencia frente a los tratamientos de MS. La eficacia de los tratamientos de MTS estuvo directamente relacionada con la termorresistencia de la bacteria esporulada objeto de estudio. El tratamiento combinado (MTS) mostro un efecto sinérgico para la inactivación de las tres bacterias esporuladas. El mayor porcentaje de sinergia fue detectado en la especie microbiana que mostró un mayor valor z_T (*B. mycoides*) mientras que en la especie microbiana más termotolerante (*B. weihenstephanensis*) se registró la temperatura más alta a la que se produjo sinergia. Este estudio demuestra que el tratamiento de MTS es capaz de inactivar bacterias formadoras de esporos y que la eficacia letal de los tratamientos combinados está relacionada con la resistencia térmica de las especies microbianas.

6.2 INTRODUCTION

As pointed out previously the industry of ready-to-eat brown crab based products use traditional procedures in their production Therefore, there is a niche for process optimisation and/or for the incorporation of new technologies to enhance the processing of this 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). The main mechanism responsible for the microbial inactivation, by high power ultrasound, 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, 2015 a,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.

6.3 MATERIAL AND METHODS

6.3.1 Microorganisms and treatment media

The three spore forming microorganisms used in this study were the three most isolated spore forming bacteria isolated from Irish brown crab (*Cancer pagurus*) meat after a cooking step at 75°C of 50 min and subsequent 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 48h at 25°C. A broth subculture was prepared by inoculating a tube containing 10mL 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. 250mL Duran bottles containing 50mL of sterile TSBYE were inoculated with the overnight subculture and incubated at 25°C. Once the stationary phase of growth was achieved (after 48h for *B. mycoides* and *B. weihenstephanensis* and 72h for *P. psychrodurans*), 1mL of the suspension was spread onto the surface of TSAYE agar plates containing 3ppm (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 25ºC was selected as sporulation temperature, since the natural sporulation temperature in the environment inhabited by these crabs is lower (i.e. temperature of the North Atlantic). 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, in each case the washing was followed 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.

6.3.2 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 350mL 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 for sample extraction. Once a stable target temperature was attained (±0.1°C), 0.2mL of the spore suspension was inoculated into the treatment medium: pH 6.8 McIlvaine citrate-phosphate buffer. After inoculation, 0.1mL 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 it use. Figure 6.1 shows a diagram of the improved equipment. Modifications included:

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, this change not only facilitated the assembly and disassembly of the equipment but also its maintenance and cleaning.

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.

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.

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).

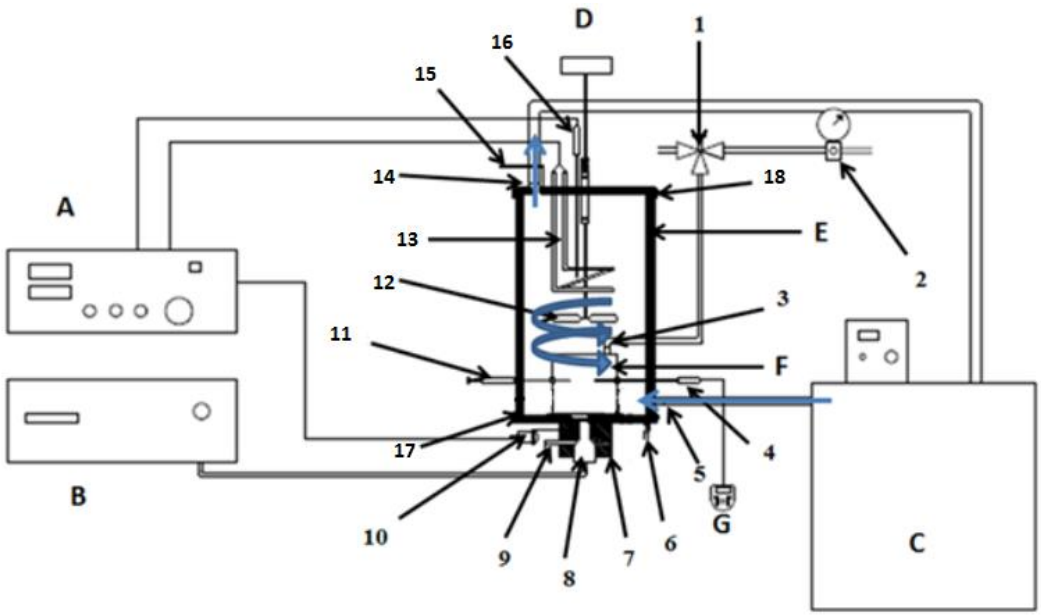


Figure 6.1: Diagram of the MTS resistometer. A, MTS resistometer main unit; B, ultrasound generator; C, heating/cooling water bath; D, agitation motor; E, Main vessel; F, treatment chamber; G, temperature data logger; 1, two way valve; 2, mano-reducer; 3, pressure/vacuum valve; 4, treatment chamber thermocouple; 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.

The ultrasonic generator used was a 2000W Branson Sonifier (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) which operates at a constant frequency of 20kHz. Survival curves to ultrasound treatments were obtained at different temperatures ranging from 35 to 95°C at a constant pressure (100kPa) and ultrasonic wave amplitude (90µm). 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 (i.e. ±0.2 °C), 0.2mL of the spore suspension was injected into the 23mL treatment chamber containing pH 6.8 McIlvaine buffer. After injection 0.1mL samples were collected and immediately pour-plated. All experiments were conducted at least in triplicate on independent working days.

6.3.3 Incubation of treated samples and survival counting

Survival curves to heat and MTS treatments were obtained by plotting the $\log_{10}$ 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, a log-linear regression plus shoulder model was used (Geeraerd *et al.*, 2000) as explain in section 2.3.6.2 using Equation 2.4. Those survival curves which showed a log-linear profile were fitted to Bigelow & Easty's model (1920), as it is also explaint in section 2.3.6.2 calculating the traditional decimal reduction time value (D_T value) using Equation 2.5. The parameter 4*D*, provided by ginafit software, was also used to compare the resistance among microorganisms.

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.

6.4 RESULTS

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 (Figure 6.2A and 6.2B respectively) followed a log-linear kinetic, the survival curves of *P. psychrodurans* (Figure 6.3A and 6.3B) and *B. weihenstephanensis* (Figure 6.4A and 6.4B) 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 6.1 and 6.2 show the mean value of the parameters calculated with the model including the shoulder length (*Sl*), the inactivation rate (k_{max}) and the 4*D* 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.

Table 6.1: Heat resistance parameters from the fitting of the Geeraerd log-linear plus shoulder model or log-linear modela to the survival curves of *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* spores treated in pH 6.8 citrate-phosphate buffer.

Microorganism	T	*Sl* mean (*SD*)	k_{max} mean (*SD*)	4*D* mean (*SD*)	*RMSE*	R^2
B. mycoides	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
B. weihenstephanensis	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
P. psychrodurans	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; 4*D*, time for a 4-Log reduction (min); *SD*, standard deviation; *RMSE*, root mean square error; R^2, determination coefficient.

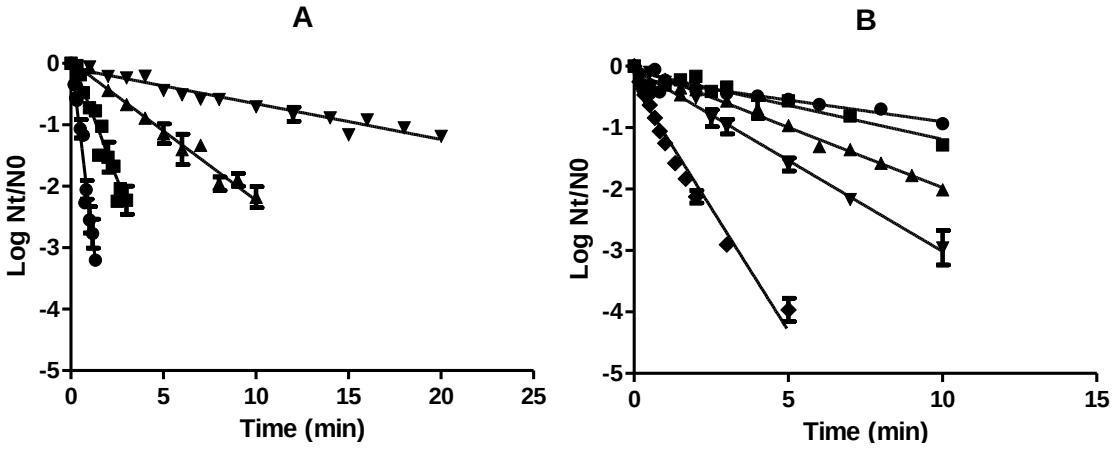


Figure 6. 2: Survival curves of *Bacillus mycoides* to heat (A) at 80°C (▼), 85°C (▲), 90°C (■) and 95°C (●) and mano-sonication (B) (●) and mano-thermo-sonication (B) at 50°C (■), 65°C (▲), 80°C (▼) and 90°C (◆) in pH 6.8 citrate-phosphate buffer.

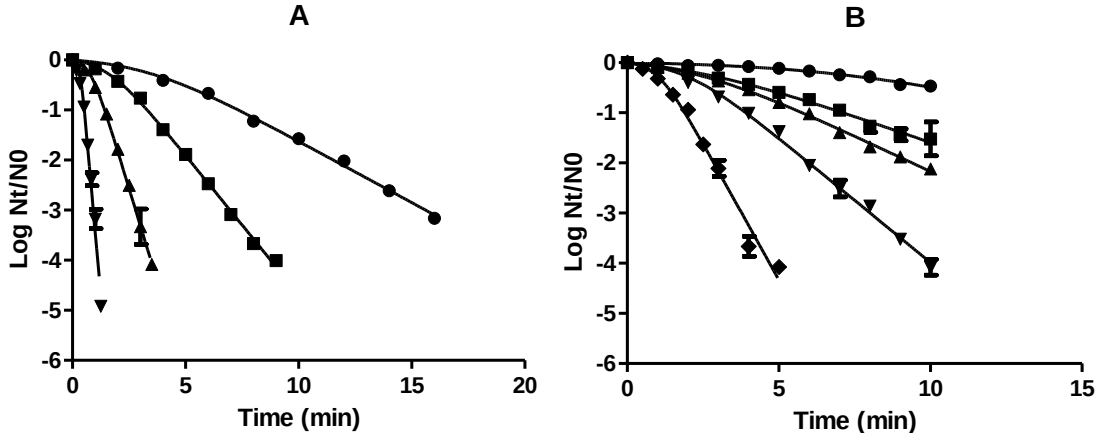


Figure 6. 3: Survival curves of *Psychrobacillus psychrodurans* to heat (A) at 90°C (●), 93°C (■), 96°C (▲) and 100°C (▼) and mano-sonication (B) (●) and mano-thermo-sonication (B) at 80°C (■), 85°C (▲), 90°C (▼) and 95°C (◆) in pH 6.8 citrate-phosphate buffer.

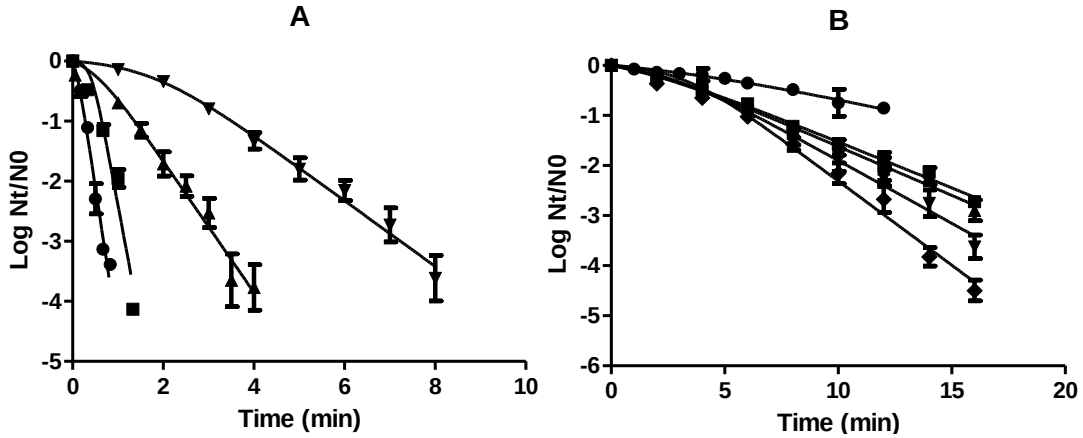


Figure 6.4: Survival curves of *Bacillus weihenstephanensis* to heat (A) at 102.5°C (▼), 105°C (▲), 107.5°C (■) and 110°C (●) and mano-sonication (B) (●) and mano-thermo-sonication (B) at 80°C (■), 85°C (▲), 90°C (▼) and 95°C (◆) in pH 6.8 citrate-phosphate buffer.

6.4.1 Spore resistance to heat

As pointed out above, the survival curves of *P. psychrodurans* and *B. weihenstephanensis* to heat (Figures 6.2A and 6.3A) showed shoulders in all the conditions tested (Table 6.1). Figure 6.5 shows the relationship between the logarithms of the shoulder length (min) and the treatment temperature (°C) for *B. weihenstephanensis* (Figure 6.5A) and *P. psychrodurans* (Figure 6.5B).

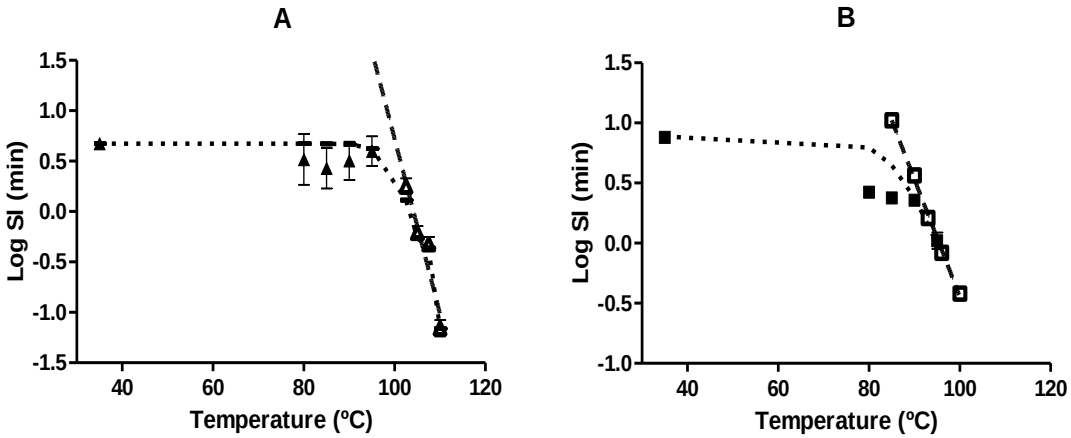


Figure 6.5: Influence of the temperature on the shoulder length calculated with the Geeraerd log-linear regression plus shoulder model for *B. weihenstephanensis* (A) and *P. psychrodurans* (B) by heat (open symbols) and MTS (solid symbols) in pH 6.8 citrate phosphate buffer. Dotted line represents the theoretical DRT curves to MTS calculated with Equation 6.6.

In both cases the *Sl* was an exponential function of the treatment temperature and a linear relationship between both parameters was found (Equations 6.1 and 6.2):

For *B. weihenstephanensis*: $Log\ Sl = -0.173 \times T + 18.07$ (Eq. 6.1)

For *P. psychrodurans*: $Log\ Sl = -0.098 \times T + 9.335$ (Eq. 6.2)

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°C) and *P. psychrodurans,* (z_{Sl} = 10.2°C). A t-test revealed that these z values were significantly different ($P \leq 0.05$).

As can be deducted 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 4*D* value (that incorporates the *Sl* and D_T values) was calculated for each spore species for each treatment (Table 6.1). 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-4*D*) were obtained by plotting $\log_{10}$ 4*D* values versus the corresponding treatment temperatures (Figure 6.6).

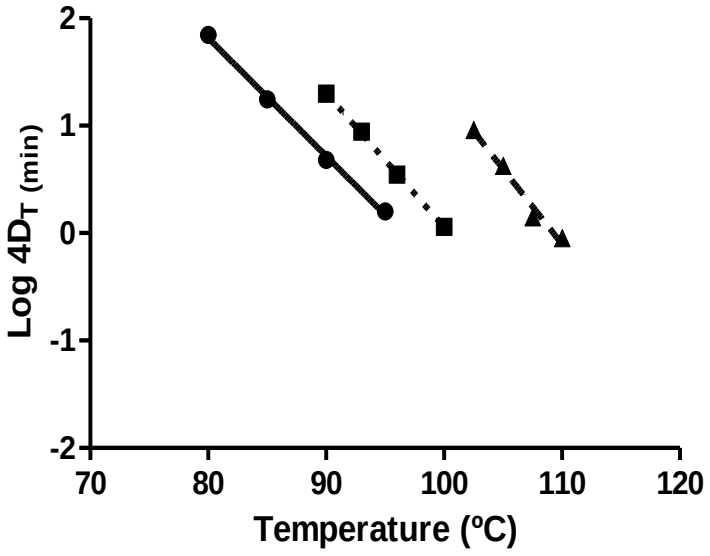


Figure 6.6: Influence of temperature on the 4*D* values calculated with the Geeraerd log-linear regression plus shoulder model or log-linear model for *B. mycoides* (●), *P. psychrodurans* (■) and *B. weihenstephanensis* (▲) inactivation by heat in pH 6.8 citrate-phosphate buffer.

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 4*D* 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.

6.4.2 Spore resistance to MS/MTS

Like for the thermal inactivation*, P. psychrodurans* (Figure 6.3B) and *B. weihenstephanensis* (Figure 6.4B) showed shoulders in the survival curves to MS/MTS treatments at all temperatures tested (Table 6.2). By contrast, shoulders were not observed at any temperature for *B. mycoides* (Figure 6.2B, Table 6.2). Therefore and similar to the heat treatments, the comparison of the resistance of the three spores to MS/MTS can be done by means of the 4*D* values (Table 6.2). When the resistance to MS (35°C) was compared, *B. mycoides* was the most resistant species with a 4*D* of 51.2 min, followed by *B. weihenstephanensis* with a 4*D* of 40.6 min while *P. psychrodurans* was the most sensitive with a 4*D* of 25.6 min.

Table 6.2: 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	*Sl* mean (*SD*)	k_{max} mean (*SD*)	4*D* mean (*SD*)	*RMSE*	R^2
B. mycoides	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
B. weihenstephanensis	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
P. psychrodurans	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.

When heat was included in the process (i.e. MTS), a change in tolerance was observed. At 80°C no significantly different $4D_{MTS}$ values (P>0.05) were calculated for *B. weihenstephanensis* (23.4 min) and *P. psychrodurans* (21.5 min) but a lower $4D_{MTS}$ was calculated for *B. mycoides* (3.7 min) (P≤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 (Figure 6.7).

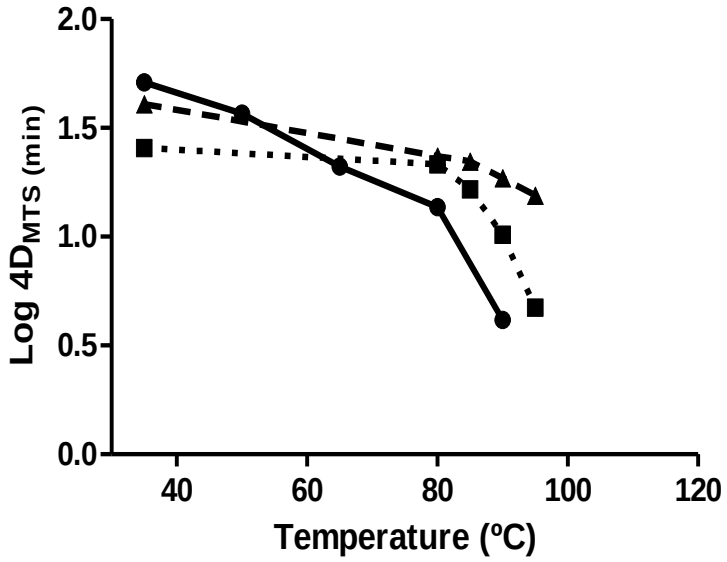


Figure 6.7: Influence of temperature on the $4D$ values calculated with the Geeraerd log-linear regression plus shoulder model or log-linear model for *B. mycoides* (●), *P. psychrodurans* (■) and *B. weihenstephanensis* (▲) inactivation by MS/MTS in pH 6.8 citrate-phosphate buffer.

As it is shown in Figure 6.7 and as opposed to the results for heat treatments, there was no exponential relationship between the $4D_{MTS}$ values and the temperature since the MTSDT curves showed concave downward profiles for the three bacterial spores. This result did not allow for the

calculation of *z* values. Overall, Figure 6.7 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. Figure 6.7 also demonstrated that the thermal dependence was not related with the resistance to MS treatments at 35ºC.

6.4.3 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 than the addition (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 pressurised ultrasonic 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, for the calculations, 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 4*D* value (Equation 6.3) was employed.

$$Theoretical\ 4D_{\mathrm{MTS}} = \frac{(4D_{\mathrm{T}})\times(4D_{\mathrm{MS}})}{(4D_{\mathrm{T}})+(4D_{\mathrm{MS}})} \qquad \text{(Eq. 6.3)}$$

In order to quantify the magnitude of the synergistic effect the percentage of synergism was calculated with Equation 6.4.

$$\%\, Synergism = \frac{Theoretical\ value - Experimental\ value}{Theoretical\ value} \times 100 \qquad \text{(Eq. 6.4)}$$

A synergistic effect was detected for the inactivation by MTS of all spore suspensions. Figure 6.8 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.

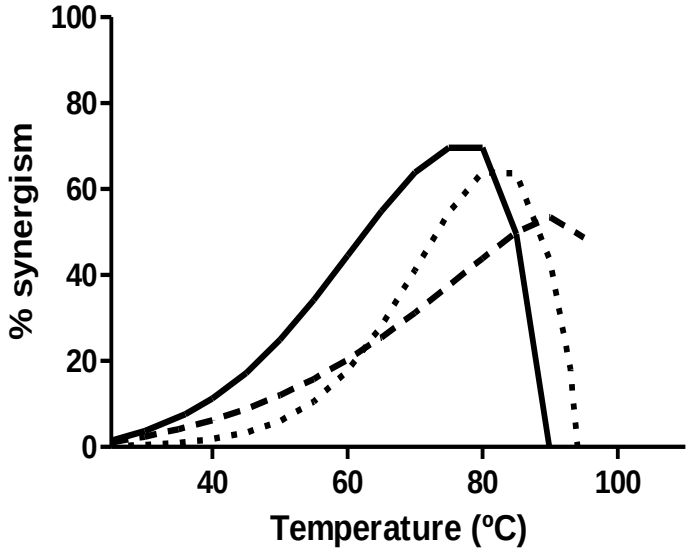


Figure 6.8: Percentage of synergism calculated with Equation 6.6 for the inactivation by MTS of 4 Log_{10} cycles (4*D* values calculated with Geeraerd log-linear regression plus shoulder model) of *B. mycoides* (block line), *P. psychrodurans* (dotted line) and *B. weihenstephanensis* (dashed line).

For each spore species there was a defined range of temperatures 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 $\log_{10}$ cycles (Figure 6.8).

6.5 DISCUSSION

In this study the resistance to heat, ultrasound under pressure and the combined process of ultrasound with heat and pressure (i.e. 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 energy 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 are usual (Baril *et al.*, 2011, 2012; Coton *et al.*, 2011). These shoulders have been associated with 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 among the studies which have been performed 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 6.1 and 6.2). These results are in agreement with data obtained for 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 (Figure 6.5). 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, a 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* (Figure 6.4A) 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 (Figure 6.8).

The results indicated that the maximum synergistic 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*., 1999b; 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.

6.6 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 is 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*).

Chapter 7

The inactivation of psychrophilic spore forming bacteria isolated from crab meat by electron beam ionizing radiation

This chapter is as published in Food Microbiology, with some minor modifications to avoid duplication between chapters.

Condón-Abanto, S., Pedrós-Garrido, S., Cebrián, G., Raso, J., Condón, S., Lyng, J. G., & Álvarez, I. (2018). Crab-meat-isolated psychrophilic spore forming bacteria inactivation by electron beam ionizing radiation. Food Microbiology, 76, 374-381.

7.1 ABSTRACT/RESUMEN

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 6*D* values. *B. weihenstephanensis* was the most resistant, requiring 7.3-7.6kGy to inactivate 6 Log_{10} cycles of this spore forming bacterium, while for *B. mycoides* and *P. psychrodurans* 6.1-6.3 and 5.4-5.3kGy 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 10kGy.

RESUMEN

Este trabajo se realizó para evaluar el potencial de la radiación ionizante de electrones acelerados para inactivar bacterias esporuladas psicrotrofas (*Bacillus mycoides*, *Bacillus weihenstephanensis* y *Psychrobacillus psychrodurans*) aisladas de buey de mar (*Cancer pagurus*) cocinado listo para comer. Se realizaron las curvas de inactivación de las tres bacterias esporuladas en la carne blanca y la carne marrón de buey de mar. También se evaluó el efecto del pH y la actividad de agua (a_w) sobre la eficacia letal de la radiación ionizante. El efecto del pH, a_w y sus interacciones se evaluó en tampón citrato-fosfato de pH entre 7 y 4 y a_w entre >0,99 y 0,80. La reducción de la a_w de >0,99 a 0,90 incrementó significativamente la resistencia de todas las bacterias esporuladas mientras que la reducción de la a_w de 0,90 a 0,80 tuvo un mínimo impacto sobre su resistencia. Contrariamente al efecto de la a_w, el pH del medio de tratamiento mostró un efecto más variable dependiendo de la especie esporulada. Mientras que el pH del medio de tratamiento no afectó a la resistencia de *B. weihenstephanensis* a ninguna a_w, *B. mycoides* mostró un aumento en su resistencia a pH 5,5 a las a_w de 0,90 y 0,80. Por otro lado el pH mostró un efecto significativo en la resistencia de *P. psychrodurans*. En el caso de los dos tipos de carne de cangrejo se observaron mínimas variaciones en los valores 6D. *B. weihenstephanensis* fue el esporo más resistentes, requiriendo 7.3-7.6kGy para producir los 6 ciclos logarítmicos de inactivación mientras que *B. mycoides* y *P. psychrodurans* requirieron 6.1-6.3 y 5.4-5.3kGy respectivamente para alcanzar niveles similares de inactivación. Se observó una resistencia similar de las bacterias esporuladas en la carne de cangrejo y los medios de laboratorio con pH y a_w similares a los de la carne de cangrejo. Los resultados obtenidos en esta investigación demuestran el potencial de la radiación ionizante de electrones acelerados para alcanzar niveles de inactivación adecuados de las bacterias esporuladas presentes en el buey de mar aplicando dosis de radiación menores a 10kGy.

7.2 INTRODUCTION

The use of ionizing radiation for food decontamination was proposed in the 19th century, and since then a wide range of research has been performed to evaluate the potential of this technology for microbial inactivation (De Lara *et al.*, 2002; Grant and Patterson, 1992; Jeong and Kang, 2017; Sarrías *et al.*, 2003; Thayer and Boyd, 1993), and assess its influence on food properties (Byun *et al.*, 2000, 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 10kGy 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 spores. 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*). 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 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*).
- to analyse if the obtained inactivation results in lab media allows to predict the results obtained in the food matrix.

7.3 MATERIALS AND METHODS

7.3.1 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 section 6.3.1. 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 121ºC for 20 min and stored under refrigeration (4±1ºC) until required for use.

Immediately before treatments, the different media were distributed in 24-well plates. Each well was filled with 2mL 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 95ºC 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 10mL, and 0.1mL 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.

7.3.2 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 15kGy. 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 16.4kGy, 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.

7.3.3 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 25ºC 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).

7.3.4 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. 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) as explain in section 2.3.6.2 using Equation 2.4. Those survival curves which showed a log-linear profile were fitted to Bigelow & Easty's model (1920), as it is also explain in section 2.3.6.2. Based in k_{max} the traditional decimal reduction value (D_{10}) of each survival curve was calculated using Equation 2.5. In this case, the D_{10}value corresponds to the necessary dose (kGy) to produce a 90% reduction in the spore population.

To determine the treatment parameters and compare the resistance between the three spores under study, 6*D* values were calculated. In this case 6*D* is defined as the necessary dose to inactivate 6 Log_{10} cycles of the initial spore population, and is calculated by Equation 7.1.

$$6D = Sl + 6*D_{10} \qquad \text{(Eq. 7.1)}$$

Where *Sl* is the shoulder length duration and D_{10} is the inactivation parameter calculated from Equation 2.4.

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≤0.05. The standard deviations (*SD*) are given in the Figures as the error bars.

7.4 RESULTS

7.4.1 Spore inactivation kinetics by electron beam irradiation: Effect of pH and water activity (aw).

Figure 7.1 (A-C) shows 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) (similarly inactivation curves at pH 5.5 and pH 4 are represented in Figure 7.2A-C and 7.3A-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 show shoulders. As indicated in the Materials and Methods section, 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 (*Sl*), and decimal reduction doses (D_{10}). Figures 7.1. 7.2 and 7.3 also presents the line obtained from modelling (black line) to show the goodness of fit. Model parameters are shown in Table 7.1 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 *Sl* 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 *Sl* of *B. mycoides*, ranged between 0 and 0.6kGy and D_{10} values ranged from 0.8 to 2.1kGy, 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 *SI* ranged from 1.1 and 2.5kGy, it being the species which showed the longest *SI*. When a_w was reduced from >0.99 to 0.90 it induced increases of 72%, 90% and 66% in the *SI* at pH 7.0, 5.5 and 4.0, respectively, while the reduction from 0.90 to 0.80 only increased the *SI* by 26%, 19% and 17%, respectively, at the same pHs. On the other hand, D_{10} values ranged from 0.8 to 1.6kGy, 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, an a_w variation from >0.99 to 0.90 induced 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 *SI* values to *B. weihenstephanensis* with a reduction in a_w leading to an increase in the *SI*. However, the influence of pH was more noticeable. At pH 5.5 and 4.0, *SI* 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 *SI* values, showing the lowest values compared to other pHs. On the other hand, D_{10} values ranged from 0.7 to 1.9kGy 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.

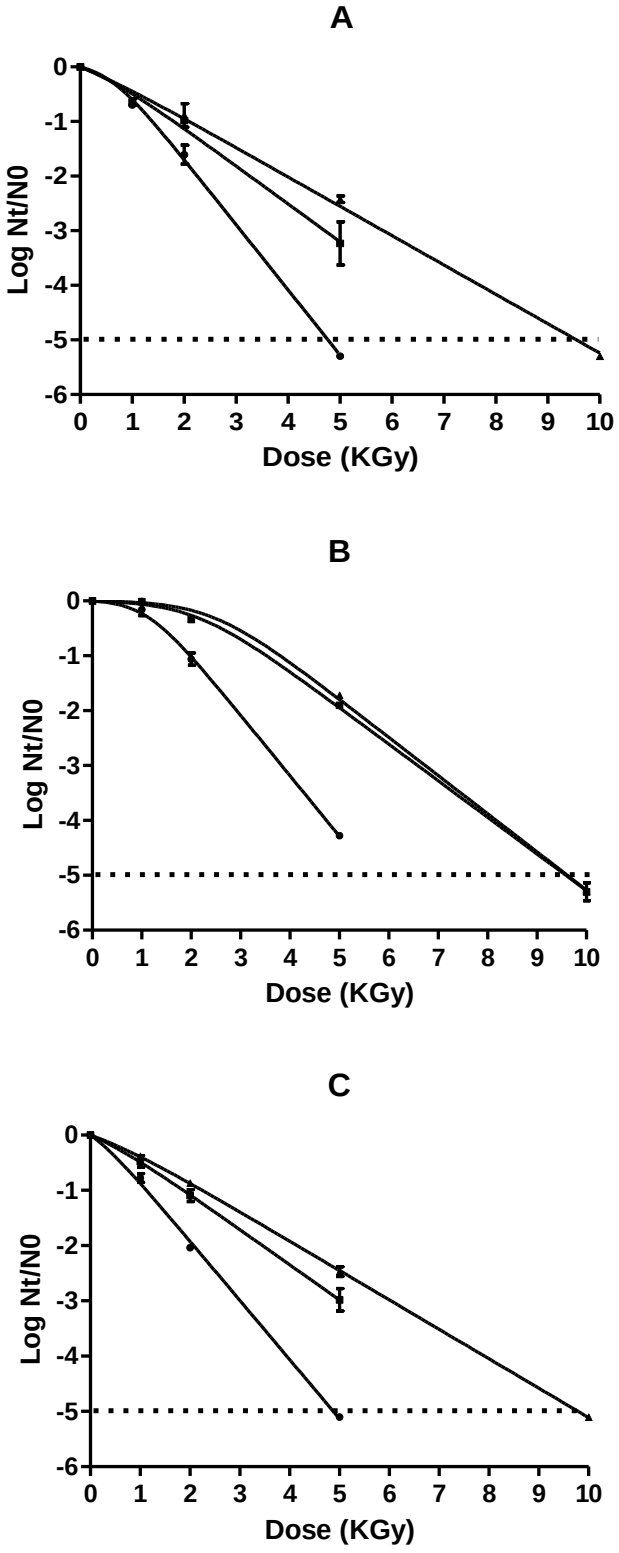


Figure 7.1: Survival curves to electron beam ionizing radiation at room temperature of *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate buffer of pH 7 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars represent standard deviation of three replicates.

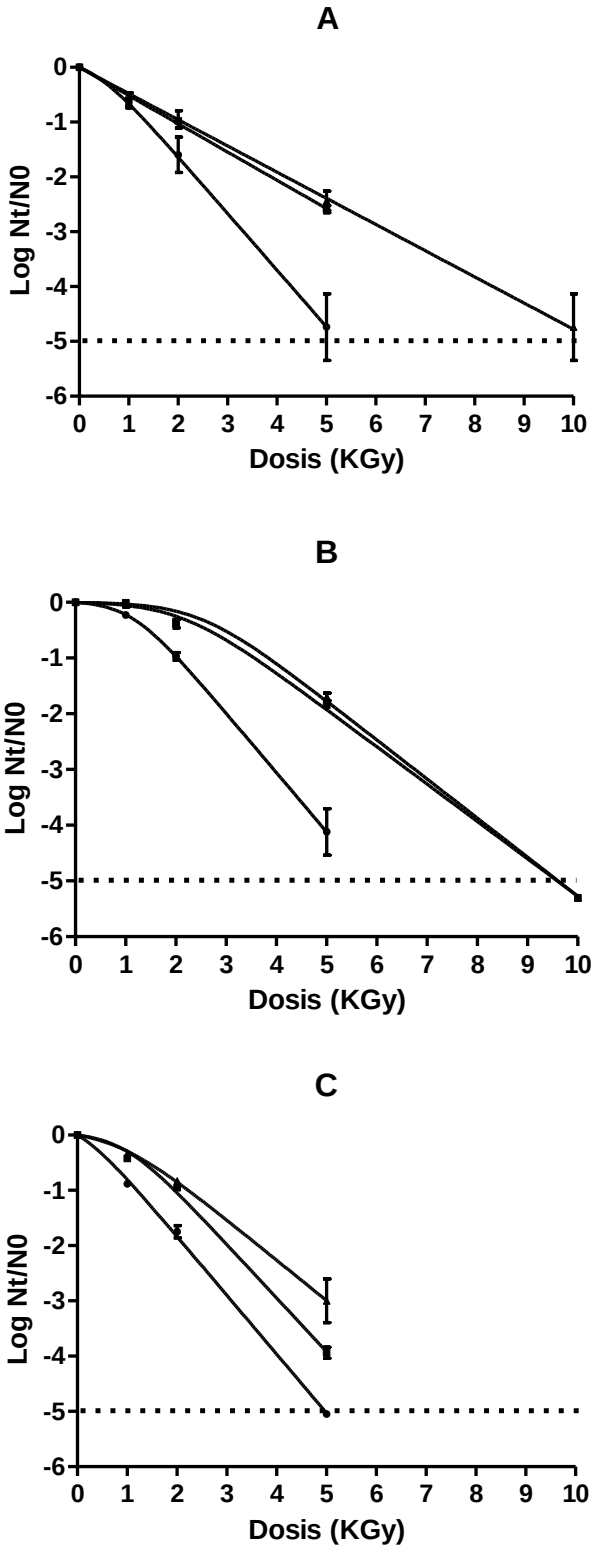


Figure 7.2: Survival curves to electron beam ionizing radiation at room temperature of *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate buffer of pH 5.5 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars represent standard deviation of three replicates.

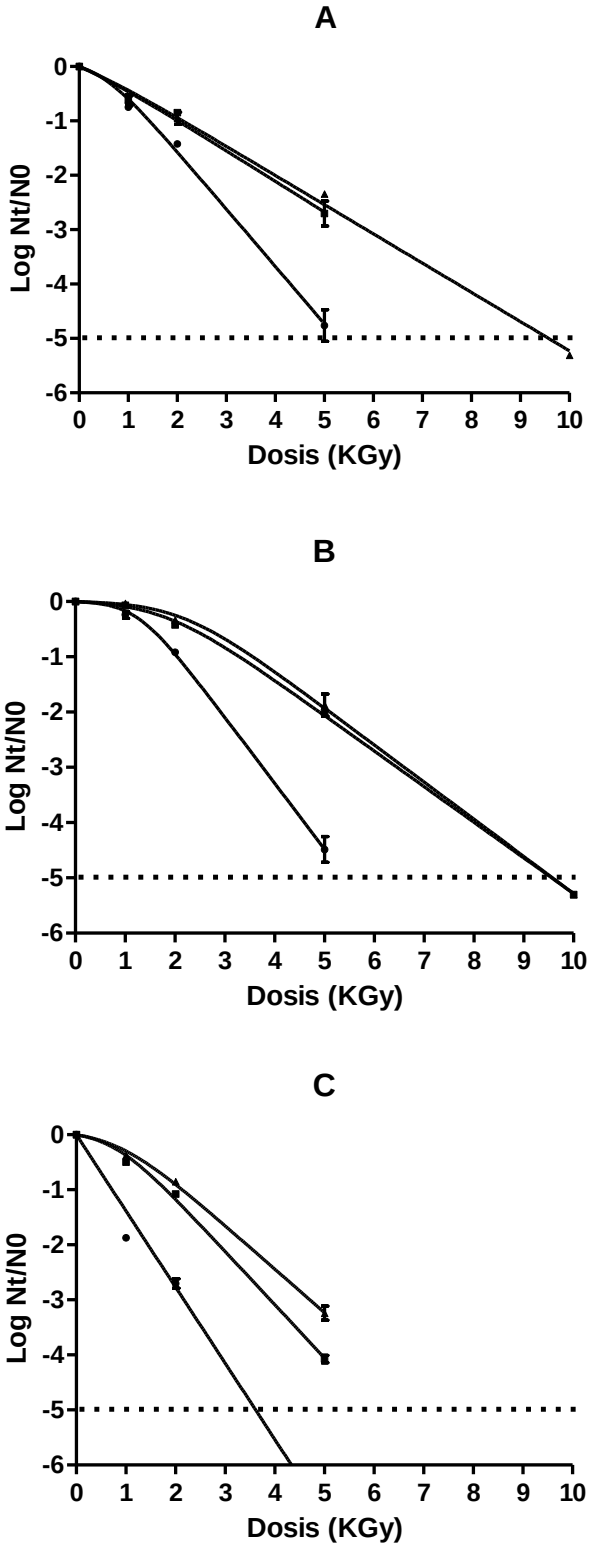


Figure 7.3: Survival curves to electron beam ionizing radiation at room temperature of *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) in citrate-phosphate buffer of pH 4 and water activity (a_w) of >0.99 (●), 0.90 (■) and 0.80 (▲). Error bars represent standard deviation of three replicates.

Table 7.1: Electron beam radiation resistance parameters obtained from the fitting of the Geeraerd log-linear plus shoulder model (Equation 7.1) to the survival curves of *B. mycoides*, *B. weihenstephanensis* and *P. psychrodurans* in citrate-phosphate buffers of different pH and a_w.

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

a_w, water activity; *Sl*, shoulder length; D_{10}, decimal reduction dose calculated from k_{max} with Equation 7.2 ; *RMSE*, root mean square error. Numbers in brackets represent standard deviation of three replicates. Letters show differences within columns for each spore specie ($p<0.05$).

In summary, the inactivation curves obtained for the spore forming bacteria evaluated showed a great variability with regard to the shoulder length duration. Also pH and a_w effects varied notably respect to the studied species: in *B. weihenstephanensis* both factors seemed to be independent of each other, but for *P. psychrodurans* an interaction between these two factors was

observed. The differences detected in D_{10} values between species and also the effects of pH and a_w were smaller than those detected in the *Sl* parameter. The most noticeable difference was observed in case of *P. psychrodurans* where a reduction in resistance was detected at acid pHs.

To define the irradiation treatment intensity required to apply at industrial level, both *Sl* and D_{10} values should be taken into account. To evaluate more clearly the effect of pH and a_w on the irradiation resistance of the investigated spores, 6*D* values were compared. The advantage of using this value is that it comprises both inactivation model parameters, *Sl* and D_{10}. Therefore, it is possible to compare directly the resistance between spores at all investigated conditions. In addition, 6*D* is the inactivation level of the target microorganism to ensure the safety in processed ready-to-eat seafood products (FDA, 2011). Figure 7.4 shows the effect of a_w and pH on the 6*D* values for *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C). As observed, pH had a lower effect on the spore resistance than the effect observed for a_w. For *P. psychrodurans* (Figure 7.4C), 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 (Figure 7.4A).

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

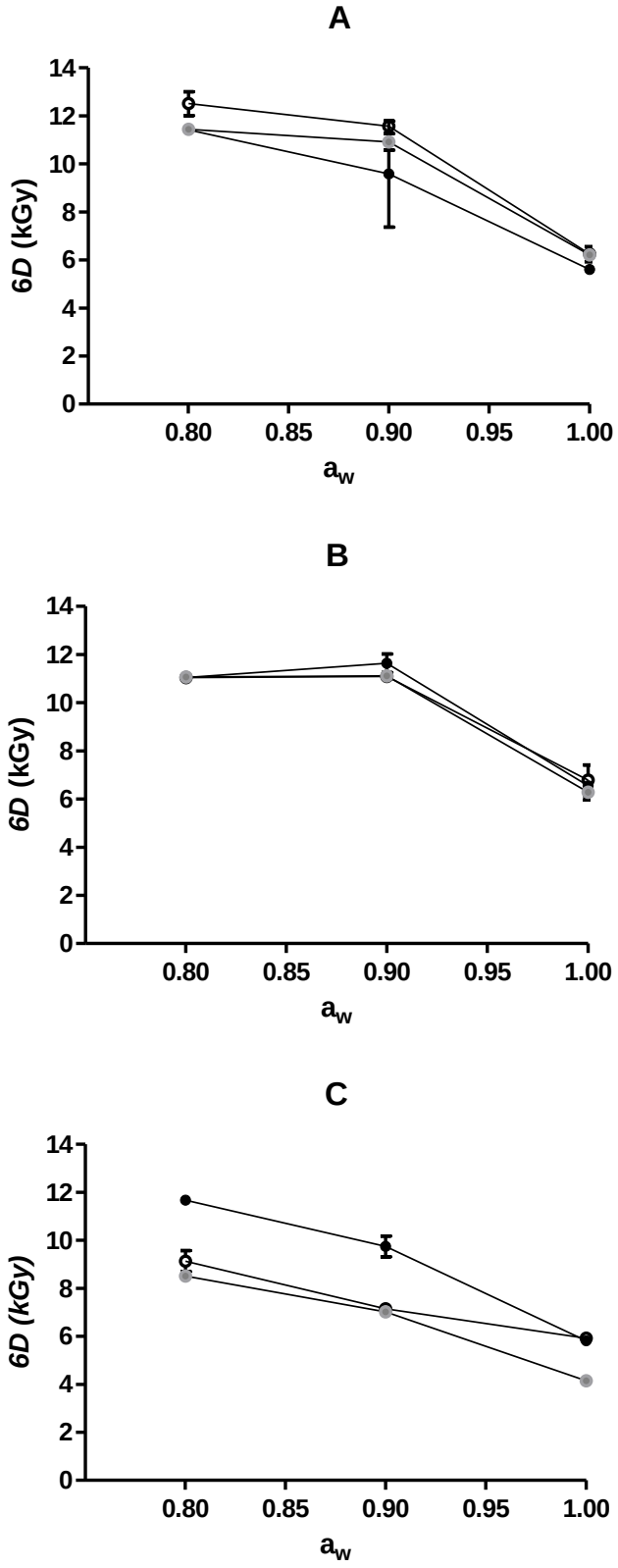


Figure 7.4: Effect of the water activity (a_w) on the dose necessary to reduce 6 Log_{10} cycles of *B. mycoides* (A), *B. weihenstephanensis* (B) and *P. psychrodurans* (C) at pH 7.0 (●), 5.5 (○) and 4.0 (●). Error bars represent standard deviation of three replicates.

From Figure 7.4, besides the influence of pH and a_w, differences in radiation resistances between species can be observed. *B. mycoides* showed the lowest resistance (5.5kGy) in pH 7.0 and a_w of >0.99, and the highest (12.6kGy) at pH 5.5 and an a_w of 0.80. *B. weihenstephanensis* showed the lowest resistance (6.2kGy) at an a_w of >0.99 and pH 4, and the highest (11.0-11.1kGy) at all pH and both an a_w 0.90 and 0.80. *P. psychrodurans* showed the lowest resistance (4.2kGy) at pH 4.0 and a_w >0.99, while the highest resistance (11.7kGy) 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.

7.4.2 Spore inactivation in crab meats

Table 7.2: Electron beam radiation resistance parameters obtained from the fitting of the Geeraerd log-linear plus shoulder model (Equation 7.1) to the survival curves of *B. mycoides, B. weihenstephanensis* and *P. psychrodurans* in white and brown crab meats.

		D_{10} (kGy)	*Sl* (kGy)	6*D* (kGy)	R^2	*RMSE*
White meat	*B. mycoides*	0.8 (0.13)^{a,b}	1.3 (0.22)^{a}	6.1 (0.04)^{a}	0.99	0.026
	B. weihenstephanensis	1.0 (0.06)^{a}	1.0 (0.07)^{a,d}	7.3 (0.05)^{b}	0.99	0.021
	P. psychrodurans	0.8 (0.01)^{b}	0.6 (0.03)^{b}	5.4 (0.02)^{c}	0.99	0.039
Brown meat	*B. mycoides*	0.9 (0.05)^{a}	0.8 (0.03)^{c}	6.3 (0.12)^{d}	0.99	0.012
	B. weihenstephanensis	1.1 (0.06)^{a}	1.0 (0.01)^{a}	7.6 (0.11)^{e}	0.99	0.023
	P. psychrodurans	0.7 (0.01)^{c}	0.9 (0.01)^{d}	5.3 (0.01)^{f}	0.99	0.038

D_{10}, decimal reduction dose (kGy) calculated from k_{max} whit Equation 7.2; *Sl*, shoulder length (kGy); 6*D*, necessary doses (kGy) to reached 6 Log_{10} reductions; *RMSE*, root mean square error; R^2, determination coefficient. Numbers in brackets represent standard deviation of three replicates. Letters show differences within columns ($p<0.05$).

As occurred in lab media, the inactivation curves obtained in crab meat showed downwards profiles in all cases (Figures 7.5A and 7.5B). Therefore, the Geeraerd log-linear regression plus shoulder model (Geeraerd *et al.*, 2000) was used to describe the curves. Table 7.2 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 2.4 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 7.6kGy for white and brown meat, respectively, to reach 6 Log_{10} reductions, while *P. psychrodurans* was the most sensitive requiring 5.4 and 5.3kGy, respectively, to reach a similar inactivation level.

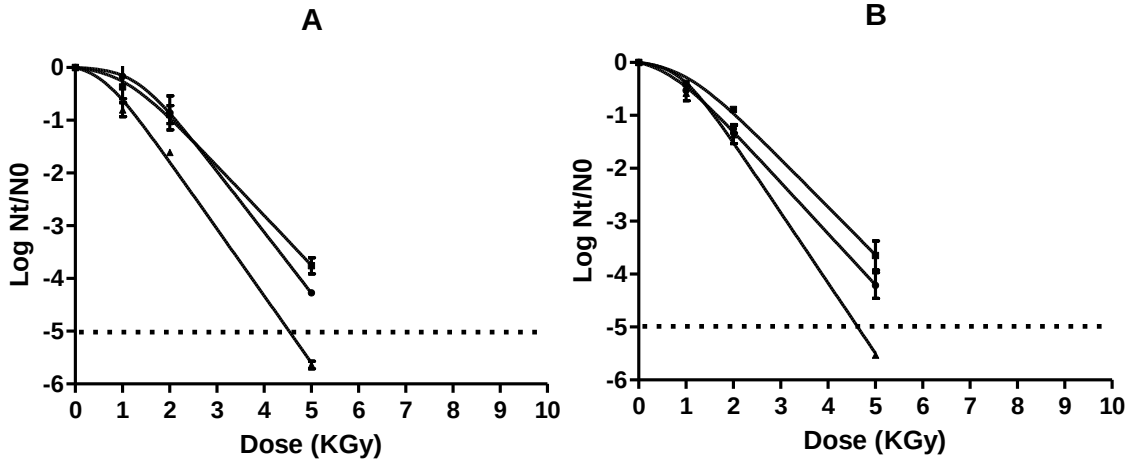


Figure 7. 5: Survival curves to electron beam ionizing radiation at room temperature of *B. mycoides* (●), *B. weihenstephanensis* (■) and *P. psychrodurans* (▲) in crab's white meat (A) and brown meat (B). Error bars represent standard deviation of three replicates.

7.5 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; 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 1-4kGy, (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 1.1kGy. These D_{10} values were lower than those

observed in other *Bacillus* species, which showed D_{10} values higher than 2kGy (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 2.1kGy.

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 damage 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).

In chapter 6 is also described the presence of shoulders in the inactivation curves when applying heat, manosonication (MS) and manothermosonication (MTS) treatments, for *B. weihenstephanensis* and *P. pshychrodurans*, but not for *B. mycoides*. These results suggested that the capacity of damage repair would depend on both the bacterial species and the main target of the applied technology in terms of mechanism of action. Considering that the main mechanism involved in the microbial inactivation produced by EBI is the

damage to the DNA, and the fact that the presence of shoulders is common in the inactivation curves obtained with other technologies which act on the same target, such as the case of UV-C light (Gayán *et al.*, 2013), it is not surprising the detection of these shoulders in the inactivation curves obtained in our research.

However, it has been postulated that pH and a_w do not affect the antimicrobial effect of UV-C light (Gayán *et al.*, 2014b), while our results suggest that the pH and more significantly a_w of the treatment medium affects the irradiation resistance. This fact would be related to the mechanism of action by which each radiation technology, UV-C or e-beam, affects DNA. While UV-C radiation induces the formation of photoproducts due to the direct absorption of photons (Gayán *et al.*, 2014b, Lopez-malo & Palou 2005), EBI reacts through two mechanisms affecting the DNA. The most simple would be comparable with the UV-C mechanisms where the damage in the DNA is produced when an energy photon or electron crash randomly with the genetic material (Dickson 2001; Goodhead 1994; Yokoya *et al.*, 2008); while the second one involved more complex reactions based on the radiation chemistry of water. EBI, in presence of water, produces reactive species, from which hydroxyl radicals (OH●) and hydrogen peroxide (H_2O_2) are considered the main factors responsible for the reactions with nucleic acids (Sutherland *et al.*, 2000; Lomax *et al.*, 2002). The protective effect of a_w observed in this investigation shows the importance of this second mechanism for the inactivation efficacy of EBI. These series of reactions would also explain the results obtained by other authors, where the radiation resistance of different microorganisms increased when microorganism were treated in frozen media, where again a_w is reduced by the freezing process (Black and Jaczynski, 2006; Fan and Sommers, 2012; Thayer and Boyd, 1993 and 2001).

De Lara *et al.*, (2000) suggested that the mechanism involved in bacterial spore inactivation by ionizing radiation would be very different from the mechanisms involved in heat destruction due to the different targets of each

technology. However, since research about the effect of a_w on spore resistance against EBI has not yet been described, 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. In contrast, 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 at al., 2014). The sorption isotherm of 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 in 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 that was 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, 1999). While the pH hardly affected 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, 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 7.6 shows a comparison between the three investigated bacterial spores against heat, MS, MTS and EBI. The variability in resistance among spores was different depending on the inactivation technology. The maximum differences in resistance among the three spores were 1.7-fold for MS, 4.4-fold for MTS, 44.4-fold for heat and less than 1.2-fold in the case of EBI.

These differences in resistance between species would be attributable to the different targets of each technology. As it has been already pointed out, while cell envelopes are the main target for ultrasound (Condón *et al.*, 2011), the most sensitive targets in heat inactivation of bacterial spores seems to be DNA, core enzymes or spore membranes (Palop et al. 1998; Setlow, 1995). On the other hand, as was suggested previously, the most sensitive target to ionizing radiation is the DNA which would explain the small differences in resistance between species as it has been previously suggested for other technologies which act on the same targets such as UV-C (Gayán *et al.*, 2013).

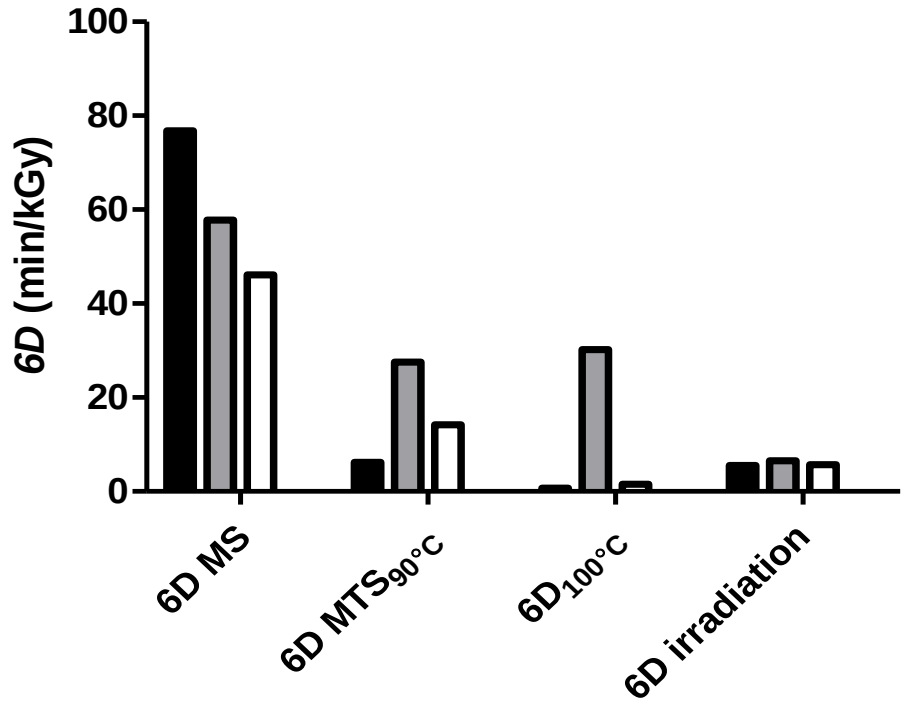


Figure 7.6: Specific resistance of *B. mycoides* (black bars), *B. weihenstephanensis* (grey bars) and *P. psychrodurans* (white bars) to different inactivation technologies in citrate-phosphate buffer of pH 7.0 and a_w >0.99 (data for MS, MTS and Heat are adapted from chapter 6).

In general, the results obtained 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 product sterilised 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 ≤ 2kGy 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.*, 2010a), 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 10kGy, 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.

7.6 CONCLUSIONS

In summary, this work covers a knowledge gap in the field of bacterial spore inactivation by electron beam ionizing radiation. The results obtained 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 a low a_w in 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 of the spore investigated. 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.

Chapter 8

General Discussoin

Crustaceans are considered as luxury, high value seafood items in coastal regions where they are landed. However, the economic evolution of society and the development of transport options have contributed to a more generalized consumption of these products in non-costal regions, often far from where they are landed. *Cancer pagurus* commoly known as Edible crab or Brown crabis a species of crab found in the North Sea, North Atlantic Ocean and in the Mediterranean Sea highly appreciated globally especially in southern European countries (Barrento *et al*., 2009a). This fact is clearly reflected in the 4-fold production volume increase, of this crustacean, in Europe over the past 6 decades with a current annual market value of over 57 million Euros (Eurostat, 2018). Traditionally the crustacean market and edible crab particularly, has been dominated by fresh live product. However, a more recent trend is the production of ready-to-eat products. For edible crab this practice allows processors reduce the loses associated with live transportation, which can become to be very significant, sometimes up to a 50% (Barrento, *et al*., 2008a, 2010b). Furthermore, ready to eat crab has considerably lower transportation costs vs live crab as it avoids shipping crab in aerated tanks of water while also adding value to the final product both of which increase profit for the producers.

As mentioned in Chapter 1, the production of ready-to-eat edible crab and edible crab based products involves an initial classification, slaughter, followed by a first heat treatment (cooking), a washing/cooling step and finally a second heat treatment (pasteurization) (Figure 1.13). The main limitations of this process are that processing parameters have been determined empirically generally from the experience of each producer. This leads to large variability between ready-to-eat products which would have originated from similar raw material. Additionally, relevant technical information on edible crab processing is not plentiful and even if it was, most crab processors are SME's who are

unable to take on the innovation challenge[1] which in turn hinders, advancement in the production of such products. Therefore there is a need in defining process parameters of each technological step (mainly those related with heat treatments) considering the main parameters involved. Once known this, innovation actions can be taken. However, previously to carry out this and in order to perform a process optimization is essential to obtain information about the raw material, the process and its impact on the product (Stoforos, 1995).

The main objective in food production is to ensure product safety. Considering that Bacteria and associated toxins joint with viruses are the principle causative agents responsible for foodborne disease outbreaks, a good knowledge of the contaminants present in the raw material is essential in order to optimise the design of a food process. In this PhD Thesis the microbiota of crabs and specifically bacteria has been investigated. Results obtained during the development of this PhD Thesis (Chapter 2) showed that the levels of microbial contamination in fresh raw edible crab differ depending on the type of meat (i.e. white or brown). In the case of white meat, the observed counts were (average±SD) 1.2±0.31 and 1.8±0.40 Log_{10} CFU/g for Total Viable Mesophilic Count (TVC_m) and Total Viable Psychrophilic Counts (TVC_p) respectively with *Pseudomonas* spp. and *Staphylococcus* spp. representing the microbial groups with the highest levels in white meat. By contrast, contamination levels detected in brown meat were 2.5±0.11 and 3±0.15 Log_{10} CFU/g for TVC_m and TVC_p respectively while the main bacterial detected were *Pseudomonas* spp., *Staphylococcus* spp. but also *Bacillus* spp. (Chapter 2, Figure 2.1).

Once characterized the raw material in terms of bacteria content, evaluation of the main steps of the process and its impact on the product was conducted. As mentioned, in the production of ready-to-eat products, brown crabs are exposed to an initial cooking step following slaughter. In commercial

[1] Joint Programming Initiative. A healthy diet for a healthy life. http://www.healthydietforhealthylife.eu/

practice, this heat treatment frequently consists of cooking crabs by submerging in water at ≈95°C for 20-30 minutes with the objective of achieving a temperature of 85°C in the crab's cold spot (Abdomen). The main purpose of this cooking step is to head induced chemical changes, i.e. protein coagulation, for characteristic flavour development of the final product. Based on the model developed in Chapter 3 (i.e. Equation 8.1), this industrial treatment would be adequate to achieve this objective for crabs in the weight range of 300-500g. However, based on the findings of the present study larger crabs, with weights over 500g, would require longer times to reach 85°C in their cold spot. Based on Equation 8.1 cooking times for such products would need to be increased by 6 min for each 100g increase in crab weight (i.e. crabs of 600g would require at least 36 minutes to achieve their cooking goal). This finding points to the importance of classifying crabs by size and weight prior to processing. Therefore, the developed Equation 8.1 is useful for RTE crab producers to enable them to establish cooking treatments while taking into account the processing temperature and also the weight of the crabs.

$$T_t = T_c - \frac{T_c - T_0}{10^{\left(\left(\frac{1}{(0.053 \times W + 4.9)} \times t\right) - \mathrm{Log}\,1.23\right)}} \qquad \text{Eq. 8.1}$$

Where T_t is the temperature (°C) of the crab 'cold-spot' at a certain time t (min), T_c is the pasteurization temperature; T_0 is the initial temperature in the crab 'cold-spot' and W is the crab weight (grams).

Another less common practice for the production of premier crab and crab based products is to apply a less severe cooking step at lower temperatures (ranging from 75 to 80°C) compared to what is used for traditional cooking treatments (95ºC). The advantage of using this cooking method at lower temperatures is to minimize the overcooking of crab claws due to the differences observed in the thermal profile between claws and crab's cold-spot (Abdomen), as this is presented in Figure 3.2 in Chapter 3. As described, differences of up to 6-fold in the time required to achieve 75ºC in the cold-spot

and inside the claws were observed. This softer cooking process is focused on *L. monocytogenes* inactivation, which is considered the most heat tolerant vegetative pathogenic cell (FDA, 2011). Using Equation 8.1 and considering a cooking temperature of 75°C, the time required to achieve the inactivation goal (i.e. 6 Log_{10} inactivation cycles) of this microorganism varied from 25 and 45 minutes for crabs of 300 and 800 grams respectively (Chapter 3). Again, an although the temperature is lower than the industrial treatment, a wide range of processing time can be applied which could explain the variability of microbial isolations of commercial and lab treated samples evaluated in Chapter 2.

Besides the organoleptic objective of the cooking step, it has to be considered that another effect of this step is its potential to inactivate microorganisms, since lethal temperatures are achieved. Theoretically and based on Equation 8.1 and Equations 3.2 and 3.3, presented in Chapter 3, the total inactivation levels, considering the recommended treatment for *L. monocytogenes* ($F_{70ºC}^{7.5ºC}$ = 2 min), in the industrial cooking step (immersion at 95ºC) would impose an equivalent *F* value of more than 100-fold bigger than that recommended by the FDA (FDA, 2011). By contrast, in relation to the recommended treatment of *C. botulinum* non-proteolytic type E ($F_{90ºC}^{7or10ºC}$ = 10 min), this cooking process barely covers 10% of this recommended *F* value. Based on these results and considering *C. botulinum* non-proteolytic type E as target, a second heat treatment is required to guarantee the safety and stability of the product as it will be discussed later on.

Since there were hardly any data concerning the lethal efficiency of the softer cooking treatments and to know the microbiological situation of commercialized crabs processed products. During the development of this PhD Thesis and in addition to evaluating the microbiota of untreated crabs already described, crabs processed at mild temperatures (i.e. 75°C for 45 min to achieve an equivalent $F_{70ºC}^{7.5ºC}$= 2 min) and also selected commercial samples were evaluated at the end of their determined microbiological shelf-life

(Chapter 2). In the samples processed at lab scale only 27% of the isolated bacteria were identified as vegetative cells while the 73% were identified as bacterial spores. In the case of commercial samples, which not only cooking but pasteurization treatments were applied, 58% of the isolated microorganisms were identified as being vegetative cells while the remaining 42% were identified as bacterial spores. From vegetative cells, *Carnobacterium divergens* and *Shewanella baltica* were the most frequently identified and *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* the most frequently detected spore-forming bacteria. Neither *Clostridium* spp. nor *Listeria* spp. were isolated or identified.

Although, it is assumed that the heat treatment at which commercial samples were subjected was more intense than the heat treatment applied at lab scale, a higher proportion of vegetative cells were isolated in the case of the commercial samples. This discrepancy may be related to the thermal resistance of the isolated bacteria and/or could be due to the different thermal treatments. However, the thermal resistance of the isolated bacteria was also evaluated in this work (Chapter 2), with *Kocuria atrinae* being identified as the most heat resistant ($6D_{60°C}$= 15 min and z = 6.6°C) of the vegetative microorganisms isolated (as indicated, *Listeria* spp. was not detected in any of crab samples in this PhD Thesis), though the reported heat resistance of *L. monocytogenes* is higher (FDA, 2011). That said, the heat penetration calculation previously presented proved that either cooking process (i.e. mild (75°C to achieve an equivalent $F_{70°C}^{7.5°C}$ of 2 min) or regular (95°C to reach 85°C in the "cold-spot") cooking temperatures) were adequate to achieve the recommended F value for *L. monocytogenes*. However, high levels of vegetative cells were still detected, which might indicate a possible recontamination after cooking. This recontamination could be a consequence of the hygienic practices during processing and meat removal. This fact is manifested in the results, since the percentage of vegetative cells isolated from lab produced samples was lower

than commercial samples, endorsing the importance of the control of hygienic practices in the crab industry.

These results indicated a possible source of re-contamination in crab processing. Cooling/washing step after cooking could be a key point since samples produced at lab scale also contained vegetative cells, where correct hygienic practices were used (i.e. workers wearing gloves, meat removed in a laminar flow cabinet, clean knives, etc.). If standard water is used, potential exists for microbial recontamination. For this reason, a washing step under hygienic conditions is essential, for the production of premier crab products. Additionally, this washing step leads to an increase in water consumption which impacts on sustainability. Therefore, developing strategies which allow the avoidance of washing or which improve its efficiency would certainly be an advantage in the production of ready-to-eat crab.

In Chapter 3 the potential for ultrasound technology to **wash and clean** crabs during cooking was demonstrated. In addition to the cleaning effect of ultrasound which removes particles or solutes from the crab, modifying the turbidity and electrical conductivity of the surrounding cooking water, this PhD Thesis has shown that ultrasound is potentially useful in removing heavy metals from crab, specifically cadmium (Chapter 4). This is the first time that this particular application for ultrasound has been ever described and if a way could be found to apply this commercially, it could be quite significant as this is an issue of major concern for the industry due to several researches have warned about the hihgh concentration of this contaminant in edible crab (Bolam *et al.*, 2016; Maulvault *et al.*, 2013; Noël *et al.*, 2011; Wiech *et al.*, 2017). The present study showed that under optimal conditions ultrasound technology enabled a 23% reduction in the cadmium content of crabs, which could be increased with further treatment optimization. The best reduction was observed at intermediate temperatures of 50°C instead of at the highest temperatures investigated when using ultrasound for cleaning. As discussed in Chapter 4 the effects of ultrasound are generally attributed to cavitation phenomena. It is

generally accepted that the intensity of cavitation decreases as the vapour tension of the liquid increases (Knorr *et al.*, 2004). Therefore, the stronger ultrasonic cavitation effects could be expected at lower temperatures. In the case of crab processing, this would appear to align with the findings observed in the cadmium removal study.

Also discussed in Chapter 3, ultrasound can be used to enhance the **heat penetration** into the cold-spot of the crab which in turn allows 12 to 17% reduction in the total cooking times while maintain the applied *F* values. On the other hand, maintaining the same cooking time, the application of ultrasound (25 kHz, 10 W/kg) enabled to increase from 2.2 to 3.2 fold the *F* values applied to the product. As also discussed in Chapter 3 this improvement in the heat transfer also showed a potential for reducing differences in the rate of heat penetration between crabs of different weights. As shown in table 3.2 when ultrasound was applied during cooking the influence of the weights of the crabs on the heating rate was reduced by 62%. This advantage could facilitate the classification step prior to processing or reduce the differences in the applied heat treatments between crabs in the same batch. Moreover, the simultaneous application of ultrasound when cooking at 80ºC reduced the posterior exudates of the crab, which would simplify or even avoid the washing/cooling step after cooking (Figure 3.7 in Chapter 3).

Based on these findings, ultrasound could be applied in the processing of edible crab for cleaning during cooking, reducing the exudates produced, enhancing the rate of heat transfer and reducing the total Cd content; although based on the results obtained in Chapter 4 the temperatures required for cooking (i.e. over 70°C) might affect the cadmium reduction induced by ultrasound. However, the cadmium reducing potential for ultrasound could be realised by applying ultrasound during the cooling phase. Alternatively pre-washing process step prior cooking, at lower temperatures, could be suggested as a method to reduce the cadmium concentration in crabs. Either way, it has been proven that ultrasound has a great potential for enhancing different

aspects of the cooking process of edible crab, though further research is necessary in order to define different ultrasonic processing parameter combination to take advantage of all ultrasonic effects.

As it has been shown in the flow diagram for RTE crab produts (Figure 1.13 in Chapter 1), after cooking and whasing/cooling step a second pasteurization of packed products is generally appplyed. This step is essential to assure their safety. Thus, although crabs produced with a single cook would be expected to have superior organoleptic characteristics, this production type is effective to inactivate a large number of the vegetative cells present in crab, but not bacterial spores. Also these minimum cooked crab products have a relatively short shelf-life (i.e. 10 days max). Therefore, in order to ensure the product safety and extend their shelf-life a second in-pack pasteurization is usually applied. This second pasteurization step ($F_{90°C}^{7or10°C}$ = 10 min) is generally focused on a 6 Log_{10} cycle-reduction in the population of *C. botulinum* non-proteolytic type E spores, which is a pathogen of great concern in food safety especially in this type of marine product, as identified by the U.S. Food and Drug Administration (FDA, 2011). Additionally, this microorganism is able to produce heat tolerant forms (i.e. bacterial spores) which can survive the heat treatment applied during cooking.

As it has been discussed before, different spore-forming bacteria were isolated in RTE crab products. However, in no case, *Clostridium* spp. were isolated or identified, but spores from *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* were isolated (Chapter 2). These results would indicate that the pasteurization treatments applied in the crab industry were effective in avoiding the presence of *C. botulinum* non-proteolytic type E spores but no of other potentially more heat resistant ones. This would be the case of *Bacillus weihenstephanensis* which showed much larger heat resistance than *C. botulinum* non-proteolytic type E (Chapter 6). Therefore, for a 6 Log_{10} cycle reduction of *B. weihenstephanensis* spores, a treatment of 821 minutes at 90°C would be required, based on the heat

resistant determined for this bacterial spores (Chapter 6). The importance of this spore-forming species is that is able to sporulate and grow at refrigeration temperatures, but more important is its potential to produce emetic and diarrheal toxins, normally associated with *B. cereus* (Stenfors and Granum, 2001; Stenfors *et al.*, 2002; Thorsen *et al.*, 2006, 2009). Therefore, the obtained results in this PhD Thesis are of relevance since *B. wehenstephanensis* could be a new key pathogen and might be considered as the new target microorganism for defining the pasteurization process of edible crab. On the other hand, from results shown in Chapter 6 an *F* value of 6 minutes at 105°C or equivalent, with a *z* value of 7.6°C, could be proposed in order to achieve a 6 Log_{10} reduction of *B. weihenstephanensis* in crab instead of the already established $F_{90°C}^{7or10°C}$ = 10 min for *C. botulinum* non-proteolytic type E.

In addition to microbial inactivation, as previously mentioned, heat treatments also induce a series of chemical reactions which can modify the attributes of foodstuffs. Ultimately, these reactions will influence consumer acceptance and, for this reason, an understanding of their development kinetics is essential in order to design thermal processes that produce products which better align with consumer expectations (Ling *et al.*, 2015; Liaotrakoon *et al.*, 2013; Zabbia *et al.*, 2011). Therefore, the selection of effective heat processing conditions, which will produce adequate microbial inactivation minimizing undesirable heat-related changes, is important for the food industry.

In the specific case of edible crab, one of the main challenges for the its pasteurization is the lack of knowledge regarding the thermal impact on its quality attributes, which makes the selection of processing parameters (i.e. process optimization) very difficult. Therefore, one of the objectives of this PhD Thesis was to characterize the effects of the FDA recommended heat pasteurization treatment ($F_{90°C}^{7or10°C}$= 10 min) on a number of quality parameters of white and brown meat from whole cooked crabs (Chapter 5). To do this, the first required step was to characterize the thermal profile of packed crabs using a pilot scale superheated water showering retort to apply the heat

treatments. Similar to the study of crab thermal profiles during immersion cooking, the cold-spot of the crabs was firstly determined (though the location was identified as being in the crab's abdomen which was in agreement with the previous findings from immersion cooking). Similarly to the study of crab cooking study a mathematical equation, based on Ball & Olson equation, to predict the thermal profile of crabs, depending on their weights, was developed, as shown in Chapter 5 (Equation 5.9).When compare Equation 8.1 (which describes the heating rate in the crab "cold-spot" during cooking) with Equation 5.9 (which describes the heating rate in the crab cold "cold-spot" during the pasteurization in a superheated water showering retort) it can be observed a different impact of the weight of the crab on the Ball & Olson equation parameters depending on the heating method the used. In case of cooking a bigger impact of the weight was observed on *fh* than in the case of the retort pasteurization. On the other side bigger *j* value was defined in case of retort pasteruization.

Based on Equation 5.9, the effect of the pasteurization temperature, on the crab quality attributes was studied by defining equivalent treatments to achieve the recommended *F* value for *C. botulinum* non-proteolytic type E ($\underline{F}_{90°C}^{7or10°C}$= 10 min) by using Equations 3.2 and 3.3 shown in Chapter 3. Based on the results obtained in Chapter 5 (Table 5.2), colour of white meat was the attribute most affected by the treatment temperature and a deeper study evaluating its thermal kinetics was subsequently performed. As discussed in Chapter 5 the colour change kinetics of white meat, considering total colour change parameter (ΔE*) as an indicator, was fitted to a pseudo-first order association kinetics and the equation parameters correlated with the treatment temperature. Finally a tertiary equation (Equation 5.12 in Chapter 5), which allowed calculating the total colour change of the crab's white meat during heat treatment was developed in Chapter 5. This is the first time in which the colour change of white meat has been defined as the critical quality parameter for heat process optimization of edible crab.

Moreover and in parallel to the colour change kinetics study, the quality perception of the white meat was correlated with a ΔE* parameter during a series of focus groups with the main edible crab producers of Ireland. Based on the obtained results white meat was classified, as "good quality" (ΔE* ≤ 7), "acceptable quality" (7< ΔE* <9) and "non-acceptable quality" (ΔE* ≥ 9) based on their colour (Figure 5.7 in Chapter 5). One of the main advantages of this study is that, the Equations developed for the colour change, could be easily transferred to other crab products such as picked white meat or crab claws.

Using the limits established above in terms of the ΔE* parameter, the thermal profile of crab claws and crab's "cold-spot", an optimization graph was constructed (Figure 5.8 in Chapter 5). As observed, depending on its weight, different heat treatments were required to achieve the goal of $F_{90°C}^{7or10°C}$= 10min, in the crab's "cold-spot". To evaluate the effect of the crab weight on the pasteurization parameters, Figure 8.1 shows the maximum temperatures at which it would be possible to pasteurize whole cooked crabs to maintain a "good quality" or "acceptable quality" in their white meat, based on the required time at each temperature to achieve an appropriate level of *C. botulinum* non-proteolytic type E inactivation. In this Figure all temperatures represented by the green line or below would produce crabs with a "good quality" in terms of white meat colour, while the temperatures in the area between green and blue line would produce an "acceptable quality". Also in the Figure it can be observed that increases in the weight of the crab suppose a reduction of the maximum pasteurization temperatures, to maintain a certain quality, in a proportion of 1°C for an increase of 100g. All of these calculi are referred to the quality associated with the colour of white meat.

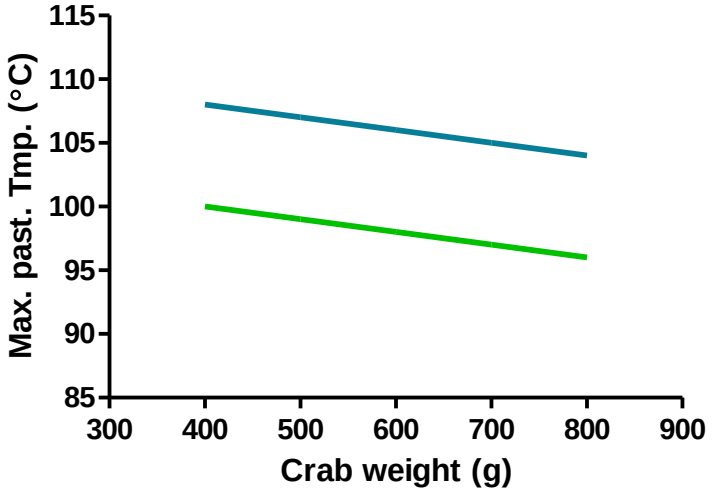


Figure 8.1: Maximum pasteurization temperatures allow to achieve the target $F_{90°C}^{10°C}$ = 10 minmaintaining "good quality" (green line) and "acceptable quality" (blue line) depending on the crab weight, based on the equations developed in Chapter 5.

The effect of crab weight on pasteurization parameters is a consequence of the different thermal profiles of crab claws (where the white meat is located) and crab abdomen (where the "cold-spot" was defined) as is represented Figure 5.1 (Chapter 5). While the crab weight did not affect the thermal profile of the claws, because small differences between claws exist regardless the size of the crabs. The impact of crab weight has a big impact on the thermal profile of the "cold-spot" (i.e. crab's abdomen). Therefore for the pasteurization of bigger crabs a reduction of the pasteurization temperature is required, based on the colour change kinetics described, due to the longer pasteurization time necessary to achieve the pasteurization objective in the "cold-spot". These results endorse, as in case of cooking, the importance of the classification step prior to processing in order to avoid the over-heating or under-heating within crabs in the same batch.

In based of the results obtained in this PhD Thesis considering microbial and quality aspects already discussed with respect of the FDA recommended pasteurization treatment, a theoretical optimisation graph for the pasteurization of ready-to-eat whole edible crab has been generated. This graph has been generated thanks to the equations developed in Chapter 5 (i.e. Eq. 5.9 and Eq.

5.12) and Chapter 3 (Eq. 3.2 and Eq. 3.3). It has to be pointed out that the mathematical Equations developed in Chapter 5 allow the definition of treatment conditions for whichever the target microorganism may be and not only centred on *C. botulinum* non-proteolytic type E. Figure 8.2 compares the treatments required for *C. botulinum* ($F_{90°C}^{10°C}$= 10 min) *vs.* those proposed for *B. weihenstephanensis* ($F_{105°C}^{7.6°C}$= 6 min) with the impact on the quality of white meat.

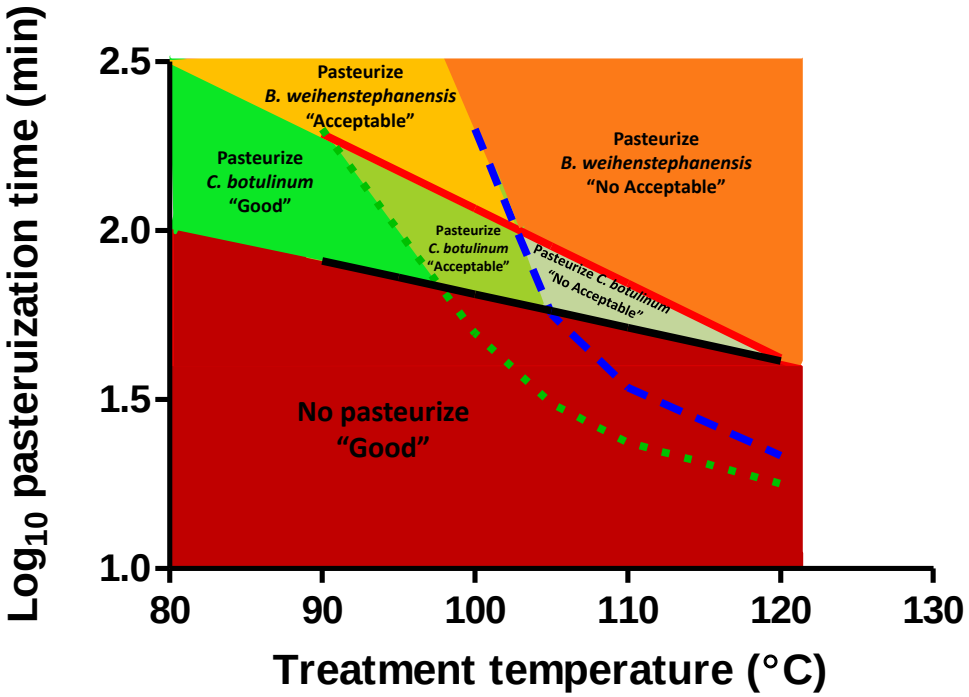


Figure 8.2: Theoretical optimisation graph for the pasteurization of ready-to-eat whole edible crab based on Equations 5.12 and 5.13 for crabs of 600g for the inactivation of 6 Log_{10} reductions of *C. botulinum* non-proteolytic type E based on U.S. FDA recommendations and *B. weihenstephanensis* based on the thermal resistance obtained in this PhD Thesis (Chapter 6).

As Figure 8.2 shows, with *B. weihenstephanensis* considered as the target microorganism the pasteurization parameters (i.e. time/temperature combinations) required to achieve a similar inactivation level to *C. botulinum* non-proteolytic type E (i.e. 6 Log_{10} cycles) would produce considerable changes in the colour of crab white meat. In fact, based on Figure 8.2 in the range of temperatures from 90 to 120°C, the required times to reduce the population of *B. weihenstephanensis* by 6 Log_{10} cycles will produce products with only "acceptable" quality. If a "good" quality is to be maintained, the pasteurization

temperatures must be ≤90°C. However, due to the high thermal resistance of *B. weihenstephanensis*, these treatments would require an excessively long treatment time (i.e. over 3 hours).

These results suggest the need of looking for strategies which enable to improve the lethal effectiveness of the current heat treatments or to find technologies which guaranty the safety and stability of the defined heat pasteurization treatments while minimally impacting crab quality. As mentioned in Chapter 1 several alternative technologies could be employed to reduce or avoid the negative heat induced impact when imposing heat treatments for the inactivation of spores. During the development of this PhD Thesis two of these alternative technologies, ultrasound in combination with pressure and heat and electron beam ionizing radiation, were explored in terms of their potential to inactivate the main bacterial spores isolated form edible crab.

As previously discussed, ultrasound has the potential to improve the cooking and cleaning of edible crab. Also in Chapter 3 the potential lethality of ultrasound during cooking was discussed (Figure 3.5 A and B). The results obtained suggest that ultrasound can increase microbial inactivation during cooking. However, under atmospheric pressures and the low ultrasonic power density employed in the present study, this effect is more likely due to ultrasonically induced improvements in heat transfer. Using the same ultrasonic conditions, but at room temperature (i.e. 20°C) no microbial inactivation was observed. Under certain conditions ultrasound is considered as one of the new alternative microbial inactivation technologies to conventional heat treatments (U.S. FDA, 2000) when applied at temperatures similar to those used in the present study. However, for effective microbial inactivation the ultrasonic power density required is considerably higher than that employed in the present study and also ultrasound is generally applied under pressure. Due to the low bactericidal efficacy of ultrasound at room temperature (Lee *et al.*, 2013; Meullemiestre *et al.*, 2017; Jambrak, *et al.*, 2017), most researchers have tried to design combined processes to enhance the overall lethal efficacy of

ultrasound (López-Malo *et al.*, 2005; Lee *et al.*, 2013; Raso *et al.*, 1998a). Some of the combined ultrasound processes proposed to date which increase the lethal effect of ultrasound are thermosonication (TS), manosonication (MS) and manothermosonication (MTS) (Chemat *et al.*, 2011; Piyasena *et al.*, 2003; Sala *et al.*, 1995).

Results obtained in Chapter 3 showed that the applied TS treatment (i.e. cooking at 75°C in presence of ultrasound in an ultrasonic bath) did not produce a substantial increase of microbial inactivation, though MS and MTS treatments proved to be effective for the inactivation of spores isolated from crab, in liquid media (Chapter 6). It has been reported that the bactericidal efficacy of ultrasound is directly correlated with the ultrasonic power transferred to the media (Mañas *et al.*, 2000). In Chapter 6 all trials were carried out in a specific device specially designed to evaluate the bactericidal potential of TS, MS and MTS treatments, which allows the application of ultrasonic power densities ranging between 1600 and 8000W/L at 20kHz. In Chapter 6 an ultrasonic power of 4800W/L and 20kHz was used to evaluate the potential of MS and MTS treatments while for the TS treatment, which was applied in an ultrasonic bath for crab cooking at atmospheric pressure, a maximum ultrasonic power density of 10W/L and 35kHz was used. Although, ultrasonic frequency could have an influence on the microbial inactivation, the difference on the applied ultrasonic power would more than likely be the main reason why in case of TS no bactericidal effect was observed while MS and MTS treatments proved to be effective for bacterial spore inactivation.

Although the results obtained in Chapter 6 show great potential for MTS treatment (using temperatures over 80°C and an ultrasonic input power of 4800 W/L) to inactivate the main bacterial spores isolated from crab, to this process is generally only considered for pumpable liquids and is not readily adaptable for whole crab, though its advantages could be exploited only for liquid products such as crab soups. However, the high ultrasonic energy required for bacterial spore inactivation using MTS treatments, (4800W/L)

compared to other ultrasound applications such as assisted freezing (from 7.3 to 25.89W/L) (Li and Sun, 2002) or marinating (from 800 to 1200W/L) (Turhan *et al*., 2013) makes its industrial application difficult due to the high energies involved. Either way, considering the results obtained for bacterial spores inactivation using ultrasound in combination with heat plus pressure (MTS) (Chapter 6), if enough ultrasonic power is applied during the pasteurization process , with *B. weihenstephanensis* considered as a target microorganism, a substantial reduction, of over the 80%, of the pasteurization time could be reached as shows Figure 8.3.

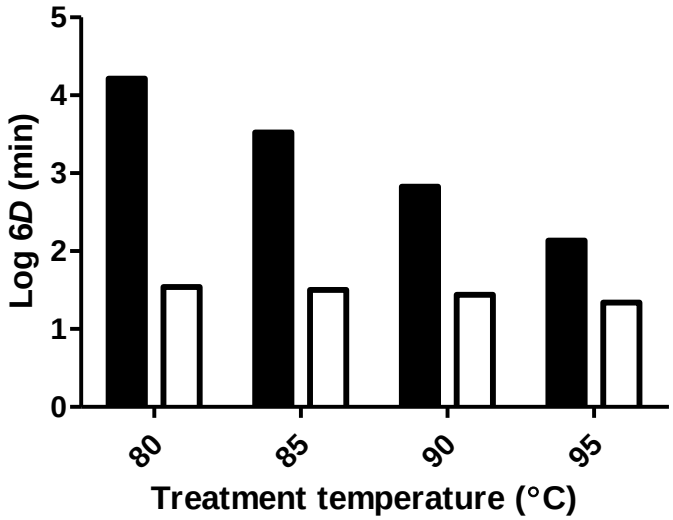


Figure 8.3: Log of the time required to achieve 6 Log_{10} reductions of the population of *B. weihenstephanensis* by heat (black bars) and MTS (white bars) at different temperatures in pH 6.8 McIlvaine citrate-phosphate buffer.

Besides MTS technology, in this PhD Thesis Electron Beam Ionizing radiation (EBI) was also evaluated. Initially, this technology would have more viability, if legally permitted, since it could be applied to cooked whole crab and crab products in pack. As it is discussed in Chapter 7 one of the major advantages of ionizing radiation is its capability to inactivate the main bacterial spores present in crab at low temperatures (even frozen) which in turn would avoid the adverse effects produced during heat pasteurization. From the results shown in Chapter 7, Figure 8.4 shows the required dose to reduce the population of the main three bacterial spores isolated from edible crab by 6 Log_{10} cycles. Based on Figure 8.4, ionization treatments below 8kGy appear to

be sufficient to achieve a proper inactivation level of the three main spores isolated from edible crab, identified in this PhD Thesis. The results obtained also showed that despite the different composition and chemical characteristics of the two types of crab meat (Anacleto *et al.*, 2011; Barrento *et al.*, 2010a), the resistance of the bacterial spores was barely affected by the meat type. Therefore, if the penetration depth, which depends on the radiation source, is sufficient these results indicate that a homogeneous inactivation will be produced during the ionization treatment within the crab. Additionally, the specific resistances of the bacterial spore species in crab meat were similar to those detected in lab media at similar pH (7) and a_w (>0.99). Therefore, considering the results obtained in the present PhD thesis, the radiation dose calculated in lab media for a specific microorganism could be used as reference to calculate the necessary treatments to achieve a certain level of safety or stability for a specific foodstuff.

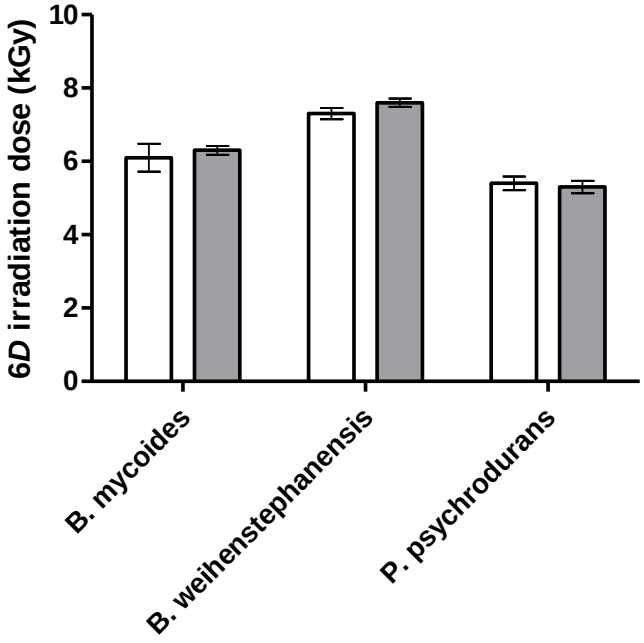


Figure 8.4: Required radiation dose to reduce 6 Log_{10} cycles the population of the main three bacterial spores isolated from edible crab in the two types of crab meat.

Considering that the maximum permitted radiation dose for foods is10 kGy (WHO 1981), the results obtained endorsed the usefulness of this technology for the pasteurization of edible crab, since an adequate level of inactivation of

the bacterial spores isolated from crab could be reached by applying radiation doses below 10kGy. However, the downside is that although more than 60 countries worldwide now have regulations regarding the use of ionizing radiation for food products (IAEA, 2017) its use in Europe is still very limited for food pasteurization especially for seafood products. Only Belgium, Czech Republic and United Kingdom have authorized the irradiation of fish and seafood and only up to a maximum dose of 3kGy. It has been also reported that a radiation dose ≤ 2kGy produces a significant extension of the shelf-life of different crab products though; treatments over this dose might produce significant impact on the sensory perception of these products (Chen *et al.*, 1996; ICGFI, 1998). Therefore in order to finalise this study, future work would involve a sensory evaluation to determine the impact of irradiation on the crab meat quality and the maximum applicable doses to avoid possible undesirable changes on its sensory characteristics, though this was beyond the scope of the present study.

Considering the results obtained during the development of this PhD Thesis, a number of different alternative processes have emerged which can improve the production of ready-to-eat edible crab and crab based products. Figure 8.5 shows these processes for crab products based on different alternative technologies at different stages in crab processing. As Figure 8.5 shows, different alternative processes can be applied for the production of ready-to-eat crab products using novel technologies. Blue shadow boxes represent the traditional steps currently applied in the crab industry while, white boxes represent the possible alternative applications, at different stages of the production line. On the other side, black lines connect the common steps applied to all crab products or early processing stage, independent of its format. While green lines connect the specific processes depending on the different formats i.e. whole cooked crab (Green block lines), picked white and brown meats or claws (Green dashed lines) or transformed crab products (Green dotted line).

On one side, the results obtained in this PhD Thesis proved the possibility of improving the traditional processes considering the obtained data regarding the microbiota present in edible crab (Chapter 2), cooking process (Chapter 3) and the effect of second pasteurization on quality characteristics of edible crab. Regarding the use of the different alternative technologies the results obtained in this PhD Thesis showed that ultrasound technology could be used at different stages with different goals. This technology could be applied in the early processing stages as a pre-treatment prior to cooking to reduce the Cd content of the crab (Chapter 4) or could be also implemented in the cooking process (Chapter 3) where, on one side would enhance heating rates in the crab cold-spot and on the other side would allow to avoid the washing step, after cooking, which in turn would limit the recontamination of the cooked crab (Figure 8.5). Additionally ultrasound technology could be also used in combination with heat and pressure (MTS) (Chapter 6), in certain type of crab based products such as crab soups. However, the down side of this technology for pasteurization purposes is the elevated energy required, which makes very difficult its industrial application. ,

Another technical option for edible crab pasteurization is electron beam ionizing radiation, which has been shown in this PhD Thesis (Chapter 7) to be an effective technology for inactivating the main bacterial spores isolated from edible crab. Unfortunately, while it is very effective, its use for seafood pasteurization is currently not permitted in most EU jurisdictions. In countries where its use is approved a maximum treatment dose of 3kGy is all that is permitted though the results obtained in Chapter 7 of the present study suggest that treatments of between 6 and 8kGy are necessary in order to attain sufficient inactivation of bacterial spores isolated from crab.

Thus, new strategies based on either the optimization of traditional processes or on the retrofitting of alternative technologies into traditional processes, could be used to for process intensification and for enhancing competitiveness in the crab processing sector.

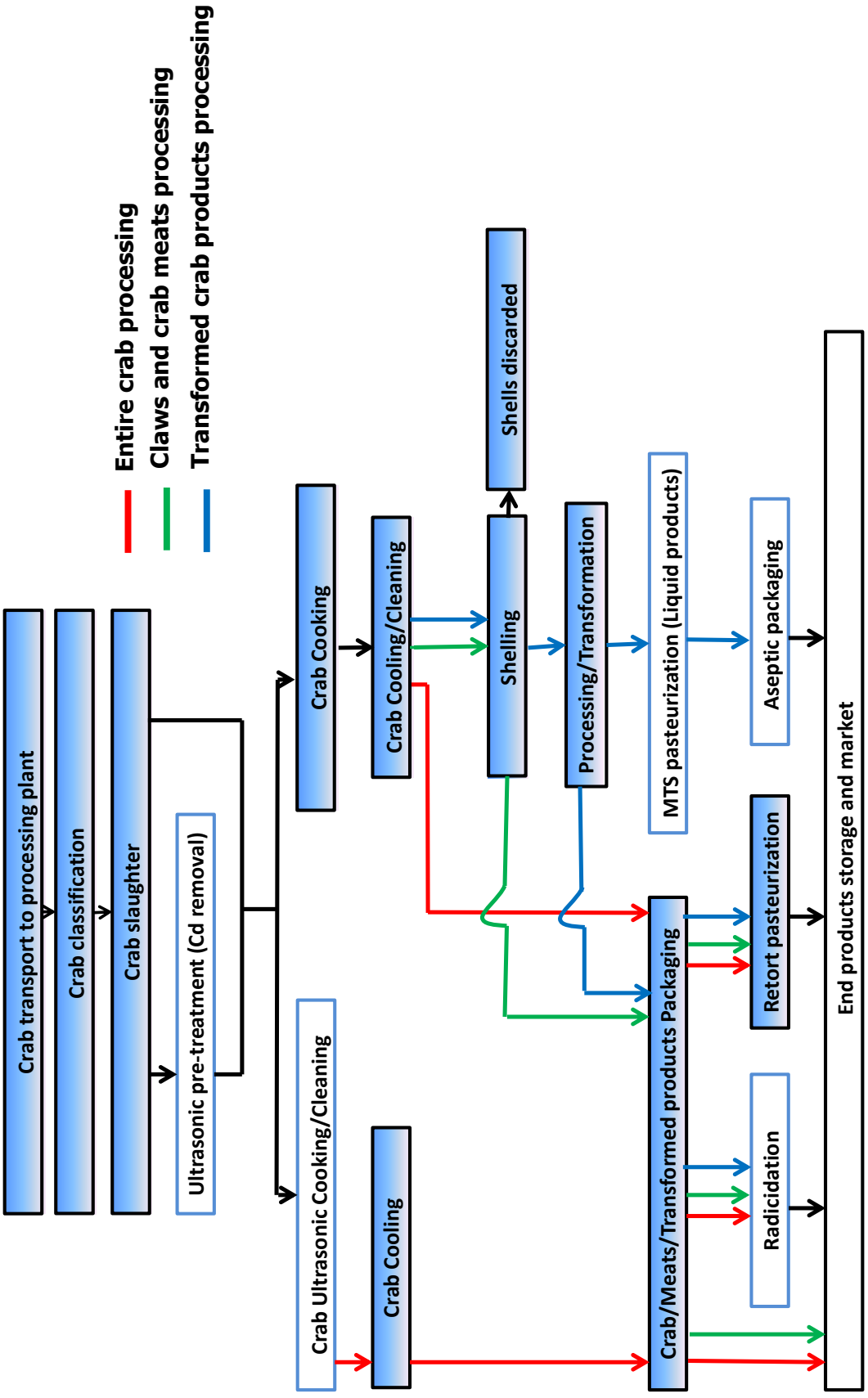


Figure 8. 5: Flow diagram of the different alternative processes proposed, using different alternative technologies.

Chapter 9

Summary and conclusions

In case of edible crab (*Cancer pagurus*), few studies have been focused on the main microorganisms responsible of crab spoilage and none have specified the main microorganisms present in this crustacean. The first part of this work (Chapter 2) consisted of the characterization of the main microorganisms present in both raw and cooked crab. The results obtained showed that the main microbial groups present in raw meat are *Pseudomonas* spp., followed by *Bacillus* spp. and *Staphylococcus* spp*.* In cooked crab the main microbial groups present are *Bacillus* spp. and *Staphylococcus* spp*.,* although their proportions varied depending on the storage temperature. From the isolated microorganisms, 18 bacterial genus and 31 species were identified by 16S rRNA sequencing. *Carnobacterium divergens* and *Shewanella baltica* were the most frequently isolated non-spore forming species and *Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans* the most frequently detected spore-forming bacteria. Neither *Clostridium* spp. nor *Listeria* spp. were isolated or identified.

The results obtained in the thermal resistance study showed *Kocuria atrinae* to be the most heat resistance vegetative cell from the microorganisms identified, though the standard recommended treatment for *Listeria monocytogenes* ($F_{70°C}^{7.5°C}$ = 2 min) is effective to achieve an adequate level of inactivation for this microorganism. For spore forming species, *B. weihenstephanensis* showed the highest heat resistance followed by *P. psychrodurans* and *B. mycoides* (Chapter 6). When the recommended heat treatments, for edible crab products were evaluated, the most severe treatment to inactivate *C. botulinum* non-proteolytic type E ($F_{90°C}^{8.6°C}$= 57 min in Dungeness crab (*Metacarcinus magister*)), was sufficient to achieve an adequate inactivation level (i.e. 6 Log_{10} reduction) of *B. mycoides* and *P. psychrodurans* but not the more heat resistant *B. weihenstephanensis.* These results prove the importance of *Bacillus* spp. in ready-to-eat edible crab products in terms of thermal resistance. Moreover, consideration has to be in mind with *B. weihenstephanensis* due to its potential to produce emetic and

diarrheal toxins, normally associated with *B. cereus* (Stenfors and Granum, 2001; Stenfors *et al*., 2002; Thorsen *et al*., 2006, 2009).

Heat treatments induce a series of chemical reactions which modify the attributes of foodstuffs. In the present PhD Theses, the effect of pasteurization conditions on quality attributes of brown crab were evaluated and optimal pasteurization treatment conditions were then proposed based on mathematical Equations (Chapter 5). From the results it can be concluded that the time necessary to achieve a target *F* value, in the crab's cold-spot, is dependent on the crab weight. The pasteurization temperature did not affect moisture, water holding capacity or the colour of the crab brown meat. Colour change of white meat was found to be the quality attribute most affected by thermal pasteurization and therefore was chosen as the indicator parameter for heat process optimization.

The colour change kinetics, of crab white meat, revealed that the degradation of the colour followed a pseudo-first order kinetic during thermal treatment. Both the maximum colour change achieved and the colour change rate, increased exponentially with treatment temperature. Prior to the optimization calculations, an industry focus group was used to define white meat colour change *vs*. product quality defining different grades (i.e. "good" ($\Delta E^* \leq 7$), "acceptable" ($7 < \Delta E^* < 9$) and "unacceptable" ($\Delta E^* \geq 9$) quality).

Based on the developed Equation (Eq. 5.12) the maximum suitable temperatures to obtain "good" or "acceptable" quality, when pasteurizing whole edible crab were between 96 and 100°C and between 104 and 108°C (depending on crab weight). As explained in Chapter 5 (Figure 5.7) these pasteurization temperatures are defined based on the less severe heat treatment proposed by FDA of $F_{90°C}^{7or10°C}$=10 minutes (FDA, 2011). However, as it has been pointed out in this PhD Thesis, other spore forming bacteria which are more heat resistant than *C. botulinum* non-proteolytic type E, such as *B. weihenstephanensis*, can be present in ready-to-eat crab and crab based products.

In this PhD Thesis in order to solve some of the limitations of the traditional edible crab processing, alternative technologies have been evaluated with different purposes: ultrasound for improving energy and mass transfer; combination of ultrasound with pressure (MS) and temperature (MTS) or the application of Electron Beam Ionizing radiation (EBI) as an alternative to traditional heat processing.

As indicated, one of the alternatives proposed for improving the processing of edible crab was the use of ultrasound technology. The obtained results proved that the application of ultrasound during cooking process enhanced heat transfer to the crab's cold spot (abdomen) during cooking and also proved to be useful to reduce the impact of the variable crab weight on crab heating rate (Chapter 3). This effect would allow for (a) a reduction of the total cooking time while the same *F* value is applied or (b) 2.2-3.2 fold increase in the total *F* value reached when applying the same treatment time. Also the application of ultrasound proved its efficiency to enhance the release of substances (environmental dirt, cook loss deposits and ionic compounds) from the crab to the cook water which would also allow for the omission of the cleaning prior packaging, reducing the risk of recontamination of the processed crab and reducing the water consumption of the process.

Since ultrasound proved its usefulness for releasing substances from brown crab during the cooking process, its ability to reduce cadmium content of this crustacean was also assessed at different temperatures (Chapter 4). Treatment temperature did not bear any influence on the release of Cd in absence of ultrasound, but proved to be a very important variable when ultrasound assisted the process. Without the use of ultrasound the Cd release rate was independent of the treatment temperature within the studied range, obtaining a maximum reduction of a 3% of Cd. By contrast, in the combined treatments (i.e. ultrasound+temperature), ultrasound technology was able to increased Cd release rates 8.7-, 2.1- and 2.7-fold at 50, 65 and 80°C, respectively. The

maximum percentage of Cd extracted of 22.8% was observed at 50°C for an ultrasound input power of 200W or 25W/L.

Based on the results obtained in this PhD Thesis, ultrasound could be implemented in the crab industry during cooking or washing steps or could be even proposed as a pre-treatment prior to initial cooking in order to reduce the Cd content of crab meats, thereby attaining an overall reduction of the risk associated with Cd intake for consumers who eat crab meat on a regular basis. Overall this work has demonstrated for the first time that the application of ultrasound during the crab-cooking process could serve as an effective physical procedure for reducing the Cadmium content of crabs.

As indicated, the potential of ultrasound in combination with pressure (mano-sonication, MS) and with heat and pressure (mano-thermo-sonication, MTS) to inactivate the main bacterial spores present in crab (*Bacillus mycoides*, *Bacillus weihenstephanensis* and *Psychrobacillus psychrodurans*) was also evaluated (Chapter 6). 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 present. When shoulders were detected in the thermal inactivation curves 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. *B. mycoides* 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 spore inactivation. The higher percentage of synergism, under the studied conditions, corresponded to the spore species with higher z_T value (*B. mycoides*), that is to the microorganisms less thermo-dependent. On the other hand the highest temperature (90°C) at which this synergy was detected corresponded to the

most heat tolerant spore species (*B. weihenstephanensis*). Independently of the synergistic effect of ultrasound, pressure and heat, *B. weihenstephanensis* would be also the target microorganism for MTS treatments.

These results show the potential for ultrasound technology to pasteurize liquid based crab products (e.g. crab bouillon or soups), allowing a reduction in the total treatment time, from 13% to 85% depending on the target microorganism, or a reduction in the treatment temperature. This in turn, would induce lower product impact while maintaining similar inactivation levels.

Finally, the potential of Electron Beam Ionizing radiation (EBI) for the pasteurization of ready-to-eat crab and crab based products was also evaluated. More specifically, the potential of electron beam ionizing radiation (EBI) for the inactivation of psychrophilic spore-forming bacteria isolated from ready-to-eat brown crab in lab media, under different conditions of pH, ranging from 4 to 7 and a_w ranging from 0.80 to >0.99, and edible crab meats were evaluated.

From the results it can be concluded that, the pH of the treatment media could affect the spore resistance, although the effect is dependent on the specific spore under study. 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 target bacterial spore. The larger protective effect of the reduction on a_w was observed in the range from >0.99 to 0.90, regardless the spore investigated. For example in case of *B. weihenstephanensis* a reduction of a_w from >0.99 to 0.9 supposed an increase in 6*D* value from 6.5kGy to 11.5kGy while further reductions of the a_w did not influence the total dose required to achieve similar spores reduction.

For the two types of crab meat (white and brown), slight differences were observed in EBI 6*D* values. *B. weihenstephanensis* was identified as the most resistant, requiring 7.3-7.6kGy to inactivate 6 Log_{10} cycles while doses of 6.1-6.3 and 5.4-5.3kGy were necessary to achieve similar inactivation levels for *B.*

mycoides and *P. psychrodurans* respectively. Also from the results obtained it can be deducted that an agreement between spore resistance in crab meats and lab media, with similar characteristics in pH and a_w, exist which opens the possibility to extrapolate the results obtained in-vitro to different ready-to-eat crab based products

Overall, from the inactivation results obtained it can be concluded that the resistance variability among the main three spore formers species against heat treatments is much higher than the variability observed for MTS or EBI treatments. EBI treatment showed the lowest variability among bacterial spores resistance (20%) followed by MS (70%), MTS (440%) and finally heat (4400%). Therefore, the use of these alternative technologies would reduce the potential risk associate with the pasteurization of products due to the wrong choice of the target microorganism.

The results presented in this PhD thesis point out the possibility of improving the production of ready-to-eat brown crab and brown crab based products by introducing different technologies at different stages of the production process or by optimizing current practices, to solve the challenges associated with the processing of this type of products.

Chapter 10

Resumen y Conclusiones

La industria alimentaria está en continua competencia y evolución, lo que requiere una gran actividad innovadora apoyada de una gran labor de investigación y desarrollo. En el caso del sector pesquero y más concretamente en la producción de buey de mar y sus productos listos para el consumo, la I+D+i se ha convertido en una parte fundamental. El desarrollo de la presente Tesis Doctoral da respuesta a muchas necesidades del sector permitiendo obtener avances considerables para el sector al introducir nuevas tecnologías de procesado en su cadena productiva.

En la producción de buey de mar y sus productos listos para el consumo se llevan a cabo diversas etapas que a continuación se detallan y en las que se pueden abordar nuevos desarrollos. Así, el procesado requiere de los siguientes pasos: una vez sacrificado el cangrejo, estos son cocinados, generalmente en agua hirviendo durante 20-30 minutos (primer tratamiento térmico). Tras el cocinado, se realiza un proceso de enfriamiento y lavado necesario para retirar la suciedad y los exudados del caparazón del cangrejo, producidos durante el cocinado. Una vez enfriados los cangrejos que van a ser destinados a la venta como enteros, estos son envasados, generalmente a vacío, y se les aplica un segundo tratamiento térmico, en este caso de pasteurización. Por otro lado, los cangrejos destinados a la producción de productos elaborados o simplemente para la venta de las carnes de cangrejo por separado, se trasladan a la zona de descascarillado tras el enfriamiento con el fin de retirar ambos tipos de carne (la carne blanca de las pinzas y patas y la carne marrón del interior del caparazón). Este proceso se realiza principalmente de manera manual. Finalmente, las carnes de cangrejo son envasadas, de diferentes formas y utilizando diferentes formatos según convenga, antes de aplicarles un tratamiento de pasteurización. Aunque estos pasos son comunes para todos los productores de buey de mar listo para el consumo, existe una gran variabilidad en la calidad entre productos de similares características y formatos. Esta variabilidad se podría atribuir a una falta de estandarización de los procesos debido al pequeño tamaño de las compañías (que generalmente son empresas

familiares) y a su reducida inversión en tecnología y optimización de los procesos. Para realizar la optimización de un proceso, se requiere un conocimiento adecuado tanto de la materia prima como de las variables involucradas en el procesado. Además, es también necesario identificar los riesgos y requerimientos de la materia que deben ser cubiertos por el proceso (Stoforos, 1995).

En el caso del buey de mar listo para el consumo, muy pocos estudios se han centrado en los microorganismos responsables de su deterioro y ninguno de ellos los ha identificado. La primera parte de esta Tesis Doctoral (Capítulo 2) se centró en la caracterización e identificación de los principales microorganismos presentes en el buey de mar crudo y cocinado listo para el consumo así como la identificación de los microorganismos responsables de su alteración y el estudio de su termorresistencia con el fin de valorar la eficacia de los actuales tratamientos térmicos aplicados a estos productos según la FDA de los EE.UU. Los resultados obtenidos mostraron que los grupos bacterianos presentes en mayor proporción en el buey de mar crudo fueron *Pseudomonas* spp., *Bacillus* spp. y *Staphylococcus* spp. Por otro lado, los grupos bacterianos de mayor importancia en el buey de mar cocinado fueron *Bacillus* spp. y *Staphylococcus* spp. aunque sus proporciones variaron dependiendo de la temperatura de almacenamiento. De las bacterias aisladas durante el mismo estudio, se identificaron mediante la secuenciación del ARNr 16S un total de 18 géneros y 31 especies bacterianos diferentes. De las bacterias no formadoras de esporas, *Carnobacterium divergens* y *Shewanella baltica* fueron las aisladas con más frecuencia y *Bacillus mycoides*, *Bacillus weihenstephanensis* y *Psychrobacillus psychrodurans* las bacterias formadoras de esporas más frecuentes.

Los resultados del estudio de termorresistencia de los microrganismos aislados revelaron a *Kocuria atrinae* como la bacteria vegetativa más termorresistente de todas las aisladas del buey de mar; sin embargo, el tratamiento térmico recomendado para inactivar *Listeria monocytogenes*, en

productos del mar procesados ($F_{70°C}^{7.5°C}$ = 2 min) fue suficiente para conseguir un nivel de inactivación adecuado de este microorganismo. De las bacterias formadoras de esporas, *B. weihenstephanensis* mostró la mayor tolerancia al calor seguido por *P. psychrodurans* y finalmente *B. mycoides* (Capítulo 6). Al comparar los tratamientos térmicos requeridos para alcanzar unos niveles de inactivación adecuados de estas esporas bacterianas con el tratamiento térmico más severo recomendado para inactivar *Clostridium botulinum* no proteolítico tipo E ($F_{90°C}^{8.6°C}$ = 57 min) en estos productos, se detectó que el tratamiento era adecuado para conseguir la inactivación de *P. psychrodurans* y *B. mycoides*, pero no para inactivar *B. weihenstephanensis*. En general, estos resultados demuestran la importancia de *Bacillus* spp. en el buey de mar tanto por sus niveles de contaminación y prevalencia como por su elevada resistencia a los tratamientos térmicos.

Como se ha indicado, en el procesado convencional de buey de mar listo para su consumo, es necesario un proceso de lavado del cangrejo, antes del envasado, para retirar la suciedad del caparazón del cangrejo procedente del ambiente marino así como los exudados proteicos producidos durante el cocinado. Este proceso de lavado puede suponer una recontaminación del cangrejo cocinado lo cual hace necesaria una pasteurización del mismo tras el envasado con el fin de garantizar su seguridad sanitaria así como para alargar la vida útil del producto. Además, este proceso de lavado supone un tiempo considerable en la producción y un elevado consumo de agua.

Además, de los riesgos microbiológicos asociados a los pescados y mariscos, en el caso concreto del buey de mar varios estudios han alertado del riesgo de sobrepasar la ingesta semanal tolerable de cadmio, establecida por la EFSA (2009), al consumir este marisco debido a la elevada concentración de este metal pesado, especialmente en la carne marrón del buey de mar (Bolam *et al.*, 2016; Maulvault, *et al.*, 2013; Noël *et al.*, 2011; Wiech *et al.*, 2017).

Una de las soluciones propuestas para afrontar algunos de estos problemas de procesado del buey de mar fue la incorporación de la tecnología de ultrasonidos durante el cocinado. La eficacia de los ultrasonidos para mejorar los procesos de transferencia de calor y de masa, así como para la inactivación microbiana han sido probados extensamente en diferentes procesos utilizando distintas matrices alimentarias (Chandrapala, *et al.*, 2012; Chemat, *et al.*, 2011). Es por ello que se evaluaron las posibles ventajas del uso de esta tecnología en el procesado del buey de mar. Los resultados obtenidos en esta Tesis Doctoral prueban que la aplicación de ultrasonidos durante el cocinado del buey de mar mejora la transferencia de calor en el punto frio del animal (abdomen) además de ser útil para reducir el efecto del tamaño y peso de los cangrejos sobre las velocidades de calentamiento (Capítulo 3). Este efecto permitió por un lado reducir el tiempo total de cocinado (hasta un 15%) manteniendo el un valor *F* actualmente aplicado o por otro lado aumentar entre 2.2 y 3.2 veces el *F* equivalente total aplicado manteniendo el mismo tiempo de cocinado. Esta tecnología también probó su efectividad para incrementar la salida de compuestos (tales como la suciedad natural, los exudados producidos durante el cocinado y compuestos iónicos) desde el cangrejo al agua de cocción lo cual en un principio podría permitir omitir el lavado de los cangrejos tras el cocinado. Además, la aplicación de ultrasonidos evito la incorporación de sal a la carne de cangrejo durante el cocinado en agua con un 5% p/v de NaCl, al contrario de lo que sucede en el cocinado convencional.

Dado el potencial que mostraron los ultrasonidos para eliminar sustancias del buey de mar, también se evalúo su potencial para reducir la concentración de Cd de este crustáceo (Capitulo 4). Para realizar este estudio, se trataron cangrejos hembra a diferentes temperaturas (50, 65 y 80°C) en presencia y ausencia de ultrasonidos, monitorizando la concentración de Cd en el agua de tratamiento. Además, tras el correspondiente tratamiento, se cuantificó la concentración de este metal pesado en las diferentes carnes del buey de mar. La temperatura de tratamiento no mostró una influencia significativa en la

velocidad de salida de Cd desde el cangrejo en aquellos tratamientos sin ultrasonidos, pero mostró una gran importancia en los tratamientos en presencia de ultrasonidos. En los tratamientos convencionales (en ausencia de ultrasonidos), la velocidad de salida de Cd también fue independiente de la temperatura determinándose como máximo una reducción del 3% de Cd en el cangrejo. Por otro lado, en los tratamientos combinados, la presencia de ultrasonidos incrementó la salida de Cd de los cangrejos 8,2, 2,1 y 2,7 veces a las temperaturas de tratamiento de 50, 65 y 80°C, respectivamente. La mayor reducción de Cd detectada en los cangrejos fue de un 22,8% tras el tratamiento combinado a 50°C utilizando una energía ultrasónica de 200W. Estos resultados muestran el potencial del uso de la tecnología de ultrasonidos para reducir el contenido de Cd del buey de mar, aunque se requeriría una investigación más en profundidad para optimizar las condiciones ultrasónicas de tratamiento para maximizar la reducción de este metal pesado. En base a los resultados obtenidos en esta Tesis Doctoral, la tecnología de ultrasonidos se podría implementar en la industria del buey de mar durante los procesos de cocción o lavado o incluso se podría proponer un tratamiento previo a la cocción con el objetivo de reducir el contenido en Cd, reduciendo así los riesgos de ingesta asociados con el consumo de este producto. De cualquier manera, en esta Tesis Doctoral se ha demostrado por primera vez que la tecnología de ultrasonidos es un procedimiento físico efectivo para reducir la concentración de Cd durante la cocción del buey de mar.

Como ya se ha expuesto, los tratamientos térmicos inducen una serie de modificaciones físico-químicas en los alimentos que alteran sus características. Esta circunstancia también se produce en el buey de mar y sus productos. Por esa razón, en esta Tesis Doctoral, se estudió el efecto de los tratamientos térmicos de pasteurización sobre diferentes parámetros que determinan la calidad del buey de mar y, según los resultados obtenidos, se propusieron diferentes condiciones de pasteurización basadas en ecuaciones matemáticas desarrolladas (Capítulo 5).

La temperatura de pasteurización no afectó en el contenido en agua ni en la capacidad de retención de agua de ninguno de los tipos de carne del cangrejo ni tampoco afectó al color de la carne marrón siendo el color de la carne blanca el parámetro más afectado por el tratamiento térmico. Por ello, el color de la carne blanca fue seleccionado como indicador para realizar la optimización del tratamiento de pasteurización del buey de mar.

El estudio de la cinética de cambio de color de la carne blanca reveló que la degradación del color de este tipo de carne del buey de mar sigue una cinética de pseudo-primer orden durante el tratamiento térmico. Además, tanto el máximo cambio de color producido por los tratamientos como la velocidad del cambio de color aumentaron exponencialmente con la temperatura de tratamiento. Con el fin de establecer los límites de calidad en los que basar la optimización y antes de llevar a cabo dichos cálculos de optimización, se realizó un "focus group" con industriales del sector para definir la calidad de los productos en base al color de la carne blanca. Gracias a este trabajo, se ha podido establecer por primera vez un ranking de categorías habiéndose definido los siguientes rangos de calidad en base al cambio de color: "Buena calidad" ($\Delta E^* \leq 7$), "calidad aceptable" ($7 < \Delta E^* < 9$) y "calidad inaceptable" ($\Delta E^* \geq 9$).

En base a las ecuaciones desarrolladas las temperaturas de pasteurización máximas aceptables para el buey de mar entero serían entre 96 y 100°C y entre 104 y 108°C (dependiendo del peso de los cangrejos) para obtener calidades buenas o aceptables, respetivamente. Como se detalla en el Capítulo 5, estas temperaturas de pasteurización se propusieron en base al tratamiento recomendado por la FDA de los EE.UU. para inactivar *C. botulinum* no proteolítico tipo E ($F_{90^oC}^{10^oC}$= 10 min). Sin embargo y como se ha demostrado en esta Tesis Doctoral, otras especies de bacterias formadoras de esporas, más termotolerantes que *C. botulinum* tipo E, como *B. weihenstephanensis* pueden estar presentes en el buey de mar listo para su consumo y sus productos derivados.

Por esta razón en esta Tesis Doctoral se evaluó el potencial de tecnologías alternativas al calor en concreto, los ultrasonidos y radiaciones ionizantes para la inactivación microbiana con el objetivo de la pasteurización del buey de mar listo para su consumo y sus productos.

En el caso de los ultrasonidos, además de las aplicaciones relacionadas con los procesos de transferencia de masa y de energía, esta tecnología es también considerada como una de las nuevas tecnologías útiles para la inactivación microbiana y ha sido sugerida como alternativa a los tratamientos térmicos convencionales para la pasteurización de alimentos (U.S. FDA. 2000). Como regla general, la resistencia bacteriana a los tratamientos de ultrasonidos disminuye con el tamaño celular siendo mayor en las de forma cocoide (Alliger, 1975; Condón *et al*., 2005). Sin embargo, la mayoría de los datos publicados indican que la eficacia bactericida de la tecnología de ultrasonidos es reducida (Lee, *et al*., 2013; Meullemiestre *et al*., 2017; Jambark, *et al*., 2017). Por ello, se ha tratado de mejorar la eficacia bactericida de esta tecnología diseñando procesos combinados (López-Malo *et al*., 2005; Lee, *et al*., 2013; Raso *et al*., 1998a). Algunos de los procesos de ultrasonidos combinados que mejoran la eficacia letal de la tecnología propuestos hasta la fecha son la termosonicacion (TS), manosonicacion (MS) y manotermosonicacion (MTS) (Chemat, *et al*., 2011; Piyasena, *et al*., 2003; Sala, *et al*., 1995).

En esta Tesis Doctoral, se evaluó el potencial de los tratamientos combinados de ultrasonidos con presión (manosonicacion) y la combinación de ultrasonidos con temperatura y presión (manotermosonicacion) para inactivar las principales bacterias esporuladas aisladas del buey de mar (*B. mycoides*, *B. weihenstephanensis* y *P. psychrodurans*) (Capítulo 6). De los resultados obtenidos, puede deducirse que los perfiles de las curvas de supervivencia, tanto para los tratamientos térmicos como para los tratamientos de ultrasonidos son dependientes de las especies bacterianas. En aquellas especies en las que se observaron hombros en las curvas de inactivación frente a los tratamientos

térmicos también se observaron hombros en los tratamientos MS/MTS, aunque la aplicación de ultrasonidos redujo su duración.

La eficacia letal de los tratamientos de MTS estuvo directamente relacionada con la termorresistencia de las especies esporuladas. *B. mycoides* mostró la mayor resistencia frente a los tratamientos de MS y la menor a los tratamientos térmicos y como resultado fue la especie esporulada más sensible a los tratamientos de MTS. El tratamiento combinado de ultrasonidos bajo presión con temperatura manifestó un efecto sinérgico para la inactivación de todas bacterias esporuladas estudiadas. El mayor porcentaje de sinergia se detectó en la especie bacteriana con el mayor valor de z_T (*B. mycoides*) mientras que la mayor temperatura a la que se detectó el efecto sinérgico se cuantificó en la especie esporulada más termorresistente (*B. weihenstephanensis*). Los resultados obtenidos demuestran por tanto el potencial de la tecnología de los ultrasonidos para pasteurizar productos líquidos a base de buey de mar (como sopas o caldos) permitiendo reducir los tiempos de tratamiento, entre un 13% y un 85%, dependiendo del microorganismo diana, o las temperaturas del procesado. Esto permitiría mantener los niveles de inactivación afectando en menor medida a las propiedades del producto final.

En el caso de las radiaciones ionizantes, se evaluó el potencial de las mismas aplicadas con electrones acelerados para la inactivación de estos esporos bacterianos en carne de buey de mar y en medios de diferentes pH y a_w. De los resultados, se dedujo que el efecto del pH sobre la resistencia frente a las radiaciones ionizantes depende de la especie esporulada más que de las condiciones de tratamiento. Por otro lado, se detectó un importante efecto protector de las bajas a_w frente a la radiación ionizante aunque la magnitud de su impacto también dependió de la especie esporulada. Además, el mayor efecto protector de la reducción de la actividad de agua se detectó en el rango de >0,99 a 0,90, siendo este efecto en este rango también independiente de la especie esporulada tratada.

En el caso de las carnes de buey de mar, se detectaron pequeñas diferencias en las resistencias entre ambos tipos de carnes. *B weihenstephanensis* mostró la mayor resistencia, necesitándose 7.3-7.7kGy para alcanzar 6 ciclos logarítmicos de inactivación de esta especie. Mientras que para *B. mycoides* y *P. psychrodurans* fue necesario aplicar tratamientos de 6.1-6.3 y 5.4-5.3kGy para alcanzar unos niveles de inactivación similares. Estos resultados demuestran el potencial de este tipo de radiación para pasteurizar buey de mar dado que es posible alcanzar unos niveles de inactivación adecuados de los esporos bacterianos, presentes de manera natural en el buey de mar, aplicando dosis de tratamiento menores al límite de 10kGy establecido por la Organización Mundial de la Salud para el procesado de alimentos. Además, de los resultados también se deduce que existe una concordancia en los datos de resistencia entre las carnes de cangrejo y los medios de laboratorio con condiciones de pH y a_w similares. Lo cual abre la posibilidad de extrapolar los datos obtenidos in-vitro a diferentes productos.

Finalmente, de los datos de resistencia frente a diferentes tecnologías puede deducirse que las mayores diferencias entre especies esporuladas se produce en los tratamientos térmicos mientras que las diferencias son muy reducidas en el caso de estas tecnologías alternativas (MTS y radiaciones ionizantes). Por lo tanto, el uso de estas tecnologías podría reducir los riesgos asociados a los productos pasteurizados debidos a un error en la definición del microorganismo diana.

En resumen, los resultados presentados en esta Tesis Doctoral muestran la posibilidad de mejorar el proceso productivo del buey de mar listo para el consumo y sus productos derivados, introduciendo diferentes tecnologías en diferentes etapas del proceso u optimizando las condiciones de las prácticas habituales para resolver los retos de la producción de estos productos. Destacar que en el Capítulo 8 de la Tesis Doctoral, se ha incluido un esquema de las líneas de procesado de los distintos productos a base de buey de mar indicando los distintos puntos de las mismas en las que se podría incluir las tecnologías

investigadas. Todo ello puede contribuir a potenciar este sector gracias a actividades de I+D+i mediante la implantación de nuevas tecnologías de procesado.

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Annex I

Joint-PhD agreement