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Departamento de Química

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Armazenamento hiperbárico de produtos cárneos à temperatura ambiente

Hyperbaric storage of meat products at room temperature

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Quimica da Universidade de Aveiro

Aos meus pais por todas as oportunidades e apoio ao longo de todos estes anos.

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Palavras-chave Fiambre, Tempo de Prateleira, Microrganismos, Deterioração, Alta pressão, Armazenamento Hiperbárico, Carne Picada

Resumo

O armazenamento hiperbárico (AH) é uma metodologia de conservação de alimentos na qual a pressão é usada como fator determinante no retardamento da deterioração. A esta nova metodologia de conservação poderão estar associadas poupanças energéticas significativas, nomeadamente quando o armazenamento ocorre à temperatura ambiente (TA). Desta forma, o objetivo deste estudo focou-se na avaliação do AH como alternativa à refrigeração na conservação de fiambre fatiado e de carne picada de porco utilizando diferentes combinações de pressão (0.1-150 MPa), temperatura (4-37 ºC) e tempo (4-24 h).

No geral observou-se um aumento da carga microbiológica em pelo menos 1 Log CFU/g para o fiambre bem como a carne picada armazenados à TA e 0.1 MPa enquanto que sob refrigeração a carga microbiológica manteve-se igual ou ligeiramente superior à inicial. Por outro lado, as amostras sujeitas a AH apresentaram cargas iguais ou menores do que as amostras iniciais, independentemente da temperatura de armazenamento empregue. Contudo verificou-se que pressões mínimas de 50 MPa são necessárias de forma a inibir o crescimento microbiológico similarmente à refrigeração. No caso do fiambre, não foram verificadas diferenças significativas nos parâmetros físico-químicos analisados (pH, capacidade de retenção de água, oxidação lipídica e cor) entre as diferentes condições de armazenamento e as amostras iniciais. Por outro lado, na carne picada o AH inibiu a oxidação lipídica quando comparado ao armazenamento a 0.1 MPa à mesma temperatura.

Assim, o AH demonstra-se eficaz na prevenção da deterioração de produtos cárneos, por inibição do crescimento microbiológico, com igual ou maior eficiência do que a refrigeração, dependendo da pressão de armazenamento usada. Como tal, estes resultados apontam o uso do AH como uma alternativa eficiente à refrigeração, na conservação de produtos cárneos.

Keywords Cooked ham, Shelf life, Microorganisms, Spoilage, High pressure, Hyperbaric storage, Minced meat

Abstract

Hyperbaric storage (HS) is a preservation methodology of food products in which pressure is used as a determining factor in spoilage inhibition. With this new preservation methodology significant energy saving might be achieved, namely when the storage occurs at room temperature (RT). As such, the objective of this study focused on the evaluation of HS as an alternative to refrigeration for sliced cooked ham and minced pork meat preservation by using different combinations of pressures (0.1-150 MPa), temperatures (4-37 ºC) and storage times (4-24 h).

In general, it was observed an increase of the microbial counts of at least 1 Log CFU/g for both sliced cooked ham and minced pork meat stored at RT and 0.1 MPa whereas under refrigeration the counts remained equal or slightly higher than before storage. On the other hand, the samples stored under HS conditions presented equal or lower counts than the initial samples, regardless of the storage temperature employed. Nevertheless, a storage pressure of at least 50 MPa is required in order to inhibit microbial growth similarly to refrigeration. In the case of sliced cooked ham, no significant differences were observed between the different storage conditions and the initial samples concerning physicochemical parameters analysed (pH, water holding capacity, lipid oxidation and colour) whereas for minced pork meat HS inhibited lipid oxidation when compared to the storage at 0.1 MPa at the same temperature.

Therefore, HS shows to be effective in preventing meat products ham spoilage, by microbial growth inhibition, as or more efficiently than refrigeration, depending on the storage pressure used. As such, these results points towards the use of HS as an efficient alternative to refrigeration in meat products preservation.

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List of Abbreviations

a_w	Water activity
CFU	Colony forming units
DFD	Dark, firm and dry meat
ENT	*Enterobacteriaceae*
HHP	High hydrostatic pressure
HP	High pressure
HPP	High pressure processing
HS	Hyperbaric storage
LAB	Lactic acid bacteria
MAP	Modified atmosphere packaging
MDA	Malondialdehyde
MRS	Man, rogosa and sharpe
MUFA	Mono unsaturated fatty acids
PA-PE	Polyamide-polyethylene
PCA	Plate count agar
PSE	Pale soft and exudative meat
PUFA	Poly unsaturated fatty acids
RBCA	Rose-bengal chloranphenicol agar
RT	Room temperature
SFA	Saturated fatty acids
TAM	Total aerobic mesophiles
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TMP	1,1,3,3-tetrametoxipropane
UHP	Ultra high pressure
VP	Vacuum-packaging
VRBDA	Violet red bile dextrose agar
WHC	Water holding capacity
YM	Yeast and moulds

Contextualization and thesis structure

This thesis is divided in four chapters. Chapter I comprises a literature review in what concerns 1) high pressure technology, 2) hyperbaric storage (HS), 3) Cooked ham production, 4) Meat products microbiological and chemical spoilage and 5) Current preservation methodologies for meat products. This chapter discusses the production process of sliced cooked ham in order to contextualize the different physicochemical and microbiological characteristics that this product has in comparison to minced meat, also studied in this work. In Chapter II information can be found concerning the storage conditions used in the experiments carried out in a laboratory scale high pressure equipment (sliced cooked ham) and experiments carried out in an industrial scale high pressure equipment (sliced cooked ham and minced meat) and the methodologies used for microbiological and physicochemical analyses. The experiments in the industrial scale high pressure equipment were developed in the last month of the schedule and so only a lower number of microbiological and physicochemical analyses were carried out. Chapters III and IV consist in the results obtained and the respective discussion, correlating with the available literature studies. These Chapters are divided into two parts: I) microbiological analysis and II) physicochemical analysis. A third section can be found in chapter IV concerning the post-HS stability of the products. Following this chapter, the final conclusions are present with respect to this work as well as suggestions for future work.

Chapter I - General Introduction

This chapter includes a part of the information published in the publication:

Fernandes, P. A. R. *, Moreira, S. A. *, Fidalgo, L. G., Santos, M. D., Queiros, R. P., Delgadillo, I. & Saraiva, J. A. 2014. Food Preservation Under Pressure (Hyperbaric Storage) as a Possible Improvement/Alternative to Refrigeration. *Food Engineering Reviews*, 1-10.

* these authors contributed equally to the work

1. High pressure

Currently, the thermal preservation methods are highly optimized regarding the foods microbial load reduction and the minimization of nutritional quality losses. However, these methods still not fully meet the consumers demands for safer food products that have longer shelf life than the fresh ones but with similar characteristics **(San Martín et al., 2002)**. Owing to recent consumer preferences, impetus has been given to de development of novel technologies that also promote a more sustainable food industry due to the lower energy and water consumption when compared to the conventional methods **(Knorr et al., 2011)**.

High pressure processing (HPP), ultra high pressure (UHP), high hydrostatic pressure (HHP) are common terms used to define one of the most promising technologies for gentle preservation of food that potentially addresses many, if not all, of the most recent challenges faced by the food industry **(Knorr et al., 2011, Mújica-Paz et al., 2011)**. High pressure (HP) technology offers several advantages over thermal processing technologies since treatment times can be shortened, scaling of laboratory and pilot plant findings to commercial production are both simple and safe, and changes in the equipment or product packaging do not require new pressure and time conditions and process redesign **(Mújica-Paz et al., 2011)**.

The first experiments regarding the HPP of food were developed by Hite in 1899 describing an increase in shelf life of products such as milk and fruits **(Hite, 1899)**. Since these experiments the acquisition of new knowledge about HP technology and the costs reduction of HP equipments brought the possibility to develop new food products and the commercial exploration of this technique in Japan, USA and Europe. As seen in **Figure 1**, it is possible to find fruit juices, seafood, cooked ham, dairy products, and other commodities processed by HP that are available worldwide. In addition, the number of installed units increased from 40 to 230 in only ten years (2003 to 2013), and it is estimated that this number will grow, most part due to its efficiency in microbial inactivation and preserving the nutritional and sensorial qualities, independently of the size and geometry of the product **(Mújica-Paz et al., 2011)**. Despite of these advantages, there are some limitations regarding the HP inactivation of microorganisms and the stability of the product. Some enzymes and spores are highly resistant being necessary pressures up to 1200 MPa to efficiently inactivate them. Besides storage at low temperature is required in

order to safeguard the product quality along its lifetime **(Cheftel, 1995)**. Still, recent studies shown that the consumers are now perceiving the naturalness and improved taste of HP products, although their knowledge about this new technology is reduced **(Nielsen et al., 2009)**.

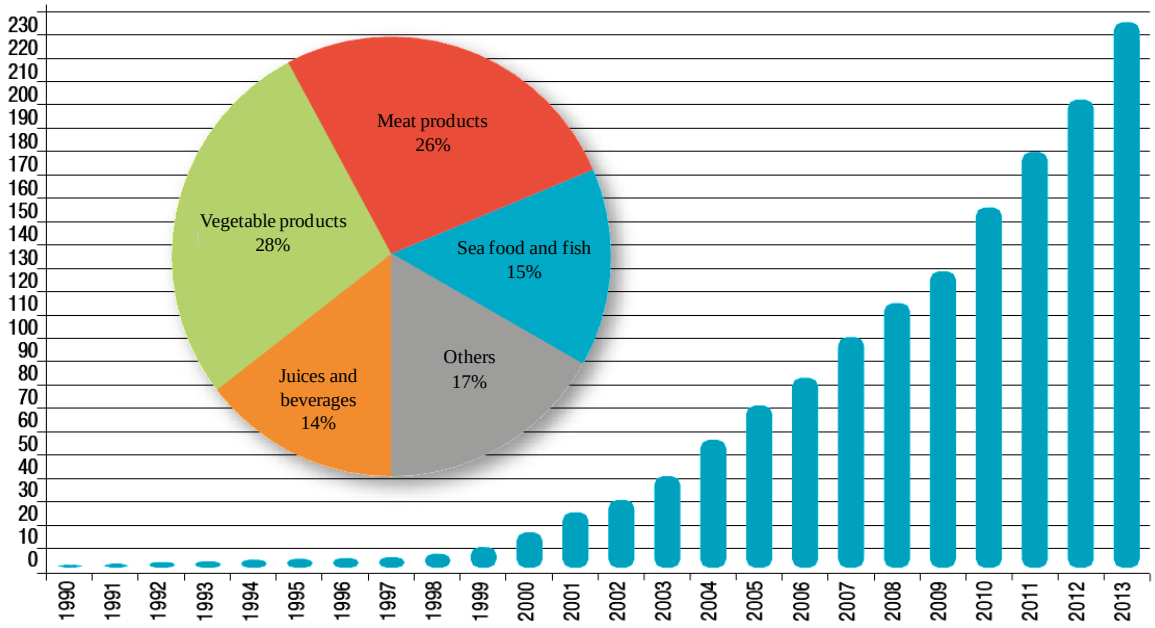

Figure 1 - Food products processed under pressure and number of installations over the years. Information provided by Hiperbaric Company.

1.1 Engineering concepts of HP

The typical HP units consists on a HP vessel where the (un)packaged food is deposed, a pressure chamber and a pressure generating device. The pressure is exerted by a volume reduction induced by pumping the medium (usually water or a mixture of fluids) to the pressure chamber. When the desired pressure is achieved, the pumping is stopped and no further energy is required to hold the pressure during the process. Nowadays, a HP equipment can subject food to pressures above 100 MPa up to 900 MPa being the values commonly used at commercial level around 400-700 MPa **(San Martín et al., 2002, Knorr et al., 2011)**. Inherent to the HP technology, two fundamental scientific principles must be taken into account. The first is the isostatic principle which defines that pressure is uniformly and instantaneously distributed over the product, regardless of its size and shape. The second principle, responsible for several consequences around food biochemistry and microbiology, is the Le Chatelier principle. This principle states that when pressure is

applied to a system at equilibrium, it tends to evolve towards the minimization of the disturbance. This means that several phenomenon such as phase transitions, molecular configuration modifications and chemical reactions accompanied by a decrease in volume are enhanced by pressure **(Norton and Sun, 2008, Rastogi et al., 2007)**. Another phenomenon that must be also taken into account during pressurization processes is the adiabatic heating and cooling which is defined as the variation of temperature as a result of compression and decompression of the treated food and pressure transmitting medium. The increase of temperature is around 3-9 ºC per 100 MPa depending on the food composition, processing temperature and pressurization rate and it can be used to thermal sterilization processes under milder temperatures **(Otero et al., 2007, Ramirez et al., 2009)**. Moreover, HP also displaces the equilibrium associated to the foods pH, usually towards more acidic values, contributing to the microbial inactivation. Once the pressure is released the pH value and the initial temperature are re-established **(Mathys et al., 2008, Knorr et al., 2011)**.

1.2 HP effect on microorganisms and spores

HP does not affect the covalent bounds which have low compressibility. Instead, HP affects the ionic bounds and hydrophobic interactions, responsible for the secondary and tertiary structure of proteins causing a multiplicity of morphological and biochemical complex damages on cells, which depend on the pressure applied and holding time **(Malone et al., 2006, Cheftel, 1995)**. These damages, accumulated in different cell structures, mainly in the membrane are summarized in **Table 1**.

Several studies indicate that in general Gram positive organisms are more resistant to pressure followed by yeasts and Gram-negative **(San Martín et al., 2002, Ramirez et al., 2009, Knorr et al., 2011)**. Nonetheless, the microorganisms' resistance within related taxonomic groups or even strains is highly variable and may depend on the chemical composition of the cell membrane and their ability to adapt. For instance, piezophilic and piezotolerants microorganisms have higher amounts of unsaturated fatty acids requiring higher pressures to be inactivated than surface mesophilic microorganisms **(Smelt, 1998)**.

In addition, microorganisms that are naturally susceptible to HP may acquire adaptive responses by the activation of certain genes. For example, the ability of some microorganisms to synthesize proteins that protect against adverse conditions (e.g. heat

shock proteins), increases the resistance to pressure as opposed to those who lacked it **(Malone et al., 2006, Iwahashi et al., 1997)**. The physiological status of microbial populations is also an important parameter regarding the resistance to HP, since microorganisms during the logarithmic phase are more susceptible than in stationary phase. Extrinsic factors such as temperature, pH, carboxylic acids, a_w, ionic solutes and others also affect the microorganisms' susceptibility to HP **(Rendueles et al., 2011, Knorr et al., 2011)**.

Table 1 – HP effect on the structure and biochemistry of living cells.

Proteins and enzymes	Unfolding of proteins. Several enzymes that participate in metabolic pathways are partially or completely inactivated inducing cell death	**(Knorr et al., 2011)**
Membranes	Primary target of HP. Membrane undergo a phase transition causing several perturbations, including the detachment of membrane proteins.	**(Schlüter, 2003, Winter, 1996)**
Ribossomes	Disintegration of ribossomes in their subunits causing cell death	**(Niven et al., 1999)**
pH	Intracellular pH modifications related to the inactivation of enzymes and membrane modifications responsible for the acidity control	**(Molina-Gutierrez et al., 2002)**

Contrarily to vegetative cells, spores are highly resistant to HP, requiring pressures up to 1200 MPa to be inactivated. In the standard HP pasteurization, vegetative cells are inactivated while spore remain able to grow without any competition. By so, several strategies have been adopted in order to inactivate these structures. One strategy is based on the germination of spores by using low pressure or mild heating processes. The germinated spores can then be destroyed later in a new cycle using HP. Another strategy is based on the combined use of HP and mild temperatures **(San Martín et al., 2002, Rendueles et al., 2011, Ramirez et al., 2009)**. Nonetheless, some of the cells/spores with sub lethal damages, under appropriate conditions can be resuscitated since the stress threshold was not surpassed. Therefore HP processed products must be refrigerated to maintain their sensory characteristics and microbiological stability/safety for longer periods of time **(Bozoglu et al., 2004, Cheftel, 1995)**.

2. Hyperbaric storage (HS)

As previously mentioned the consumers are now demanding the development of new preservation technologies that provide food products with similar characteristics to the natural but with higher shelf life **(Chen et al., 2012)**. Nonetheless, these new technologies are not capable by themselves to retard food spoilage. By so to reduce the losses during processing, storage, transportation, retailing and use by the consumer, refrigeration temperatures are required. Yet, about 30% of world production is lost and about 50% of the consumed energy by food industry is required for cooling jeopardizing its financial and environmental sustainability **(Coulomb, 2008, James and James, 2010)**. Additionally, many foods, as is the case of raw foods, are stored frozen. This process has even higher energetic costs and causes changes on solid foods texture. Therefore, there is a need to develop a new technology that ensures the products preservation with less energy expenses and with minimal impact on their quality. The storage under HP is one of the most promising alternatives.

The use of HP technology as a tool for storage arose, by chance, about 40 years ago with the recovery of well-preserved food in the 10 month sunk Alvin submarine at a depth of 1540 m (≈15 MPa). As posteriorly proved by **Jannasch et al. (1971)**, it was the combination of HP and low temperatures that inhibited the microbial growth, allowing to maintain the foods quality during the ten month period. Thus the possibility to storage food under hyperbaric conditions arose, called hyperbaric storage (HS), with the main objective to inhibit microbial growth, retarding food spoilage similarly to refrigeration. This effect on microbial behaviour led to the development of several studies: firstly by **Charm et al. (1977)** who suggested food (beef, chicken and cod) preservation at sub-zero and refrigeration temperatures under hyperbaric conditions; and more recently the storage of food products at variable (uncontrolled) room temperature (RT) and hyperbaric conditions of fruit products was suggested by two research groups **(Queirós et al., 2014, Segovia-Bravo et al., 2012, Fidalgo et al., 2014)**. These last conditions, i.e. the storage of food at and above RT, will possibly allow to achieve significant energy savings since no temperature control is necessary and energy is only required during the compression and decompression phases of the equipment. As a consequence, it is expected that HS will have significant impact on food research and has potential to be commercially applicable in the next years.

This review aims to better elucidate and comprehend this new storage method, by focusing on the current scientific knowledge about food HS at sub-zero, refrigeration and RT. **Table 2** compiles the published information regarding the storage of meat and fish products, framed on the objective of this thesis. It is worth to mention that emphasis will be given to the last condition (food storage under pressure at variable RT) since this is expected to be the major trend in the upcoming years.

Table 2 – Main results regarding the HS of meat and fish commodities

Product	Conditions	Results	Reference
Cod fish fillets, pollock, chicken and beef	22.8 MPa for 36 days at -3 °C	Stable for at least 36 days. Classified with equivalent quality as 7 days at 0.1 MPa at the same temperature	(Charm et al., 1977)
Beef	200 MPa at -20 °C	Microbial load reduction and inactivation of yeasts and some bacteria.	(Deuchi, 1990)
Chicken and carp	170 MPa for 50 days at -8 and -15 °C	Stable for 50 days. Enzymatic activity reduced	(Ooide, 1994)
Cod fish fillets	24.12 MPa for 21 days at 1 °C	Stable for 21 days. Classified with equivalent quality as 8.2 days at 0.1 MPa at the same temperature.	(Charm et al., 1977)
Pollock	24.12 MPa for 12 days at 1 °C	Stable for 12 days. Classified with equivalent quality as 6.7 days at 0.1 MPa at the same temperature.	(Charm et al., 1977)
Tilapia fillets	203 MPa for 12 h at 25 ºC	K value under 40%. Inhibition of deterioration only under pressure	(Ko and Hsu, 2001)

2.1 HS at sub-zero temperatures

The HS of food under sub-zero temperatures has as principle the shift of water physical properties when subjected to HP. By applying 209 MPa, water reaches a minimum freezing point at -22 ºC which allows to preserve food without going through the freezing and thawing processes **(Norton and Sun, 2008)**.

Deuchi (1992) was one of the firsts to investigate the effect of HS at sub-zero temperatures on the enzyme activity. These authors observed that the activity of catalase, α-amylase, cathepsin and lactate dehydrogenase from strawberries and tomatoes is reduced when subjected to pressures up to 200 MPa and -20 ºC, but not as efficiently as in frozen storage. Similar trends were observed on enzymes associated to nucleic acids degradation

present in chicken and carp muscle subjected to pressures of 180 MPa and -15 ºC **(Ooide, 1994)**.

Alongside with pressure effect on enzymes activity, the evaluation of sub-zero HS on microbial load was carried out. Concerning the hyperbaric effect at sub-zero temperatures on the microbiology of fish, chicken and beef at ≈24 MPa, it was observed that the microbial counts were maintained during the 36 days of storage (≈4 Log CFU/g) while those stored at 0.1 MPa and refrigeration temperatures had an increment (≈1-3 Log CFU/g). These results were mirrored on the quality evaluation of the products by an expert panel who classified cod fillets stored at 22.8 MPa and -3 ºC with higher and similar quality over the products stored at 0.1 MPa and -3 and -20 ºC, respectively **(Charm et al., 1977)**. In another study, the storage of beef at 200 MPa and -20 ºC leaded to the reduction of the microbial load when compared to the control at 0.1 MPa and the same temperature **(Deuchi, 1990)**. In addition, the quality of agar, strawberries, tomatoes, and raw pork subjected to HS was maintained, including the fresh colour and flavour of the commodities **(Deuchi, 1990, Ooide, 1994, Deuchi, 1992)**.

In conclusion, HS at sub-zero temperatures can be used to extend food products shelf life by inhibiting or even reducing food enzyme and microbial activity in similar or more efficient way than refrigeration and freezing. In addition, the sensorial quality of food can be improved since the freezing and thawing processes are not required. However, this process still requires temperature control and thus the inherent high costs for food preservation are maintained.

2.2 HS at refrigeration temperatures

As previously mentioned, the recovery of well preserved food (bouillon, sandwiches and apples) from the sunk submarine Alvin opened the HS potentialities, mainly at low temperatures. This submersible was exposed to about ≈15 MPa and temperatures around 3-4 ºC that leaded to the enzyme activity and microbial growth inhibition as posteriorly explained by **Jannasch et al. (1971)**. These authors showed that the exposure of mix and pure cultures at depths of 5300 m did not give rise to turbid cell suspensions when compared to the control at 3 ºC and 0.1 MPa due to a possible inhibitory effect of pressure. Transposing this effect to food matrixes, food samples stored under pressure were more stable than those stored at atmospheric pressure. In another study, **Charm et al. (1977)**

showed that the quality and shelf life of pollock and codfishes could be increased by pressure at low temperature storage. While the samples stored at 24 MPa and 1 ºC remained consumable after 21 days of storage, those stored at 0.1 MPa were unacceptable. In this study, the effect of pressure on two key enzymes related to food quality: trypsin and peroxidase was also evaluated combining different temperatures (-3, 0, 4 and 23 °C) and pressures (0, 27.6, 34.5 and 41.3 MPa). In general, the authors observed that increase of temperature and pressure caused a decrease of the enzyme activity at constant pressure and temperature, respectively and that each enzyme has a critical temperature point below which the pressure reduces the reaction rate and above it increases the reaction rate. The storage of rice at a depth of 30 m for one year showed significant effects on its biochemistry during storage. In this experiment the seed moisture, fatty acids, vitamin B_{12} and reducing sugars changes were less pronounced than those stored at 0.1 MPa **(Mitsuda et al., 1972)**.

In summary, HS at low temperatures is also efficient in enzyme and microbial activity inhibition. Nevertheless, under these conditions temperature control is still required and thus the high energetic costs still remain associated to food preservation.

2.3 HS at RT

The study of HS at RT is focused on the increase of food shelf life by inhibiting microbial growth, similarly to refrigeration. The main advantage associated to this new preservation methodology is the possible reduced energy consumption since it is only required energy during the compression and decompression phases of the equipment and no temperature control is required **(Segovia-Bravo et al., 2012)**.

The first study regarding the HS at RT was developed by **Robitaille and Badenhop (1981)** by compression air (O_2, N_2 and CO) to 3.6 MPa into a HP unit in order to increase the mushrooms shelf life. These authors observed that the moisture loss and browning degree were reduced when compared to the storage at 0.1 MPa. It was also verified that the control sample allowed larval forms growth during the storage period, while in the mushrooms stored under pressure the larval development was verified only 1 week after depressurisation and subsequent storage at 0.1 MPa. A similar inhibitory effect on the growth of rotting agents was observed in tilapia fillets stored 101 MPa and 25 ºC for 12 hours. After storage it was observed that the total plate counts remained similar to the

initial value and when the fillets were subjected to 203 MPa, a microbial load reduction of about to 2.0 log CFU/g was achieved **(Ko and Hsu, 2001)**. The same authors also evaluated the K value (a freshness quality index that indicates putrefaction when its value is above 60%). Tilapia fillets stored at 203 MPa showed a higher freshness than controls (K value below 40% and up to 92%, respectively).

The HS of strawberry, melon (**Figure 2**) and watermelon (**Figure 3**) juice at and above RT was also proposed by two research groups **(Segovia-Bravo et al., 2012, Queirós et al., 2014, Fidalgo et al., 2014)**. **Segovia-Bravo et al. (2012)** studied the storage of raw strawberries juice under HS conditions (25-250 MPa) for 15 days at 20 ºC, raw juice at 0.1 MPa and 4 and 20 ºC as well as pasteurized juice at 4 ºC. These authors observed that the control kept at 20 °C and 0.1 MPa for 15 days had a microbiological load increased around 3 log units for total aerobic mesophiles (TAM) and yeast and moulds (YM) whereas the juice stored at 5 ºC showed a 2 Log units increase in TAM counts. On the other hand, the pasteurised juice showed a microbiological load below the detection limits after the same storage period. The most relevant results lie in the raw juice stored under pressure (25-250 MPa) presenting microbial counts below the detection limit during the all storage period. These results thus show that strawberry juice preservation in possible at RT without going through the pasteurization and refrigeration storage processes.

Similar results were observed in melon juice stored under HS conditions that showed a higher stability than that stored at 0.1 MPa and the same temperature **(Queirós et al., 2014)**. As seen in **Figure 2** pressures of at least 50 MPa are needed in order to achieve a microbial growth inhibition for TAM, *Enterobacteriaceae* (ENT) and YM, similarly to refrigeration. This effect was also observed at 75 MPa. On the other hand, the increase of the storage pressure to 100 and 150 MPa showed a reduction of the initial microbiological load in about 0.5-2.0 Log CFU/mL of juice for TAM, ENT and YM, better results than those observed for refrigeration. In another study developed by **Fidalgo et al (2014)**, watermelon juice was stored at 100 MPa and variable RT (18-21 ºC) and 30 ºC for 60 and 8 hours, respectively. As seen in **Figure 3**, in general the watermelon juice stored at an above RT and 0.1 MPa presented an increase of the microbial load in the first 24 and 8 hours, respectively (from 4.28, 3.00 and 2.50 to above 6.00 Log CFU/mL of juice for TAM, ENT and YM, respectively).

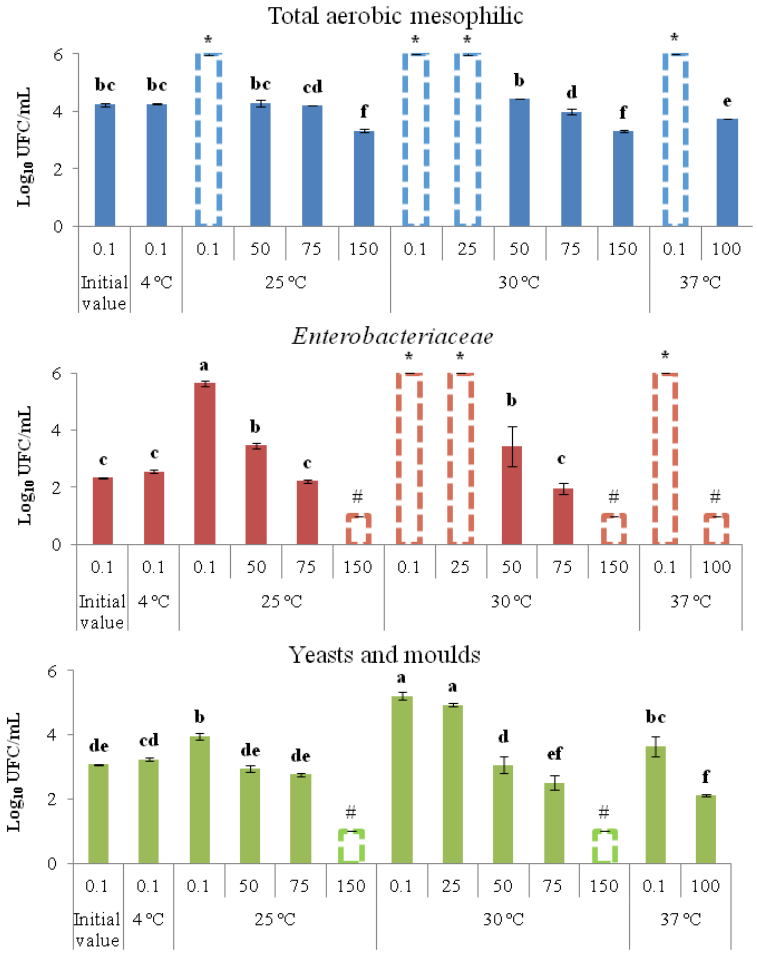

Figure 2 - TAM, ENT and YM counts in melon juice, initially and after 8 hours of storage, at different pressure and temperature conditions. Bars with * and # are indicative of higher than 6 and lower than 1 Log CFU/mL, respectively. Different letters between (a-g) indicate significant differences (p<0.05). Adapted from **(Queirós et al., 2014)**

On the other hand, the juice stored at 0.1 MPa and 5 ºC showed no changes in microbial counts when compared to the initial samples, except for YM that increased from 2.5 to around 5.0 Log CFU/mL of juice. The most noteworthy result was observed in the juice stored under pressure, at RT or above (30 °C), showing a microbial load decrease in the first 8 hours of storage (down to ~3.0 and <1.0 Log CFU/mL of juice for TAM and both ENT and YM, respectively) which remained stable along the remaining storage period.

The post HS stability of some of these food products was also assessed. After HS, the strawberry juice was placed at 0.1 MPa and 4 ºC for more 15 days and it was observed that the microbial remained unaltered while the juice only stored at 0.1 MPa and the same temperature had an increase in the microbial counts **(Segovia-Bravo et al., 2012)**.

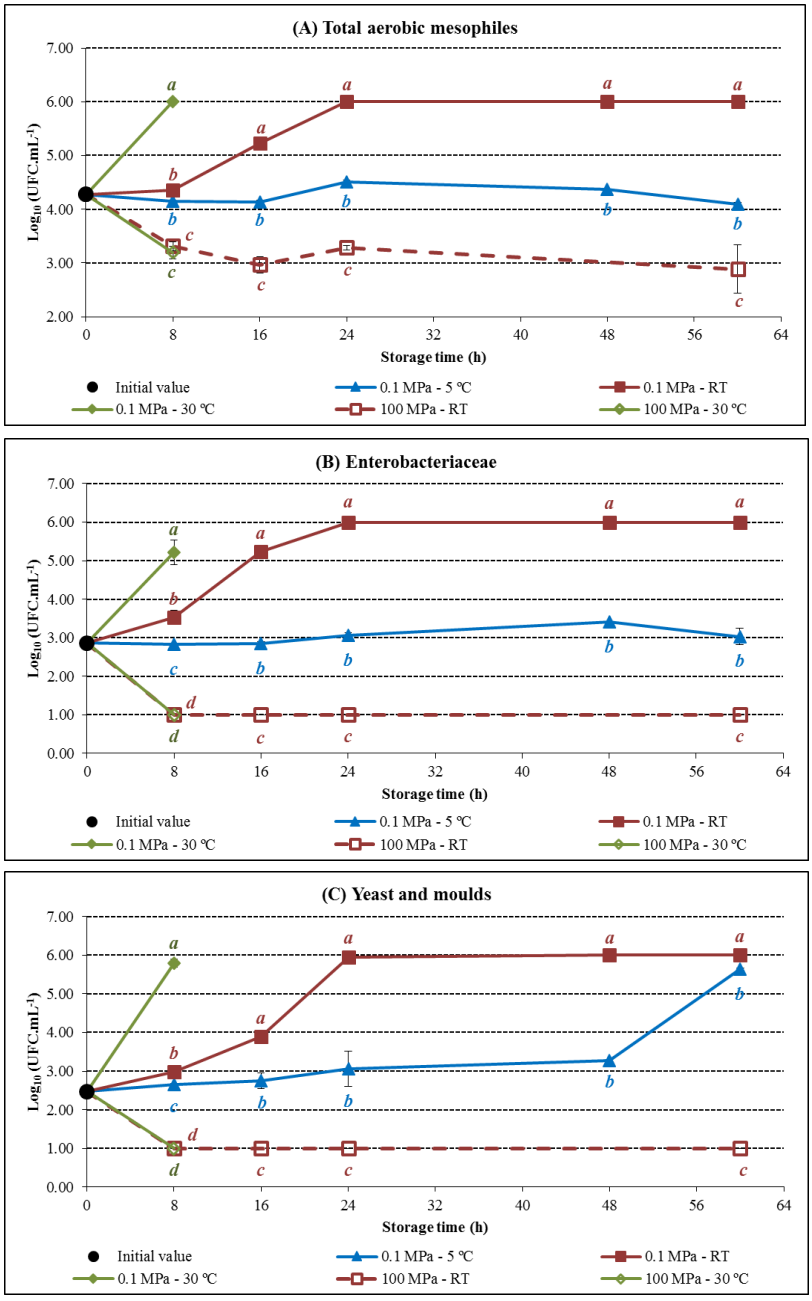

Figure 3 - TAM (A), ENT (B) and YM counts (C) (expressed in Log CFU/ml) of juice stored during 8, 16, 24, 48, and 60 hours at 0.1MPa and refrigerated temperature (5 ºC), 0.1 MPa and RT (18-21 ºC), 0.1 MPa and above 30ºC, 100 MPa and RT, and at 100 MPa and 30 ºC. Adapted from **Fidalgo et al (2014)**.

A similar behaviour was observed for watermelon juice, stored at 0.1 MPa and 4 ºC, after HS, for more 7 days. It was observed after this period an increase on YM counts from <1.0 to 3.57 Log CFU/mL of juice while the TAM and ENT loads remained stable. Contrarily, the juice stored only under refrigeration presented higher microbial loads that increased along storage time (**Fidalgo et al., 2014**). Nonetheless it is important to mention the HS effect on the colour parameter of fruit juices is diversified. While **Segovia-Bravo et**

al. (2012) observed delayed colour decay in the samples stored under HS conditions when compared to those stored at 0.1 MPa, **Fidalgo et al. (2014)**observed a more accentuated decay in the juices stored under pressure. These authors stated that this difference is possibly related to the different pH of the matrixes.

In summary, these results show that HS at RT is efficient in retarding food spoilage, at least taking into account microbial loads. Thus, HS at RT imposes itself as an alternative to refrigeration as a food preservation methodology.

2.4 Concluding remarks

As it was possible to observe throughout this literature review, HS, a new preservation methodology, shows very promising results, allowing to take several observations: 1) this new preservation methodology allows to inhibit microbial growth, even at and above RT, retarding food spoilage similarly to refrigeration; 2) in some conditions an inactivation effect is observed apart from the inhibitory action on microbial growth; 3) some products show a higher stability when placed at 4 ºC and 0.1 MPa, after HS, than those only stored at 4 ºC and 0.1 MPa; 4) very considerable energy savings can possibly be achieved since energy is only required during the compression/decompression phase of the equipment when stored at variable RT. Nevertheless further studies are required like the application of this preservation methodology to other food matrixes such as meat products, the purpose of this thesis.

3. Cooked ham and minced pork composition

Meat and meat products such as cooked ham are important elements in human diet for the most part associated to its high nutritional value, mainly in proteins, lipids, vitamins and minerals. As seen in **Table 3**, water constitutes a large part of meat and cooked ham composition, followed by protein, carbohydrate (by difference) and fat which content differs between/within different species due to genetics, sex, feed and processing procedure **(Toldrá, 2007)**. These nutrient categories will be further discussed except for carbohydrates since meat and meat products are not relevant sources of this kind of nutrients.

3.1 Proteins

Meat and meat products are known as a valuable source of several essential amino acids and nitrogen needed to synthesize non-essential amino acids and other nitrogen-containing compounds that take part in human biological functions **(Jiménez-Comenero, 2006, Insel et al., 2013)**. As seen in **Table 3**, minced pork and cooked ham protein content is around 18-21 %. These nutrients also take part on the development of flavour, as in the case of cooked ham, due to the release of free amino acids throughout the raw material post-mortem resolution that participate in Maillard and Strecker degradation reactions during the cooking process **(Flores et al., 1998, Robert, 2012)**.

3.2 Lipids

Lipids are hydrophobic and/or amphipathic molecules that play an important role in physiology of living species acting as structural elements of cell membranes, energy storing and signalling molecules **(Insel et al., 2013)**. As seen in **Table 3**, the minced pork fat content can reach 4-9 % whereas cooked ham presents values around 2 % being one of the delicatessen products with the lower fat per gram of product. In these products prevail monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). Moreover, cooked ham is also composed by PUFA (0.31%) used by the human body to synthesize eicosanoids **(Insel et al., 2013, USDA, 2013)**. The cholesterol content in cooked ham is also the lower when compared to other delicatessen products (e.g. salami) **(Hui, 2012)**. To this sterol, harmful effects are associated such as the development of cardiovascular diseases when excessive amounts are accumulated in the blood **(Insel et al., 2013)**.

Table 3 – Main nutrients present in 100 g of cooked ham and minced pork meat. The information use for building this table was adapted from **(USDA, 2013, Insel et al., 2013)**.

Nutrients	Cooked ham	Minced pork*	Benefit
Water (g)	69.9	73.62	Participation in metabolism, pH balance, temperature regulation.
Protein (g)	17.9	21.10	Source of essential amino acids and nitrogen needed for non essential amino acids synthesis and other nitrogen containing compounds
Carbohydrate, by difference (g)	7.27	0	-
Total lipid (fat)	2.37	4	Energy source
Lipid (mg)			
SFA^{+}	1.17	1.31	Structural elements of cell membrane. Energy source. Signalling molecules.
MUFA^{++}	1.67	ND	
PUFA^{+++}	0.31	ND	
Cholesterol	0.022	59	Cell membrane structure, Bile salts synthesis, Steroidal hormones.
Vitamins (mg)			
Thiamine	0.402	0.414	Action as cofactors in energy yielding reaction and nucleic acid synthesis.
Riboflavin	0.101	0.368	Action as cofactors in oxidation-reduction reactions in metabolic reactions
Niacin	2.27	7.914	Catabolism of lipid, carbohydrate and protein. Anabolism of fatty
Vitamin B6	0.231	0.668	Amino acid metabolism, gluconeogenesis and lipid metabolism
Folates	0.002	ND	Action as cofactors in nucleic acids synthesis and amino acid catabolism
Minerals (mg)			
Na	900	67	Blood volume, pressure regulation and osmotic equilibrium.
K	165	310	Muscle contraction, electrolyte balance and cell transfer systems.
Ca	6	15	Bone structure, muscle contraction, blood clotting
P	384	190	Teeth, bone and cell structure and energy balance.
Mg	8	19	Nucleic acid synthesis, energy balance and cell membrane stability
Zn	0.91	1,93	RNA and DNA metabolism, gene expression, apoptosis.

*composition taking into account 96 % lean and 4 % fat (the content in fat can reach 9 % in minced pork); ND – Not determined; $^{+}$Saturated fatty acids; $^{++}$Monounsaturated fatty acids; $^{+++}$Poly unsaturated fatty acids

3.3 Vitamins

Vitamins are low-molecular weight organic components to which is associated a high relevance in biological processes as cofactors components. These nutrients are divided in two main groups: 1) fat soluble vitamins (A, D, E and K) and 2) water soluble vitamins (B and C) **(Insel et al., 2013)**. Regarding the cooked ham and minced pork composition in vitamins, it is observed in **Table 3** higher contents of niacin and thiamine when compared to other vitamins.

Niacin is a coenzyme component that yields NAD^+ and $NADP^+$ which participate in oxidation-reduction reactions essential to the production of ATP and to the synthesis of several compounds, respectively **(Insel et al., 2013)**. Oppositely, thiamine is a structural component of coenzyme thiamine pyrophosphate (TPP) that participate in energy yielding reactions such as glucose metabolism and citric acid cycle and in the synthesis of nucleic acids **(Insel et al., 2013, Lonsdale, 2006)**.

3.4 Minerals

Minerals are important elements in human nutrition due to their role as enzyme activators and structural components and are usually divided in two categories: 1) macrominerals which daily intake must be at least 100 mg and 2) trace minerals which daily requirements are in very low amounts **(Gropper and Smith, 2012)**. As it can be seen in **Table 3**, cooked ham and minced pork is especially rich in sodium, potassium and phosphorus, being also present small amounts of iron, zinc, magnesium and calcium.

Potassium is essential in human nutrition due to its importance in muscle contraction and cell transfer systems while phosphorous participates in cell structure and in energy balance, among others **(Gropper and Smith, 2012, Anderson, 1991)**. The high content in sodium of cooked ham arises from the brine that is injected in raw meat and it is mainly directed to the preservation and development of flavour by the product **(DeSimone et al., 2013, Campbell and Reece, 2003)**.

4. Cooked Ham Processing Technology

Cooked ham is a product that can be made from pork or poultry meat and which quality depends on several characteristics that are related to the raw material, the brine ingredients composition (polyphosphates, starches, carrageenans and others), technological yield and ham presentation (boneless, bone-in, whole leg, pieces among others) **(Frentz, 1982, Motzer et al., 1998)**. The microbiological load present in the cooked ham is also important in the final quality and stability of the product.

The main stages in cooked ham processing, summarized in **Figure 4** are: 1) raw material reception; 2) brine injection and tumbling/massaging; 3) cooking and cooling and are briefly described in order to comprehend the origins of the spoilage bacteria, how the process affects its shelf life and physicochemical characteristics and how it differentiates from the raw meat.

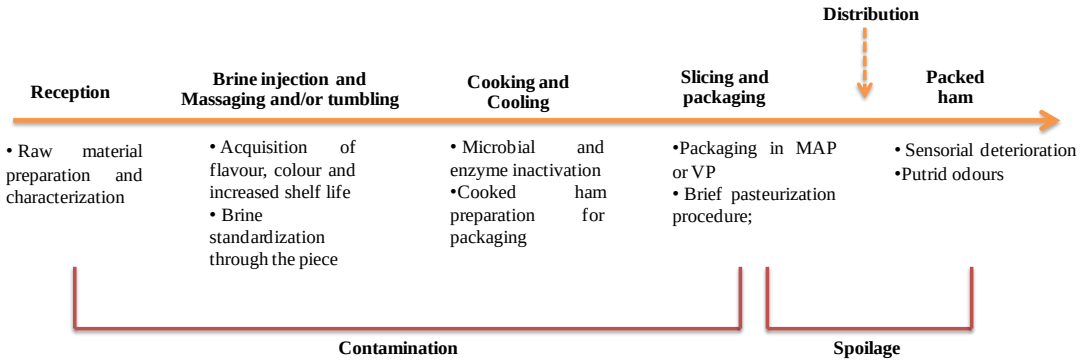

Figure 4 - Cooking ham process schematic representation and its effects. Adapted from **(Vasilopoulos et al., 2013)**. VP – Vacuum Packaging; MAP – Modified Atmosphere Packaging

4.1 Raw material reception

The first step in cooked ham production is the raw material reception where its weight and pH are measured in order to identify the type of meat, pale, soft and exudative (PSE) or dark, firm and dry (DFD), among other properties. As opposed to dry cured hams, both PSE and DFD meats are used in cooked ham production, despite the limitations inherent to each one **(Toldrá et al., 2010)**. At this stage, the microflora is mainly constituted by *Pseudomonas* (68.1%) and *Brochotrhrix thermosphacta* (26.4%), while the acid lactic bacteria (LAB), yeasts and moulds (YM) and others only represent less than 2% of the total microbiological load, as shown in **Table 4 (Samelis et al., 1998)**.

Table 4 – Microbiological diversity during cooked ham processing. Adapted from **(Samelis et al., 1998)**

	Reception	Brine injection and Tumbling	Before cooking	After cooking
TAM[1]	4.86	5.46	5.72	<3.00
LAB[2]	3.04	4.28	5.32	<3.00
Pseudomonas	4.69	4.20	3.86	<3.00
ENT[3]	2.41	3.54	3.23	<2.00
B.thermosphacta	4.28	4.96	4.89	<3.00
YM[4]	<3.00	3.26	4.51	<3.00

[1]Total aerobic mesophiles; [2]Lactic acid bacteria; [3]*Enterobacteriaceae*; [4]yeast and moulds

4.2 Brine injection and massaging/tumbling

In this process, the brine is injected in the meat and tumbled in order to standardize its distribution along the entire piece. Salt the main ingredient, is added to around 2% to ham in order to decrease its a_w preventing microbial spoilage, partially solubilize myofibrillar proteins, to increase the water retention and to attribute the salty flavour **(Toldrá et al., 2010, Toldrá, 2006, Frentz, 1982)**. Nitrites are commonly added with the purpose to contribute to the acquisition of the pink colour, associated to nitrosomyochromogen, and to extend the shelf life of the product due to its antioxidant and antimicrobial activity. Sodium ascorbate is also added with nitrite to avoid the formation of nitrosamines that have potential carcinogenic effects **(Toldrá et al., 2010, Honikel, 2010)**. Depending on the final quality of the product, the addition of polyphosphates, phosphates and pyrophosphates to improve the water retention and the addition of non-meat ingredients such as milk powder, carrageenans and others thickeners may also occur in lower quality cooked hams **(Toldrá et al., 2010, Delahunty et al., 1997)**.

During these processes changes at microbiological level are observed. Whereas during brine injection an enrichment in ENT is observed as a consequence of brine recirculation, after tumbling the LAB become the prominent bacteria corresponding to 40.4% of the total microflora while *Pseudomonas* and *B. thermosphacta* are reduced to lower levels, 1.4% and 14.8%, respectively **(Samelis et al., 1998)**.

4.3 Cooking and cooling

The aim of the cooking process is to inactivate enzymes and to destruct microorganisms, mainly those that are pathogenic, increasing thereby the shelf life and safety of the product. This is achieved by using water baths or steam where the internal

temperature of ham must reach 68-70 ºC for 30-60 min affecting the texture, colour and flavour as a consequence of several chemical reactions, as represented on **Table 5 (Toldrá et al., 2010)**.

Table 5 - Biochemical reactions affecting sensory properties of cooked hams. Adapted from **(Toldrá, 2006)**

Group of Reactions	Cooked Ham
Protein degradation	Intense by heat denaturation
Generation of small peptides and free amino acids by proteolysis	Poor
Lipid degradation	Medium by heat damage
Generation of free fatty acids by lipolysis	Poor
Oxidation of free fatty acids	Medium
Generation of volatile compounds	Medium
Strecker degradation of amino acids	Scarce
Maillard reactions	Intense
Cured colour generation	Nitrosomyochromogen

The heat induces meat tenderization by an increase in the proteolytic breakdown of myofibrillar proteins when exposed to temperatures up to 65 ºC and by destruction and solubilization of collagen when exposed to temperatures above 70 ºC. Heat also induces the acquisition of a stable pink colour due to the nitrosomyochromogen formation, which resulted from the denaturation of the nitrosylmyoglobin protein moiety **(Ann Boles, 2010)**. Concerning flavour development, the amino acids and free fatty acids that resulted from enzymatic proteolysis and lipolysis prior to cooking participate in several chemical reactions such as the Maillard, Strecker degradation and fatty acid oxidation reactions leading to the development of several volatile compounds that are responsible for the cooked ham aroma **(Toldrá et al., 2010, Toldrá, 2006)**.

After cooking, cooked ham is cooled down to temperatures below 4-5 ºC that are achieved either by using cold water baths, air blast or vacuum cooling. **(Toldrá et al., 2010, Toldrá, 2006)**. Finally the product is ready to be packaged or to be smoked according to the desired final properties. The cooked ham can be sold as an entire piece or in slices addressed to the retailing and final consumer, respectively. At microbiological level, after the cooking and cooling processes all microorganisms counts are below 3 log CFU/g **(Samelis et al., 1998)**.

5. Meat and meat products deterioration

Meat and meat products shelf life is limited mainly due pre/post-slaughter and to post-processing manipulation, like slicing and packaging, that reintroduces spoilage and sometimes pathogenic microorganisms. Furthermore, the high pH (around 6) and a_w (above 0.85) as well as the availability of essential nutrients promote spoilage, inducing the development of sensorial defects (off-flavours, discolouration, gas and slime formation) and consequently economical losses. Nonetheless, meat and meat products shelf life also depends on extrinsic parameters such as the packaging method and storage temperature (**Table 6**). For instance, cooked ham lifetime under refrigeration conditions can go from a few days when stored to air up to some weeks when stored under MAP **(Borch et al., 1996, Hu et al., 2009, Samelis et al., 1998, Dave and Ghaly, 2011)**. The two types of spoilage responsible for meat and meat products deterioration: 1) microbial spoilage and 2) physicochemical deterioration are further discussed in the next subsections.

Table 6 – Factors that affect the shelf life and native microflora in meat and meat products. Adapted from **Dave and Ghaly (2011)**.

Type	Factors
Intrinsic	Type of animal (bovine or porcine)
	Initial microflora
	Chemical properties (e.g. pH)
	Availability of oxygen
	Processing conditions and control
	Hygiene
Extrinsic	Temperature control
	Packaging technology
	Storage types

5.1 Microbial deterioration

Meat and meat products are an excellent growth media for the proliferation of microorganisms. In raw meat is frequently to found bacteria such as *Pseudomonas*, *Micrococcus*, *Staphylococcus*, LAB, ENT, among others and YM such as *Candida*, *Cryptococcus*, *Cladosporium* and *Penicillium* that are responsible for spoilage **(Dave and Ghaly, 2011)**. Contrarily, in cooked ham the microorganisms responsible for spoilage emerge predominantly during the slicing and packaging, as previously explained in

Section 4. Since LAB (*Lactobacillus sakei*, *L. Curvatus*, *Leuconostoc mesenteroides* and uncultured *Leuconostoc sp.*) are favoured by oxygen-restrained conditions and are not extensively inhibited by CO_2, these microorganisms become the major spoilage agents in cooked ham either in VP, MAP and air conditions as a result of these and other selective circumstances of thee commodity (nitrites and reduced a_w, etc) **(Hu et al., 2009, Samelis et al., 1998, Borch et al., 1996, Han et al., 2011)**.

The metabolic activity of the native bacteria present either in raw meat and sliced cooked ham is responsible for the unpleasant odours and flavours acquisition (acidity and putrefactive odours) due to the organic acids formation such as lactic acid, acetic, formic and compounds with a more volatile nature (ethanol, 3-methyl butanol and others). The swelling of the package may also occur as consequence of CO_2 formation. LAB and other microorganisms are also capable of forming H_2O_2 and H_2S that may cause the oxidation of myoglobin to choleomyoglobin and sulphomyoglobin, respectively, causing the formation of green spots **(Borch et al., 1996, Vasilopoulos et al., 2008, Leroy et al., 2009)**. The ability of some microorganisms to produce biogenic amines is also a matter of great concern in food safety. Some LAB, ENT and *Pseudomonas* are able to manifest decarboxylase activity, an essential enzyme in the conversion of free amino acids to biogenic amines. Nonetheless, the amounts produced depends on several factors such as the meat microenvironment, the microbial load, species and strains found in the sample and the processing of the raw material as represented on **Figure 5 (Ruiz-Capillas and Jiménez-Colmenero, 2005, Bardócz, 1995)**.

5.2 Chemical deterioration

The chemical deterioration of meat and meat products is responsible for the alteration in colour, texture and flavour, three important quality characteristics that define the consumer acceptance. Lipid and pigments (myoglobin) oxidation and autolytic enzyme spoilage are the main responsible for these changes **(Addis, 1986)**.

Lipid oxidation is defined as a chain reaction between unsaturated fatty acids and free radicals that generates a great variety of volatile and non-volatile compounds where aldehydes, ketones, alcohols and acids are the most frequent. Hydroperoxides are produced due to lipid oxidation of highly unsaturated fatty acids and usually decompose in secondary products such as malondialdehyde (MDA) that are often used as indicators of

these reactions **(Min and Ahn, 2005, Esterbauer et al., 1991)**. Several factors such as raw meat pH and composition, aging time, cooking/heating, additives, oxygen availability and prolonged storage influence the lipid oxidation rate. Heme proteins and "free iron" can also act as catalysts **(Morrissey et al., 1998, Min and Ahn, 2005)**.

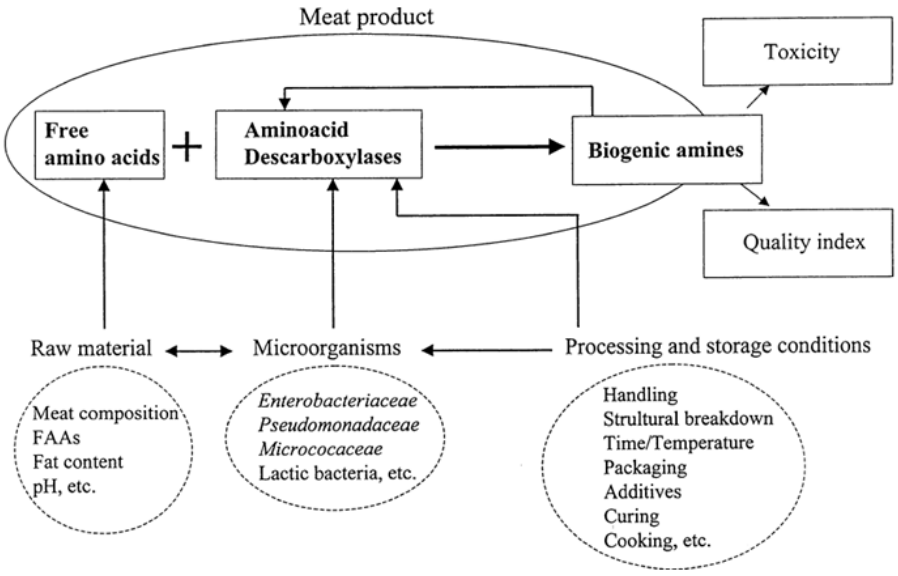

Figure 5 – Factors that affect biogenic amine formation. Adapted from **(Ruiz-Capillas and Jiménez-Colmenero, 2005)**.

In the particular case of meat and meat products, the triglyceride and phospholipids fractions are susceptible to lipid oxidation, especially the latter group of compounds that is richer in unsaturated fatty acids than the former. Nonetheless these reactions are not completely undesirable since they contribute to the cooked ham aroma. It is however the increasing concentration of some lipid oxidation products during storage that leads to the development of undesirable flavours and consequently the product rejection by the consumer **(Vasilopoulos et al., 2013, Dave and Ghaly, 2011)**.

Myoglobin, a muscular sarcoplasmatic heme protein, is the primary responsible for the colour of meat despite the contribution of hemoglobin and cytochrome in a lesser extent **(Livingston and Brown, 1981)**. In meat and meat products, myoglobin may exist in different redox forms: deoxymyoglobin, oxymyoglobin, carboxymyoglobin, metmyoglobin and nytrosilmyoglobin. These different redox forms can be interconverted to each other depending on endogenous (pH and lipid oxidation) and exogenous factors (presence of ligands, antioxidants and prooxidants), thus defining the meat colour **(Min and Ahn,**

2005). In the particular case of cooked cured meat products, the nytrosomyochromogen responsible for the pink colour is sensitive to the presence of oxygen, light and microbial activity causing it to fade (its oxidation imposes a dull greyness) and thus decreasing the consumer acceptance of the commodity. This oxidation proceeds in parallel with rancidity indicating a possible contribution of lipid oxidation on meat decolouration **(Li et al., 2012, Munk et al., 2010)**.

Enzymatic reactions, naturally present in muscle cells are also a leading cause of spoilage. These catalysts induce de hydrolysis of carbohydrates, proteins and lipids to their monomeric units favouring the softening and greenish discolouration associated to microbial growth **(Dave and Ghaly, 2011)**. For instance, cathepsins, calpains and aminopeptidases are enzymes responsible for the proteolytic breakdown of muscular myofribrils causing the tenderization of meat and also the release of amino acids used by the microorganisms as a carbon source and also leading to the formation of biogenic amines **(Kuwahara and Osako, 2003, Dave and Ghaly, 2011)**. As a consequence of these processes, several strategies concerning the meat and meat products preservation are being studied and are further discussed in the next section.

6. Minced meat and cooked ham preservation technologies

Cooked ham has a perishable nature that is more pronounced when sold in slices that as an entire piece and it is usually preserved under VP or MAP conditions. Having as a reference the threshold value 6-8 Log CFU/g, for the TAM counts defined by several authors as the upper limit value for the product acceptability, the entire piece has a 25 days lifetime under refrigeration and VP conditions although the spoilage signals are only detected after 90 days. Furthermore, the sliced cooked ham stored under the same conditions has a lifetime between 6-30 days but consumable, i.e. without signals of deterioration till 25-35 days **(Samelis et al., 1998, Vasilopoulos et al., 2008, Hu et al., 2009).** Similar shelf life is observed for MAP sliced cooked ham due to the ability of LAB to grow, reaching 7.5-8.6 Log CFU/g after 30 days at 7-15 ºC **(Vercammen et al., 2011, Vasilopoulos et al., 2013)**. These behaviours therefore suggest that is not only the final microbial concentration that determines spoilage, but also their metabolic activity and growth phase **(Stolzenbach et al., 2009, Mataragas et al., 2006)**.

On the other hand, minced meat has an even shorter shelf life and its microbial quality mostly depends on the storage temperature and packaging atmosphere. This meat product is often preserved under MAP or VP, similarly to cooked ham, reaching a life time of 2-7 days under refrigeration conditions, taking to account the reference limits of ≈7 Log CFU/g for TAM defined by some authors and established by the Portuguese legislation **(Koutsoumanis et al., 2008, Michalczyk et al., 2012, Esmer et al., 2011, Agricultura, 1996)**.

In order to become a more competitive industry, the application of common organic acids, essential oils, bioprotective cultures and mild preservation procedures have been studied in to increase cooked ham and minced meat shelf life **(Chaillou et al., 2014, Vermeiren et al., 2004, Jayasena and Jo, 2013, Vasilopoulos et al., 2013)**. Nevertheless, the use of additives is perceived by the consumers as undesirable and the use of bioprotective cultures shows several disadvantages at industrial level **(Vasilopoulos et al., 2013)**. Therefore, several studies have been developed focused on multi-target preservation technologies since they allow the in-package treatment, reducing the post-cooking contamination and increasing meat products shelf life.

One of the most promising technologies in cooked ham and minced meat preservation is HP due to its ability to inactivate microorganisms and enzymes without affecting the

sensory properties of the product. In general, data in the literature suggests that depending on the conditions used (pressure, processing time and temperature), cooked ham shelf life can be extended to 56-126 days by applying treatments from 200-600 MPa. However, LAB still remain the responsible for spoilage due to their ability to resume growth after the pressure treatment **(Pietrzak et al., 2007, Vercammen et al., 2011, Han et al., 2011)**. As a consequence several other studies regarding a hurdle approach combining HP treatment with natural antimicrobials is being developed in order to extend cooked ham shelf life **(Vercammen et al., 2011, Jofré et al., 2008)**.

In the case of minced meat, high pressure also proved to be efficient in extending its shelf life. **Jung et al. (2013)** subjected VP ground beef to a high pressure treatment of 300-600 MPa and observed that for the higher pressures the microbial loads could be maintained below the detection limit for at least 10 days, under refrigeration temperatures.

E-beam irradiation is another alternative, despite the lower acceptance by the consumer about irradiated food. Depending on the dose used, the shelf life of cooked ham and minced meat can be increased up to 41 and 24 days, respectively, when stored under VP and refrigeration conditions although some detrimental effects in flavour and colour might be observed **(Concepción Cabeza et al., 2007, Al-Bachir and Zeinou, 2009)**. The use of pulsed light for sliced cooked ham in-packaging processing is also proposed. Sliced cooked ham shelf life is increased by 4 (12 days shelf life) and 28 days (54 days shelf life) when treated with 8.4 J/cm^2 of pulsed light and stored at air and VP under refrigeration conditions, respectively **(Hierro et al., 2011)**.

Although all these methods proved to be efficient in cooked ham and minced meat shelf life extension, they are also highly dependent on refrigeration to which is associated a high energetic cost. Therefore new preservation technologies are needed in order to reduce the costs associated to refrigeration or even substitute this preservation condition. One of the most promising alternatives is the storage of food under moderated pressures, called hyperbaric storage (HS) as described in the beginning of this introduction.

Objectives

The current published studies state that depending on the pressure applied, HS of food has an inhibitory effect on enzymes and microorganism responsible for deterioration. As a consequence it is possible to increase foods shelf life by storing it under hyperbaric conditions, with no need for temperature control, and so no refrigeration.

To our knowledge, the studies regarding the HS of food commodities are basically inexistent since this is a very recent preservation methodology. There is so a great lack of information regarding the microbial spoilage in different food matrixes that are submitted to these environments.

Therefore the objectives, scheduled in **Table 7,** defined for this work are:

1. Development of the first review article concerning HS preservation;
2. Evaluation of different combinations of pressures, temperatures and storage times on the stability of sliced cooked ham with comparison to refrigerated storage at atmospheric pressure in a laboratory scale HP equipment;

2.1 Evaluation of HS effect on sliced cooked ham native microflora (LAB, TAM, ENT and YM);

2.2 Evaluation of HS effect on some chemical physical parameters (pH, lipid oxidation, colour and water holding capacity);

3. Carry out the first study concerning sliced cooked ham and minced meat preservation under HS conditions in an industrial equipment.

Table 7 - Estimated Schedule for the objectives concretization.

2013			**2014**					
Oct	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**
1								
2.1								
				2.2				
							3	

The major objective behind this work is to contribute to evaluate the feasibility of HS to substitute refrigeration as a preservation procedure for sliced cooked ham minced pork meat and food.

Chapter II -Materials and Methods

This chapter comprises all the adopted microbiological and physicochemical analyses procedures.

1. Minced meat and sliced cooked ham sampling

The experimental procedure used is summarized in **Figure 6** in order to facilitate the understanding of the whole sample preparation and storage processes.

For lab scale storage experiments, two sliced cooked ham products, one purchased from the local supermarket and other directly from the factory, were cut in small rectangular slices (1 x 4 cm), under aseptic conditions, using a knife previously washed in 70% ethanol. This was achieved by overlapping commercial cooked ham slices, from the same brand, originating from different packages but the same batch. After slicing, sliced cooked ham samples were divided in 10 g portions and placed into low permeability polyamide-polyethylene bags (PA/PE-90, Albipack – Packaging Solutions, Águeda, Portugal), which were vacuum sealed (Vacuum packager Packamn, Albipack – Packaging Solutions). Each bag containing cooked ham was afterwards inserted into a second bag that was heat sealed under vacuum. Both packaging films were previously sterilized by irradiation with UV light for 15 min (Biosafety Cabinet Telstar Bio II Advance, Terrassa, Spain). Finally the samples were frozen and stored at -80 ºC until the experiments were carried out.

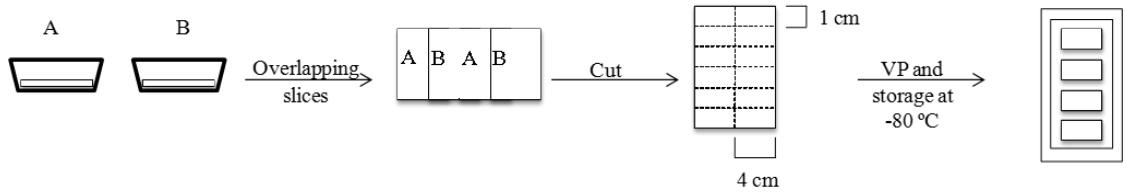

Figure 6 - Schematic representation of cooked ham samples preparation

For the experiments carried out in the industrial scale equipment, sliced cooked ham acquired directly from the factory (≈ 5 kg – five packages) was divided into portions of 100 g and packaged in low permeability polyamide-polyethylene bags (mimetizing the product available on the market). Minced meat (≈ 2 kg) was purchased in a local butchers' shop (minced at the time of acquisition) and divided in small circular portions of 100 g, under aseptic conditions. Afterwards, the samples were packaged according to the procedure adopted for sliced cooked ham samples and stored at -8 ºC for 24 hours, until the experiments in the industrial scale equipment.

2. HS experiments

2.1 Experiments in laboratory scale equipment

HS storage experiments were carried out by using a high hydrostatic press (High pressure system U33, Unipress Equipment Division, Institute of High Pressure Physics, Warsaw, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height (100 mL capacity) surrounded by an external jacket, connected to a thermostatic bath (Huber Compatible Control CC1, New Jersey, USA) to control the temperature. It was used a mixture (40:60) of propylene glycol (96% propylene glycol and 4% inhibitors and water, Dowcal N fluid, Dow Chemical Company) and water as a pressurizing fluid and to control the temperature in the external jacket. The sliced cooked ham samples were stored for 4 and 8 hours at several pressure (25, 50, 100 and 150 MPa) and temperatures (25, 30 and 37 ºC), as represented on **Table 8**. Control samples were maintained at atmospheric pressure (0.1 MPa) at the same temperature and refrigeration (5 ºC) for the same period, immersed in the same fluid and in the dark to create exactly the same conditions of the samples stored in the pressure equipment chamber, except for pressure.

Table 8 – Storage temperatures, pressures and times used for sliced cooked ham samples preservation

Temperature (ºC)	Pressure (MPa)	Time (h)
4	0.1	4
		8
25	0.1	4
		8
	100	4
		8
30	0.1	4
		8
	25	4
	50	4
	100	4
		8
	150	4
37	0.1	4
	100	4

Microbial (TAM, LAB, ENT and YM) and physicochemical analysis (pH, lipid oxidation, colour and water holding capacity (WHC)) were evaluated in order to determine the effect of the different storage conditions.

2.2 Experiments in industrial scale equipment

The HS experiments in an industrial scale equipment were carried out in a 55-litre capacity apparatus (model 55, Hyperbaric, Burgos, Spain). The packages of samples were stored at variable RT (20-21 ºC) and 100 MPa for 12 and 24 hours of storage. Control samples were maintained at atmospheric pressure (0.1 MPa) at the same temperature and refrigeration (5 ºC) condition for the same period and in the dark. Afterwards the sliced cooked ham and minced meat microbial loads were analyzed and the remaining packages of the 24 hours storage were place at 4 ºC for more 4 days (post HS). Posteriorly, the microbial load (TAM, LAB, ENT and YM) and physicochemical parameters (pH, lipid oxidation, colour and water holding capacity (WHC)) were analyzed with the exception to minced pork meat that was not analysed for LAB and water holding capacity.

3. Microbiological Analysis

All the samples were analyzed for TAM, LAB, ENT and YM. Two gram of each sample was homogenized with 18 mL Ringers solution for 4 min in a Stomacher 80 Biomaster at medium velocity. For the large scale storage experiments, 10 g of samples were homogenized in 90 mL of Ringer solution and decimal dilutions were prepared using the same solution. The microbiological analyses were made in triplicate for each storage condition.

3.1 TAM counts

Total aerobic mesophilic (TAM) counts were determined in plate count agar (PCA; Merck) taking into account the NP 4405 **(IPQ, 2003)** and ISO 4833:2013 **(ISO, 2013)**. The pour-plate method was used with 1.0 mL of diluted solutions. The plates were incubated aerobically at 30 ± 1 ºC for 72 ± 4 h and the yellow colonies were counted.

3.2 LAB counts

Mesophilic lactic acid bacteria (LAB) were determined in Man, Rogosa and Sharp (MRS; Merck) medium. The diluted solution, 1.0 mL, was plated using the pour-plate method. The plates were incubated at 30 ± 1 ºC for 5 days and the yellowish colonies were counted according to ISO 15214:1998 **(ISO, 2013)**.

3.3 ENT counts

Enterobacteriaceae (ENT) counts were determined in violet red bile dextrose agar (VRBDA, Merck), by pour plate method, incubated for 24 h at 37 ± 1 ºC in aerobic conditions. The red-pink colonies were counted according to NP 4137:1991 **(IPQ, 1991)**.

3.4 YM counts

The counts of yeast and moulds (YM) were determined on rose-bengal chloranfenicol agar (RBCA, Merck) medium, according to NP 3277-1 **(IPQ, 1987)**. The spread-plate method, using 200 µL, was used in 5 plates. The plates were incubated at 25 ± 1 ºC for 5 days, being counted the pink colonies for yeasts and filamentous colonies for moulds.

3.5 Microbial counts

For all the microorganisms analyzed, the Petri dishes containing 30-300 colonies forming units (CFU) were selected and counted with exception for YM (15-150 colonies). The microbial load was determined according to **equation 1 (ISO, 2013)**:

$$N = \frac{\sum Colonies\ formed}{V(n_1+0.1n_2)\times d} \quad \textbf{(Equation 1)}$$

Being:

N → colony forming units per gram of sample

V → volume of sample in mL

n_1 → number of plates e the 1st countable dilution

n_2 → number of plates e the 2nd countable dilution

d → 1st countable dilution

After analysis the samples were vacuum sealed in the same polyamide-polyethylene bags, heat sealed in a second bag and stored at -80 ºC.

4. Physicochemical analysis

In order to determine the physicochemical characteristics, the samples were homogenised with an Ultraturrax T25 homogeniser (Janke & Kunkel IKA-Labortechnik) in order to minimize the error.

4.1 pH determination

Firstly, the sample was blended with distilled water at a proportion of 1:10 (w/v). Afterwards the pH was determined using a pH meter (pH electrode 50 14, Crison Instruments, S.A. Barcelone, Spain), previously calibrated with a buffer solution of pH 4.0 and 7.0.

4.2 Water holding capacity (WHC)

The water holding capacity (WHC) of sliced cooked ham was determined according to the procedure developed by **(Bosco et al., 2001)** and previously to the homogenization in the ultraturrax.

One gram of sliced cooked ham sample (slices 1 x 4 cm) was wrapped with two layers of filter paper (Whatman no.1) and place inside a centrifuge tube as represented on **Figure 7**. Afterwards, the samples were centrifuged at 1500 x g and the weight (w_{cent}) was determined. Thereafter, the samples were dried for 12 ± 1 h at 75 ºC and the final weight (w_{dry}) was determined. The WHC was determined according to **equation 2**:

$$WHC\ (\%) = \frac{w_{cent} - w_{dry}}{w_{initial}} \quad \textbf{(Equation 2)}$$

Being:

W_{cent} → samples weight after centrifugation

W_{dry} → samples weight after centrifugation and drying

$W_{initial}$ → initial samples weight

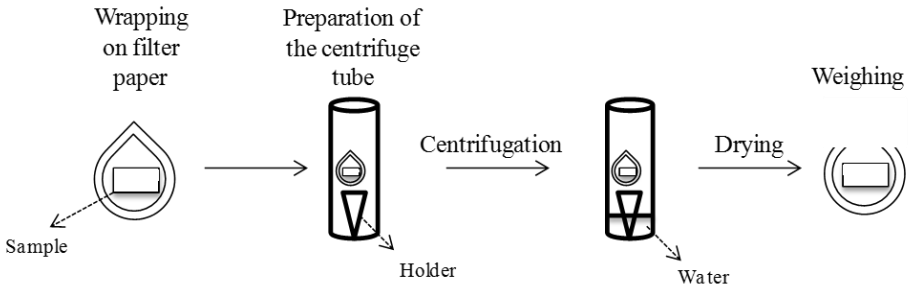

Figure 7 – Schematic representation of the procedure adopter for WHC determination

4.3 Lipid oxidation - Determination of Malondialdehyde (MDA)

The measure of lipid oxidation was adopted from **(SALIH et al., 1987)** as is described below.

A standard solution of 1,1,3,3-tetrametoxipropane (TMP) at a concentration of 10 µM in water was prepared and used as a stock solution. From this, standards from 0.5-10 µM were prepared in water and used for the construction of a standard curve represented in **Equation 3**.

$$Abs\ (532\ nm) = 0.0829 \times C_{MDA}(\mu M) + 0.0029 \quad R^2 = 0.998 \text{ (**Equation 3**)}$$

In order to extract the MDA from the samples (correspondent to initial, refrigerated, hyperbaric stored and atmospheric pressure stored samples), a proportion of 1:3 (w/v) was used and homogenized, in an ice bath, using an Ultraturrax T25 for 30 seconds. Afterwards, the extract was centrifuged at 3000 g for 5 min at 5 ºC. The supernatant was then filtered using a Whatman nº1 and used for the TBA reaction.

The TBA reaction consisted in the mixture of 1 mL of extract with 1 mL of TBA (acid thiobarbituric) solution (0.02 M TBA in distilled water) in a test tube, heated in boiling water for 30 min. Afterwards the mixture was cooled in a cold water bath and 300 µL were place in a microplate well and the absorbance was measured at 532 nm. These measurements were expressed in µg MDA/g of sample. The standards were measured using the same procedure.

4.4 Colour measurement

Colour assessment was carried out at RT, after sample homogenization to minimize colour determination variation errors. Afterwards, approximately 7 g of samples were

placed on a small dish and the colour was measured. The colour parameters were recorded using a colorimeter Konica Minolta CM 2300d, Minolta Konica, Japan. The CIELab parameters were determined using the original *SpectraMagic™ NX* Software, Konica Minolta, USA, according to regulations by the International Commission on Illumination: red/green colour (a^*) and yellow/blue colour (b^*) components, and luminosity (L^*). Measurements were done selecting six randomly spots in each sample.

5. Statistical analysis

Storage experiments were analyzed for microbial counts and physicochemical parameters in triplicate. Statistical data analysis of the results was performed using Analysis of Variance (ANOVA) and Tukeys HSD Test, at a 5% level of significance in order to identify differences between storage conditions.

The decrease of the microbial counts in the lab scale storage experiments, expressed in CFU/g of sample, with storage pressure at 30 ºC was determined in order to identify the microorganisms susceptibility to the storage pressure conditions (k). This k constant was determined from a first order equation (**Equation 4**), were *Log N* is the microbial count at a certain pressure and b the Y intercept.

$$Log\ N = -k \times P + b \qquad \textbf{(Equation 4)}$$

The decrease of the microbial counts in the large scale storage experiments, expressed in CFU/g of sample, with storage time at variable RT and 100 MPa was also assessed. This rate constant (x) was determined from a first order equation (**Equation 5**), were *Log N* is the microbial count at a certain time and b the Y intercept (initial count).

$$Log\ N = -x \times t + b \qquad \textbf{(Equation 5)}$$

Chapter III -Results and Discussion: Experiments in laboratorial scale equipment

1. Microbiological Analysis

1.1 Initial HS experiments: sliced cooked ham purchased in a local supermarket

The initial experiments regarding sliced cooked ham storage were carried out by using sliced cooked ham purchased in a local supermarket. It was observed that the commercial product microbial load was above 6 Log CFU/g, i.e. above the quality limits proposed by several authors **(Samelis et al., 1998, Mataragas et al., 2006)**, for all the storage conditions including the initial samples. Two factors explain these high microbial counts: 1) the storage temperatures abuse (from 4 to 12 ºC) to which retail subject the products **(Ruiz-Capillas et al., 2007)**; 2) the fact that that the product was 15 days from the end of its shelf life. As a consequence, we proceeded to obtain sliced cooked ham directly from the factory to ensure a lower microbial load and to assess its evolution over the time upon the storage conditions that will be subjected.

1.2 HS of sliced cooked ham: final samples

In order to assess the possibility of replacing refrigeration for HS as a preservation procedure for sliced cooked ham, different storage conditions which differed in temperature, pressure and time were used and the microbial load, before and after storage, were studied. The trials performed were designed to assess the effect of pressure on the microbial growth and physicochemical parameters at different temperatures.

As seen in **Figure 8** the sliced cooked ham initial TAM and LAB counts were 3.66 ± 0.10 and 3.66 ± 0.10 Log CFU/g, respectively, showing a high prevalence of LAB, which is in agreement with data in the literature that identify these microorganisms as the major spoilage agents in these type of products **(Samelis et al., 1998, Hu et al., 2009, Holley, 1997)**. The initial YM and ENT levels of the cooked ham used in this experiment were lower than 2 Log CFU/g (data not shown). These results are consistent with those observed by **Samelis et al. (1998)**, except for YM. These authors observed an initial count of 3.11 and 3.04 Log CFU/g for TAM and LAB, respectively, 3 Log CFU/g for YM and less than 2 Log CFU/g for ENT, in VP sliced cooked ham. This difference in YM counts is probably due to different preparation conditions of the cooked ham.

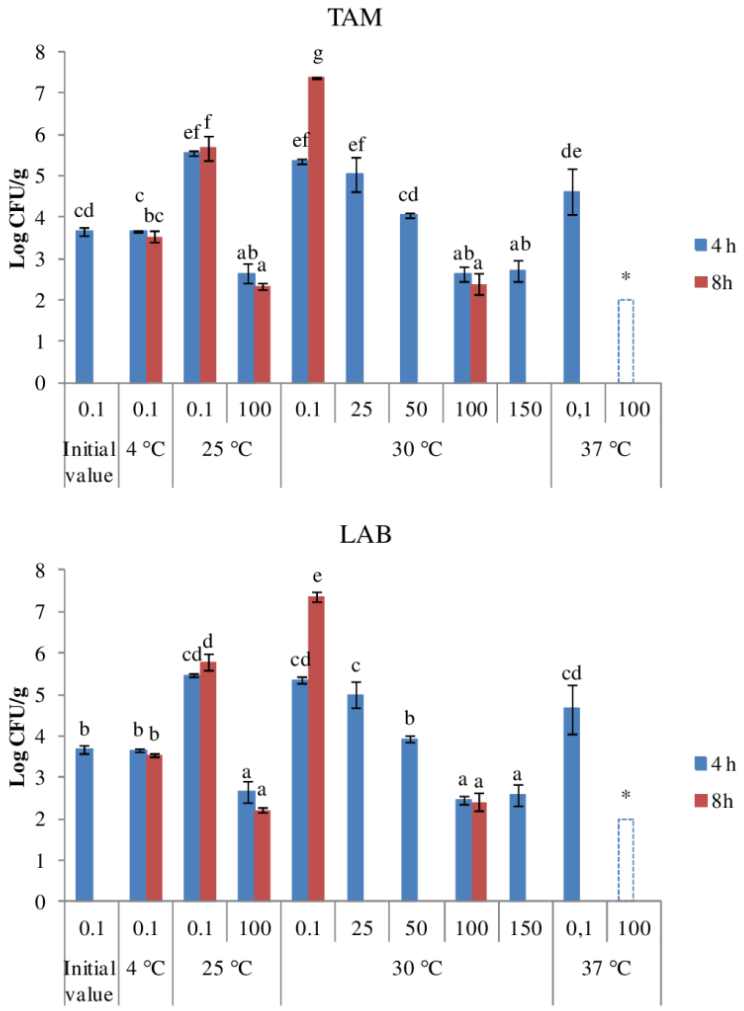

Figure 8 – TAM and LAB counts (expressed in Log CFU/g) of sliced cooked ham both before and after storage for 4 and 8 hours at the different pressures and temperature conditions. Bars with * represent samples with microbial counts below the detection limit (2 Log CFU/g). Different letters (a-g) indicate significant differences (P<0.05) between storage conditions.

Different changes on sliced cooked ham microbial loads were observed during the various storage conditions. As seen in **Figure 8**, after 4 hours of storage at 0.1 MPa the TAM and LAB counts were similar (P>0.05) at all the tested temperatures (25, 30 and 37 ºC), around 5.5 Log CFU/g. These results showed an increase of about 2 Log CFU/g compared to the initial LAB value of the sliced cooked ham. After 8 hours, the microbial counts reached a maximum at 30 ºC (7.37 ± 0.01 and 7.37 ± 0.11 CFU/g of sliced cooked ham for TAM and LAB counts, respectively), reaching the acceptable quality limits indicated by several authors of 6-8 Log CFU/g of cooked meat products (**Samelis et al., 1998, Mataragas and Drosinos, 2007**). ENT and YM remained below 2 Log CFU/g for all the tested conditions (data not shown). This increase in sliced cooked ham microbial

load is explained by its high pH (above 6) and water activity (above 0.94) that allow microorganisms to proliferate at and above RT, whereas their growth is slowed down under refrigeration, as represented in **Figure 8**. The microbial load (TAM, LAB, ENT and YM) of the samples stored at 5 ºC and 0.1 MPa remained unchanged (P>0.05) during the 4 and 8 hours of storage and no significant differences (P>0.05) were observed when compared to the initial counts. According to data in literature, it is required at least 1-8 days in order to observe an increase of 0.2-3.5 Log CFU/g of cooked ham, under refrigeration conditions **(Ruiz-Capillas et al., 2007, Vasilopoulos et al., 2008, Hierro et al., 2011)**.

Contrarily to the storage at atmospheric pressure, different behaviours such as growth, growth inhibition and inactivation of microorganisms were observed when the sliced cooked ham samples were stored under HS conditions. In general, pressure had a significant effect on the final microbial load, which was statistically lower (P<0.05) from those obtained at 0.1 MPa, for the same storage time and temperature employed (except for TAM and LAB at 25 MPa and 30 ºC). These results are representative of an inhibitory effect of pressure on TAM and LAB growth, when compared to the results obtained at 0.1 MPa for the same temperatures and storage times. For instance, the cooked ham stored at 0.1 MPa and 30 ºC had its TAM load increased to 5.36 ± 0.06 Log CFU/g and 7.37 ± 0.01 Log CFU/g after 4 and 8 hours of storage, respectively, whereas the samples stored at 100 MPa showed TAM and LAB counts of about 2.63 ± 0.19 Log CFU/g after 4 hours of storage which remained unchanged (P>0.05) after 8 hours (a similar behaviour was observed at 25 ºC). The increase of temperature from 30 to 37 ºC at 100 MPa lead to the reduction of the microbial load to values below the detection limit (<2.0 Log CFU/g), showing a higher inactivation effect, whereas the increase of pressure from 100 to 150 MPa at 30 ºC did not cause any further significant differences (P>0.05). At 25 MPa and 30 ºC an increase of the microbial load was observed, for both TAM and LAB, reaching values similar to those observed at 0.1 MPa for the same temperature (≈5.3 Log CFU/g). Nonetheless, at 50 MPa a microbial growth inhibition was verified, being observed levels similar (P>0.05) to refrigeration and to the initial samples, whereas the increase to 100 and 150 MPa resulted in microbial inactivation, additionally to microbial growth inhibition, as just shown above. Therefore, in general the microbial growth starts to be inhibited at pressures around 50 MPa whereas an additional inactivation effect is observed at pressures

equal or above 100 MPa. At 25 MPa and 30 ºC (the only studied temperature at this pressure level) no microbial growth inhibition is verified. This way, pressures above 25 MPa seem to be necessary to achieve microbial growth inhibition in the case of HS of sliced cooked ham.

The inactivation of microorganisms and inhibition of their growth is of extreme importance in order to extend a food product shelf life. In the case of HS, pressure is the main factor responsible for food preservation, in this case of sliced cooked ham, and it has different effects (microbial growth inhibition or inactivation), as represented on **Figure 9**.

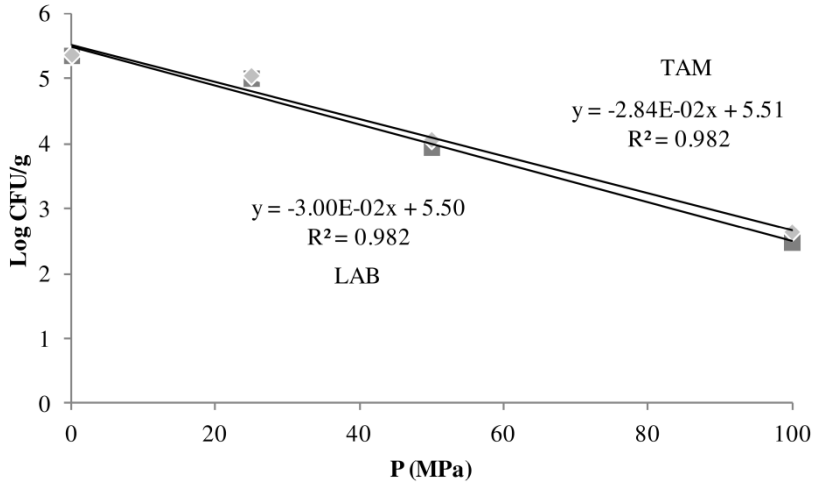

Figure 9 - Linear decrease of TAM (diamonds) and LAB counts (squares), expressed in Log CFU/g, after 4 hours of storage as a function of the storage pressure at 30 ºC.

In **Figure 9** it is represented the microbial load of sliced cooked ham, for TAM and LAB, as a function of the pressure applied (from 0.1 to 100 MPa at 30 ºC). As it can be seen from the observed slopes, -0.028 and -0.030 Log CFU/g/MPa for TAM and LAB respectively, the increase of the storage pressure leads to lower counts and shows a similar susceptibility for both the microorganisms. Taking into account the conditions applied in this study, there is only one work with a similar analysis. **Queirós et al. (2014)** observed that regardless of the storage temperature, the increase of pressure leaded to lower microbial counts being the YM more susceptible to pressure variation than TAM, taking into account the observed slopes (-0.020 and -0.011 Log CFU/mL/MPa, respectively). These data are extremely important to the extent that allows to have an awareness of the microorganisms susceptibility to pressure conditions at which the food is stored. As such,

it will be possible to determine the necessary storage conditions to delay the growth of deteriorative microorganisms without the need of the pressure levels currently used in HPP. Due to the conditions required for HPP, the capital costs of HP equipment are high making it unfeasible for food storage. However, further studies will possible foster the development of equipment which capital costs would be significantly lower than those already charged for HPP due to the lower pressures that are used in this new preservation methodology.

As far as the authors are aware, this is the first study dealing with HS of a cooked meat product. The only published study regarding a raw fish product was reported by **Ko and Hsu (2001)** concerning storage of tilapia fillets at 101 and 203 MPa and 25 ºC for 12 hours. The tilapia fillets stored at 101 MPa had similar microbial counts to the initial, inhibiting the fillets deterioration. Furthermore, for 203 MPa, a microbial load reduction from 4.7 to around 2.0 Log CFU/g of sample was observed after storage, indicating an inactivation effect of pressure apart from its inhibitory action on microbial growth. Moreover, only one temperature was considered, while in the present work three temperatures (25, 30 and 37 ºC) were studied.

These and other published data support the idea that the minimum pressures to have an inhibitory effect similar to refrigeration on microbial growth depend mostly on food physicochemical parameters **(Segovia-Bravo et al., 2012, Queirós et al., 2014, Fidalgo et al., 2014)**. **Segovia-Bravo et al. (2012)** observed that 25 MPa was sufficient to inhibit microbial growth on strawberry juice, mostly due to its acidic nature that aids in microbial growth inhibition. Nonetheless, food commodities with high pH such as melon juice require pressures up to 50-75 MPa (depending on the temperature) **(Queirós et al., 2014)**, similarly to sliced cooked ham. An inactivation effect was also observed for melon and watermelon juice with the increase of the storage pressure to 150 and 100 MPa, respectively, causing a reduction of the TAM counts in ≈1 Log CFU/g, similar to our data, whereas ENT and YM were reduced to counts below the detection limit **(Queirós et al., 2014, Fidalgo et al., 2014)**.

Since the inhibitory and/or inactivation effects observed in this work were verified for the three studied temperatures (25, 30 and 37 ºC), i.e. for temperatures at and above RT, it is possible to hypothesize the use of HS under uncontrolled (naturally variable) temperature conditions for the preservation of sliced cooked ham, and so with no need for

energy during storage. This is a new concept on food preservation and this preservation methodology is a very promising alternative for refrigeration that needs further extensive study.

2. Physicochemical analysis

2.1 Effect of HS on sliced cooked ham pH

The initial sliced cooked ham pH was 6.26 ± 0.07 (**Table 9**), similar to the values found in literature **(Samelis et al., 1998, Vercammen et al., 2011, Han et al., 2011)**. After the storage of sliced cooked ham for 4 and 8 hours at all the tested conditions, no significant differences (P>0.05) were found on pH when compared to the initial despite a minimum of 6.14 ± 0.04 and a maximum of 6.29 ± 0.01 that were observed at 25 ºC and 0.1 MPa and 30 ºC and 150 MPa, after 4 hours of storage respectively.

Table 9 - Sliced cooked ham pH after 4 and 8 hours of storage at the different temperature and pressure conditions. Different upper case letters between conditions (A-C) and different lower case letters between storage times indicate significant differences (P<0.05).

Temperature (ºC)	Pressure (MPa)	Time (h)		
		0	4	8
4 ºC	0.1	6.26±0.07 a	6.22±0.06 aAB	6.25±0.03 aB
25 ºC	0.1	6.26±0.07 a	6.14±0.04 aA	6.27±0.01 aB
	100	6.26±0.07 a	6.27±0.01 aB	6.17±0.01 aA
30 ºC	0.1	6.26±0.07 a	6.26±0.01 aAB	6.26±0.02 aB
	25	6.26±0.07 a	6.23±0.02 aAB	-
	50	6.26±0.07 a	6.22±0.01 aAB	-
	100	6.26±0.07 a	6.23±0.05 aAB	6.23±0.02 aAB
	150	6.26±0.07 a	6.29±0.01 aB	-
37 ºC	0.1	6.26±0.07 a	6.19±0.01 aAB	-
	100	6.26±0.07 a	6.18±0.01 aAB	-

Data in the literature suggest that cooked ham pH declines, reaching a decrease of 1.5 pH units in 20 days under refrigeration conditions, due to the metabolic activity of LAB **(Han et al., 2011, Samelis et al., 1998)**. Since these experiments were conducted at RT, it would be expected that significant variations occurred during the studied storage times, 4 to 8 hours, which was not observed. Therefore, the results obtained in the present work regarding the cooked sliced ham pH along storage may accrue from the shorter storage times applied that did not allow observing statistically significant pH declines.

Currently little information is available regarding the pH behaviour of hyperbaric stored food. However, the published results so far point to a slower decrease of foods pH stored under HS conditions compared to atmospheric pressure storage **(Queirós et al., 2014, Fidalgo et al., 2014)**.

2.2 Water Holding Capacity (WHC)

The metabolic activity of spoilage microorganisms is responsible for the decrease in raw meat pH during storage to values next to meat protein isoeletric point which consequently affects its WHC **(Huff-Lonergan and Lonergan, 2005)**. Therefore, this parameter was evaluated in the samples subjected to the different storage conditions.

The initial WHC of cooked ham used in these experiments was 46.46 ± 0.02 %. As seen in **Table 10** no significant differences (P>0.05) were observed between the samples stored at the different tested conditions and the initial samples with the exception of those stored at 25 MPa, 30 ºC, 4 h (39.51 ± 1.01 %) and 150 MPa, 30 ºC, 4 h (50.45 ± 4.97 %).

2.3 Lipid Oxidation

The lipid oxidation of cooked sliced ham samples, expressed as µg MDA/g of cooked sliced ham, was determined before and after 4 and 8 hours of storage at all the tested conditions. The initial MDA content of cooked sliced ham was 0.091 ± 0.005 µg/g of sample, as can be seen in **Table 11**. This value is slightly higher than that reported by **Hierro et al. (2011)**, 0.035±0.005 µg/g of sample, and lower than the one observed **Liu et al. (2012)**, around 0.25 µg/g of sample, which may result from differences in cooked ham composition, namely in lipid content.

In general, the storage of sliced cooked ham for 4 hours at atmospheric and moderated pressures conditions from 25 to 37 °C yielded MDA contents not statistically significantly different (P>0.05) to those stored under refrigeration temperatures with the exception to

the samples stored at 30 ºC and 25 MPa. Overall these values were also similar (P>0.05) to the initial samples with exception to the samples stored at 30 ºC and 0.1, 25 and 150 MPa.

Table 10 - Sliced cooked ham WHC (%) after 4 and 8 hours of storage at the different temperature and pressure conditions. Different upper case letters between conditions and different lower case letters (a-b) between storage times indicate significant differences (P<0.05).

Temperature (ºC)	Pressure (MPa)	Time (h)		
		0	4	8
4 ºC	0.1	46.46±0.02 a	44.40±2.27 aA	46.21±4.19 aA
25 ºC	0.1	46.46±0.02 a	45.45±5.22 aA	50.45±4.97 aA
	100	46.46±0.02 a	45.58±2.76 aA	46.69±9.34 aA
30 ºC	0.1	46.46±0.02 a	43.83±3.13 aA	44.02±0.55 aA
	25	46.46±0.02 b	39.51±1.01 aA	-
	50	46.46±0.02 a	43.18±3.93 aA	-
	100	46.46±0.02 a	48.59±3.13 aA	43.58±4.84 aA
	150	46.46±0.02 b	42.2±0.91 aA	-
37 ºC	0.1	46.46±0.02 a	47.21±0.50 aA	-
	100	46.46±0.02 a	45.39±1.14 aA	-

Nevertheless, the hyperbaric and refrigerated storage of sliced cooked ham for 8 hours yielded significant differences (P<0.05) when compared to the initial sample, presenting in general lower values. Notwithstanding, the MDA values did not differ significantly (P>0.05) between the storage at atmospheric pressure and under pressure at and above RT (25 to 37 °C) and the samples stored under refrigeration conditions. The only exception was observed at 25 ºC and 100 MPa after 8 hours of storage, where the highest TBARS value was observed (0.130 ± 0.004 µg MDA/g).

In meat and meat products it is generally suggested that high pressure triggers lipid oxidation although a lower susceptibility is observed for cured meat products than fresh meat **(Bajovic et al., 2012)**. Nonetheless, the studies already published are based on a pressure treatment at much higher pressures, 300-600 MPa, during few minutes for food pasteurization, while in this study the products were subjected for 4 and 8 hours at lower

pressures. Yet, our data suggest that lipid oxidative stability of cooked sliced ham stored under HS conditions is not affected being similar to refrigeration.

Table 11 - Sliced cooked ham lipid oxidation (TBARS, µg MDA/g of cooked ham) after 4 and 8 hours of storage at the different temperature and pressure conditions. Different upper case letters between conditions (A-C) and different lower case letters between storage times indicate significant differences (P<0.05).

Temperature	**Pressure**	**Time (h)**		
(ºC)	**(MPa)**	0	4	8
4 ºC	0.1	0.091±0.005 bA	0.093±0.005 bA	0.066±0.001 aAB
25 ºC	0.1	0.091±0.005 abA	0.099±0.003 bA	0.082±0.001 aB
	100	0.091±0.005 aA	0.085±0.004 aA	0.130±0.004 bC
30 ºC	0.1	0.091±0.005 bA	0.110±0.004 cA	0.052±0.002 aA
	25	0.091±0.005 aA	0.163±0.005 bB	-
	50	0.091±0.005 aA	0.088±0.019 aA	-
	100	0.091±0.005 aA	0.097±0.010 aA	0.065±0.010 aAB
	150	0.091±0.005 aA	0.110±0.001 bA	-
37 ºC	0.1	0.091±0.005 aA	0.097±0.007 aA	-
	100	0.091±0.005 aA	0.097±0.010 aA	-

2.4 Colour

The initial cooked ham samples showed a bright pinkish colour (L* value of 68.27 ± 0.72 and h* value of 1.00 ± 0.03) tendentiously red (a* value of 8.41 ± 0.47) and yellow (b* value of 12.98 ± 0.14). In general, no significant differences (P>0.05) in the colour parameter (L*, a* and b*) were observed between the samples stored at atmospheric or HS conditions for 4 and 8 hours, regardless the temperatures and the initial samples (**Table 12**). Data in the literature suggest that cooked ham colour tends to fade during storage as a consequence of the microbial activity and chemical deterioration, acquiring a dull greyness colour that decreases the consumer acceptance of the commodity (**Li et al., 2012, Munk et al., 2010**). In this particular study, the reduced changes in the colour parameters may accrue from the reduced storage times applied.

Currently little data is available regarding the colour preservation under HS conditions. The results published so far point towards the feasibility of HS to preserved food commodities colour when compared to atmospheric storage at the same temperature, and as efficiently refrigeration at least in the case of strawberry juice **(Segovia-Bravo et al., 2012)**. These authors observed that strawberry juice stored at RT and atmospheric pressure for 15 days yielded more accentuated colour changes ($\Delta E=4.5\pm0.7$) whereas for the samples stored under HS conditions at RT and refrigeration at 0.1 MPa no significant colour changes were observed ($\Delta E=1.3\pm0.1$ and $\Delta E=0.4\pm0.2$, respectively).

Table 12 - Sliced cooked ham colour parameters (*L**, *a** and *b**) after 4 and 8 hours of storage at the different temperature and pressure conditions. Different upper case letters between conditions (A-C) and different lower case letters between storage times indicate significant differences ($P<0.05$).

		T (°C)									
		4 °C	25 °C			30 °C				37 °C	
	t (h)	0.1	0.1	100	0.1	25	50	100	150	0.1	100
L*	0	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa	68.27±0.72 Aa
	4	68.27±0.73 aA	69.01±0.56 aA	68.47±1.28 aA	69.19±0.69 aA	66.89±0.60 aA	68.12±0.68 aA	67.47±0.38 aA	65.46±2.28 aA	69.31±0.48 aA	66.84±3.09 aA
	8	69.37±0.54 aBC	68.73±0.28 aBC	66.64±0.65 aA	67.00±0.57 aAB	-	-	67.99±0.23 aABC	-	-	-
a*	0	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA	8.41±0.47 aA
	4	8.44±0.18 aA	8.23±0.16 aA	8.36±0.78 aA	8.15±0.19 aA	8.43±0.07 aA	8.23±0.15 aA	8.87±0.68 aA	9.36±0.46 aA	8.95±0.17 aA	9.18±0.56 aA
	8	8.41±0.05 aA	9.04±0.28 aA	8.81±0.65 aA	8.96±0.17 aA	-	-	8.52±0.33 aA	-	-	-
b*	0	12.98±0.14 aAB	12.98±0.14 bAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 aAB	12.98±0.14 bAB
	4	13.02±0.15 aAB	13.32±0.24 bB	13.14±0.34 aAB	13.41±0.08 bB	12.80±0.67 aAB	12.63±0.33 aAB	13.33±0.16 aB	12.57±0.14 aAB	13.64±0.36 aB	12.08±0.02 aA
	8	13.14±0.07 aA	11.96±0.08 aA	12.81±0.35 aA	13.18±0.02 abA	-	-	12.56±0.14 aA	-	-	-

Chapter IV -Results and Discussion: Experiments in the industrial scale equipment

1. Microbiological Analysis

1.1 Experiments in the industrial scale equipment: sliced cooked ham

The large scale storage experiments were focused on a real scale preservation of sliced cooked ham and minced pork meat, packages of 100 g. These experiments were carried out in the last month of the timetable defined for the elaboration of the thesis, so only basic microbial and physicochemical analysis and one HS conditions were assessed.

The initial TAM, LAB, ENT and YM counts of sliced cooked ham samples were below 2 Log CFU/g, i.e. less than the limit of quantification of the method which can be explained by the minor storage time, about 2 days, from the time when the product was sliced to the reception in the laboratory, when compared to the samples used in lab scale storage experiments (3 days).

In order to determine the feasibility of HS to substitute refrigeration as a preservation procedure for cooked sliced ham at real scale, the samples were stored for 12 and 24 hours to each of the following conditions: i) 4 ºC and 0.1 MPa; ii) variable RT at 0.1 MPa; iii) and variable RT at 100 MPa. After storage it was observed that the TAM, LAB, ENT and YM remained below the quantification limit for all the testes conditions which can be explained by differences in product formulation (information provided by the factory), when compared to those used in lab scale storage. This compositional difference may thus result in additional barriers to microbial growth. As a consequence no further analyses were carried out in these samples and the large scale study was focused on another product stored simultaneously with the sliced cooked ham, minced pork meat.

1.2 Large scale storage experiments: minced pork meat

The initial microbial load of minced pork meat (**Figure 10**) was 5.24 ± 0.07, 2.69 ± 0.09 and 3.87 ± 0.30 Log CFU/g of product for TAM, ENT and YM, respectively - although LAB are also present in minced pork meat, these were not quantified since the experiments in industrial scale equipment were carried out with other five food products making the microbiological tests very time consuming. These values are within the microbiological loads observed by **Andritsos et al. (2012)**. These authors observed that the TAM, ENT and YM counts of minced pork meat purchased in butcher's shops and supermarkets can range from 4.9-8.7, 1.4-5.9 and 3.3-5.9 Log CFU/g of product, respectively.

As represented on **Figure 10**, the storage of minced pork meat at variable RT and 0.1 MPa leaded to microbial counts above 6 Log CFU/g for TAM and 4.52 ± 0.18 Log CFU/g for ENT, after 12 hours of storage. These counts reached higher values when the storage time was increased to 24 hours. Both TAM and ENT counts increased to values above 6 and 5 Log CFU/g, respectively whereas YM counts remained unchanged during the storage period, similar (P>0.05) to the initial counts.

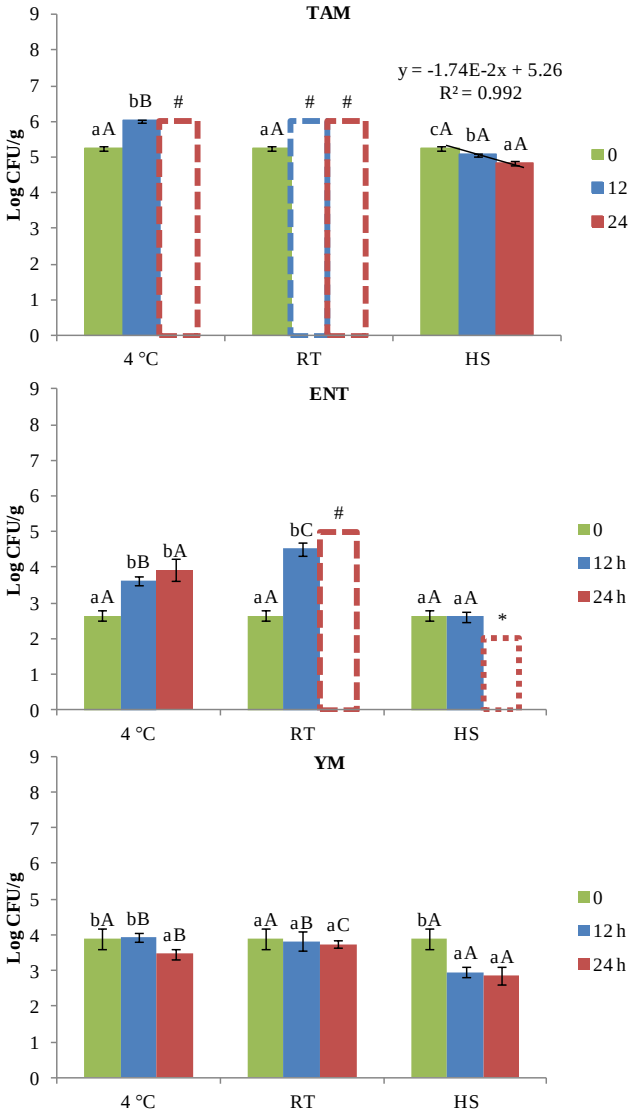

Figure 10 - TAM, ENT and YM counts expressed in Log CFU/g (mean ± SD) of minced pork meat both before and after storage for 12 and 24 hours at the different storage conditions. Traced bars with * represent samples with microbial counts below the quantification limit whereas traced bars with # represent samples with at least the represented microbial counts. Lower case letters (a-c) and upper case letters (A-C) represent significant differences (P<0.05) between storage times at the same condition and significant differences between conditions at the same storage time, respectively. Linear decrease of TAM, expressed in Log CFU/g, as a function of the storage time under HS is also represented.

An increase in the microbial counts of minced pork meat stored at 4 ºC and 0.1 MPa was also observed but at slower rate. Under this storage conditions the TAM counts increased to 6.03 ± 0.04 Log CFU/g and to values above 6 Log CFU/g in 12 and 24 hours respectively. Similarly, ENT increased to 3.63 ± 0.05 Log CFU/g after 12 hours of storage, values significantly (P<0.05) higher than the initial samples. ENT counts maintained unchanged (P>0.05) when the storage period was extended to 24 hours. On the other hand, YM counts remained similar (P>0.05) to the initial counts during the first 12 hours and then reduced to 2.85 ± 0.25 Log CFU/g. These results are in accordance with the data previously obtained in the lab scale storage experiments where the samples stored at RT had an increment in the microbial counts whereas those under refrigeration showed values similar to the initial samples, thus showing microbial growth inhibition. Published data in the literature also shows that the increase of the storage temperature of minced pork meat leads to an increase in the growth rate of the spoilage microbiota **(Koutsoumanis et al., 2008, Argyri et al., 2011)**. **Argyri et al. (2011)** observed that in minced beef, the maximum growth rate of TAM counts in air, MAP and MAP supplemented with EO increased in 3-9 times with the increase of the storage temperature from 0 to 15 ºC. In another study, **Koutsoumanis et al. (2008)** observed that the maximum growth rate of *Pseudomonads* in high and low permeability packages was increased from 0.069 and 0.035 h^{-1} to 0.323 and 0.270 h^{-1}, respectively, when the storage temperature increased from 0 to 15 ºC.

While in the samples stored at atmospheric pressure microbial growth was observable, the HS yielded a different behavior. As represented on **Figure 10**, the HS of minced pork meat at variable RT led to the inhibition of microbial growth with additional inactivation effect during the whole storage period. For instance, while in the samples stored at 0.1 MPa for 12 hours the TAM counts increased to values above 6 Log CFU/g, those stored at 100 MPa had a reduction (P<0.05) of the microbial load from 5.24 ± 0.07 to 5.06 ± 0.05 Log CFU/g. The increase of the storage time to 24 hours led to even lower (P<0.05) counts, 4.83 ± 0.06 Log CFU/g. This reduction of the TAM counts in the samples stored at 100 MPa showed a linear behavior with the storage time (**Figure 10**), presenting a reduction rate of -0.0174 Log CFU/g/h. Concerning the published data relating to HS experiments, this is the first study that shows the inactivation rate under HS conditions along the storage time. A similar inactivation effect to values below 2.0 and to 2.85 ± 0.25

Log CFU/g was observed for ENT and YM, respectively, when the samples were stored for 24 hours under HS conditions. When compared to refrigeration, HS also showed to be more effective by inhibiting microbial growth. For all the tested microorganisms, the TAM, ENT and YM counts were significantly (P<0.05) lower than those observed in the samples stored under refrigeration. For instance, after 24 hours of storage the TAM counts of refrigerated samples increased to values above 6 Log CFU/g whereas those stored under HS conditions were maintained in values around 4.9 Log CFU/g.

The obtained results are in agreement with those obtained in the lab scale storage of sliced cooked ham samples where pressures of 100 MPa inhibited microbial growth and caused an additional microbial inactivation. Similar studies concerning the storage of strawberry juice, melon juice, watermelon juice and tilapia fillets showed the ability of pressure to inhibit microbial growth and even reduce the microbial counts **(Segovia-Bravo et al., 2012, Queirós et al., 2014, Ko and Hsu, 2001, Fidalgo et al., 2014)**.

This study thus demonstrated that is feasible to store real scale minced pork meat under HS conditions at RT being the microbiological results significantly (P<0.05) better than those obtained under refrigeration. Although this new emerging preservation technology is shown to be very promising, several studies at microbiological level need to be carried out in order to ensure the safety and the quality of the products.

2. Physicochemical analysis

2.1 pH

The initial pH of minced pork meat was 6.02 ± 0.14, within the values reported in the literature **(Andritsos et al., 2012, Michalczyk et al., 2012, Skandamis and Nychas, 2001)**.

As represented on **Table 13**, the storage of minced pork meat for 24 hours yielded different pH variations that differed between the storage conditions. Concerning the storage at variable RT, it was observed a significant (P<0.05) pH decrease to 5.83±0.08 in the first 12 hours followed by an increase to 6.06±0.04 in the final storage period. This pH variation can be explained by the use of glucose that is metabolized to organic acids followed by a metabolic shift towards more basic metabolites due to the depletion of this substrate. Indeed, glucose was found to be the initial substrate supporting the growth of the microflora present in meat and its use is accompanied by an increase in the titrametric

acidity as a consequence of lactic acid formation **(Drosinos and Board, 1995b, Drosinos and Board, 1995a)**. When this carbon source is depleted, lactate and amino acids began to be metabolized being the latest responsible for the release of ammonia that increases the pH **(Drosinos and Board, 1995b, Nychas, 1998, Skandamis and Nychas, 2001)**.

Table 13 – Minced pork meat pH after 12 and 24 hours of storage at the different storage conditions. Different upper case letters between conditions (A-B) and different lower case letters between storage times (a-b) indicate significant differences (P<0.05).

Condition	Time (h)		
	0	12	24
4 ºC	6.02±0.14 aA	6.04±0.03 aB	6.06±0.04 aA
RT	6.02±0.14 bA	5.83±0.08 aA	6.06±0.04 bA
HS	6.02±0.14 aA	6.00±0.10 aB	6.35±0.06 bB

Contrarily, the storage of minced pork meat under refrigeration leaded to a steadier pH which is possibly related to microbial activity inhibition by the low storage temperatures. With respect to the samples stored under HS conditions at RT, it is observable in **Table 13** that the minced pork meat pH is stable (P>0.05) for at least 12 hours, followed by an increase (P<0.05) to 6.35 ±0.06 after 24 hours of storage. As reviewed by **Doulgeraki et al. (2012)** the spoilage microbiota and metabolic activity is highly influenced by the storage conditions applied and their competition. In this case, pressure was applied as a storage condition which may alter the metabolism or the dominant microflora of the product to another capable of producing metabolites of basic nature. This hypothesis is supported by data in the literature that shows that the yield of ethanol in alcoholic fermentation performed by *Saccharomyces cereviseae*, can be affected when this process occurs under pressure and can even be stopped when pressures about 87 MPa are achieved **(Picard et al., 2007, Galanakis et al., 2012)**. Nevertheless, metabolomics studies of hyperbaric stored food products are required in order to confirm this hypothesis.

Despite all, pressure shows a stabilizing effect on the pH of minced pork meat, at least in short term, similar to what was found in melon and watermelon juices **(Queirós et al., 2014, Fidalgo et al., 2014)**.

2.2 Lipid Oxidation

The development of rancidity in meat and meat products is recognized as a serious problem during storage, particularly in ground meats due to its porous structure and due to the disruption of muscle cell structure during the mincing process that exposes lipid components to prooxidants resulting in the generation of free radicals **(Sato and Hegarty, 1971)**.

The TBARS values for minced meat are given in **Table 14**, and these values have different variations according to the storage conditions. In the case of the samples stored at RT and 0.1 MPa it is observable the maintenance (P>0.05) of the TBARS values during the first 12 hours of storage followed by a significant (P<0.05) increase to 0.178 ± 0.022 µg/g of sample after 24 hours of storage. On the other hand, the samples stored under refrigeration at 0.1 MPa maintained low TBARS values, decreasing significantly (P<0.05) to 0.014 µg/g of product after 24 hours of storage.

Table 14 – Minced pork meat TBARS values (µg MDA/g) after 12 and 24 hours of storage at the different storage conditions. Different upper case letters between conditions (A-C) and different lower case letters between storage times indicate significant differences (P<0.05). TBARS values are presented as mean ± standard deviation

Condition	Time (h)		
	0	12	24
4 ºC	0.027±0.004 bA	0.021±0.008 abA	0.014±0.004 aA
RT	0.027±0.004 aA	0.015±0.004 aA	0.178±0.022 bB
HS	0.027±0.004 abA	0.021±0.009 aA	0.035±0.002 bA

These variations are according to data published in the literature that states that lipid oxidation is favored by the increase of the storage temperature **(Limbo et al., 2010, Rogers et al., 2014)**. **Limbo et al. (2010)** observed that the TBARS limit value for perceived rancidity, 1.0 mg/kg of product, in minced beef stored in high-oxygen modified atmosphere packaging is reduced from 8 to 2 days when the storage temperature is increased from 4.3 to 15.5 ºC. Similar observation were taken by **Rogers et al. (2014)** for high oxygen packages. Another factor that may also contribute to the increase in TBARS values is the high lypolitic activity of microorganisms **(Rubio et al., 2007)**, and their capability to produce hydrogen peroxide which can lead to the formation of radicals.

Relatively to the samples stored under HS conditions, no significant (P>0.05) variations were observed in lipid oxidation when compared to the initial samples, even after 24 hours. In addition, the values were not statistically different (P>0.05) to refrigeration during the whole storage period. When comparing HS to the samples stored at variable RT and 0.1 MPa, the TBARS values were significant (P<0.05) lower in the former than in the latter. These results are in agreement with those previously obtained in sliced cooked ham stored under HS conditions where the low storage pressures applied did not affect to oxidative stability of the products, when compared to refrigeration. Data in the literature, concercing HPP, suggest that pressures higher than 300-400 MPa are required in order to observe marked differences in meat and fat oxidative stability and that the rate of these reaction increases with the increase of pressure **(Cheah and Ledward, 1996, Cheah and Ledward, 1995, Cheah and Ledward, 1997, Bolumar et al., 2012)**. However, this threshold limit is clearly not surpassed in this preservation procedure, so the oxidative stability of HS food products might not be affected, as observed in the present study.

2.3 Colour

At the point of sale, meat colour is one of the most important attributes to the consumer **(Troy and Kerry, 2010)**. Therefore, it is one of the most important parameters to preserve during storage. As represented on **Table 15**, the storage for 24 hours of minced pork meat at RT led to an increase (P<0.05) in the L* and b* parameters while the a* value was preserved (P>0.05), when compared to the initial samples. On the other hand, refrigeration yielded the maintenance (P>0.05) of all the colour parameters after the 24 hours storage period. Contrarily to refrigeration, HS increased the L* and b* value (P<0.05), whereas the a* was maintained (P>0.05). When comparing the different storage conditions after 24 hours of storage, it is observed that no significant differences (P>0.05) are observed for the L* and a* parameters. Nevertheless, a significant (P<0.05) higher values in the b* parameters are observed in RT stored samples while HS stored samples did no differed (P>0.05), when comparing to refrigeration.

Data in the literature suggest that HP may induce colour alterations as a consequence of globin denaturation, causing a decrease in the a* value **(Carlez et al., 1995)**, which was not observable in the present study. In fact, data in the literature shows that 100 MPa do not cause significant myoglobin denaturation **(Cheah and Ledward, 1996)** and therefore it

is expected that the storage under these HS conditions do not affect negatively the colour of minced pork meat, especially the a* value.

Table 15 – Minced pork meat colour parameters (*L**, *a** and *b**) after 12 and 24 hours of storage at the different storage conditions. Different upper case letters between conditions (A-B) and different lower case letters (a-b) between storage times indicate significant differences ($P<0.05$).

	Condition	Time (h)		
		0	12	24
L*	4 °C	48.65±1.17 aA	48.74±0.69 aA	50.51±1.68 aA
	RT	48.65±1.17 aA	51.46±0.71 bB	51.33±0.83 bA
	HS	48.65±1.17 aA	51.24±0.89 aB	50.80±1.27 aA
a*	4 °C	5.26±0.31 aA	4.93±0.39 aA	5.20±0.50 aA
	RT	5.26±0.31 bA	4.17±0.29 aA	4.54±0.48 abA
	HS	5.26±0.31 aA	4.61±0.24 aA	4.94±0.53 aA
b*	4 °C	10.58±0.27 aA	11.00±0.22 aA	10.77±0.30 aA
	RT	10.58±0.27 aA	11.24±0.22 bA	11.73±0.08 bB
	HS	10.58±0.27 aA	11.20±0.25 abA	11.31±0.22 bAB

These results therefore show that HS is able to maintain minced pork meat colour, similarly to refrigeration, for 24 hours of storage. Nevertheless, large periods of storage are required in order conclude if this trend is maintained.

3. Post HS

3.1 Microbiological Analysis

The post HS monitoring is of extreme importance in order to evaluate potential behaviours in hyperbaric stored foods, such as a longer stability of the product, when compare to refrigerate and RT stored samples at 0.1 MPa. Therefore, in this study an evaluation of the microbiological, pH and oxidative stability of the samples previously stored under the different conditions was carried out at 0.1 MPa and 4 ºC. One of the most interesting parameters is the evolution of microbial counts after HS, represented in **Figure 11**.

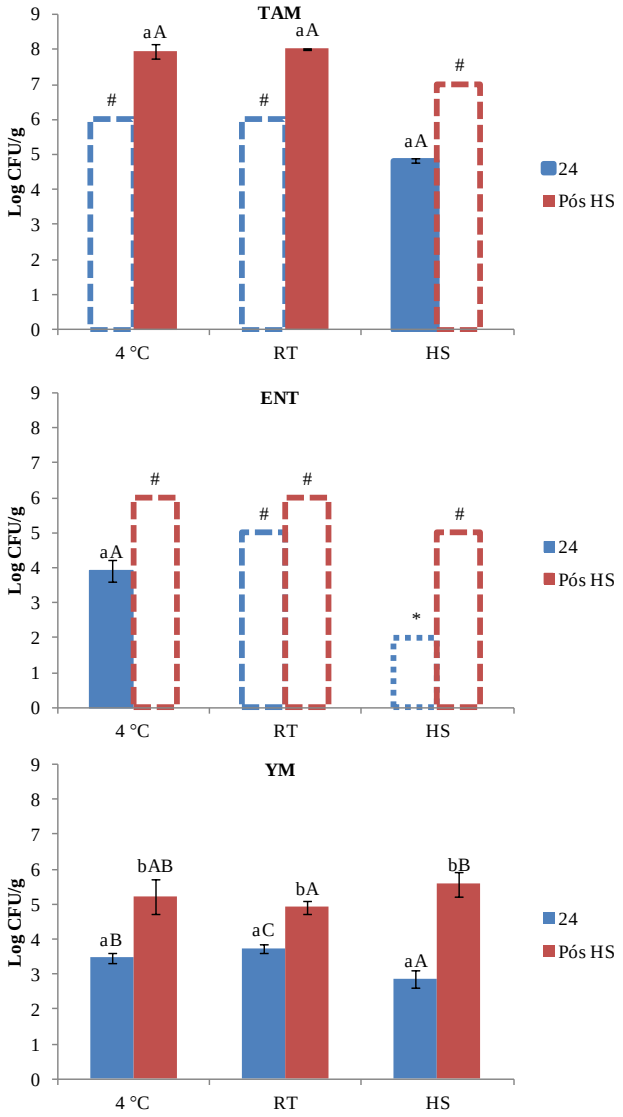

Figure 11 - TAM, ENT and YM counts expressed in Log CFU/g (mean ± SD) of minced pork meat previously stored under refrigeration, RT and HS conditions both before and after storage for 4 days at 4 ºC and 0.1 MPa. Traced bars with # represent samples with at least the represented microbial counts. Lower case letters (a-b) and upper case letters (A-C) represent significant differences (P<0.05) between storage times for the same samples and significant differences between samples at the same storage time, respectively.

As represented in **Figure 11**, after four days of storage at refrigeration temperatures and 0.1 MPa the microbial counts increased in all samples, regardless of the previous storage conditions. In the case of refrigerated and RT stored samples, the TAM, ENT and YM counts increased to values around 8, higher than 6 and around 5 Log CFU/g of sample, respectively. Similarly the samples previously stored under HS conditions suffered an increase in the microbial counts to values above 7, 5 and around 5 Log CFU/g for TAM, ENT and YM, respectively. This results point towards the loss of the benefits resulting from HS of minced pork meat along the post HS of the product, i.e., the low microbial

counts observed after 24 hours of storage increase to values close to the refrigerated and RT stored samples. Contrarily to what was observed in this study, the post HS stability of strawberry and watermelon juice was improved when compared to the samples only stored at refrigeration temperatures **(Segovia-Bravo et al., 2012, Fidalgo et al., 2014)**. **Segovia-Bravo et al. (2012)** observed that the strawberry juice stored under HS conditions maintained the microbial counts unaltered, below 1.0 Log CFU/g after 15 days of storage at 4 ºC and 0.1 MPa whereas the juice only stored under refrigeration conditions had an increase in the YM of about 1-2 Log CFU/g. Similarly, **Fidalgo et al. (2014)** observed that watermelon juice stored at 0.1 MPa and 4 ºC after HS only had an increment in the YM counts from values below 1.0 to around 3.57 Log CFU/g while TAM and ENT remained unaltered. Contrarily, all the microorganisms counts increased to values above 6 Log CFU/g in the juice only stored under refrigeration temperatures.

In summary, these results point that the post HS stability of a food commodity might be influenced by the food physicochemical characteristics and microbial counts after HS. For instance the low microbial counts after HS of strawberry juice, below 1.0 Log CFU/g, and the natural acidity of the juice that acts as a hurdle preventing microbial growth allows to achieve a longer stability. On the other hand, food matrixes such as the used in this study, minced pork meat, that showed high microbial counts even after HS and that do not pose an additional hurdle due to its high pH and a_w have a reduced stability even after HS. Therefore a careful approach regarding food post HS stability is advised and further studies are required in order to clarify this possible advantage of this new preservation methodology.

3.2 Physicochemical parameters

In order to evaluate the potential benefits from HS when the samples are replaced at 0.1 MPa and 4 ºC, the pH and TBARS values were evaluated. As represented on **Table 16** the pH of RT and HS samples declined (P<0.05) after 4 days of storage at refrigeration temperatures and atmospheric pressure whereas the pH of the refrigerated samples maintained stable.

Relatively to the oxidative stability of the different samples, it was observed that those stored at 0.1 MPa and RT yielded the highest TBARS value, 0.319 ± 0.029 µg/g of product, followed by hyperbaric stored samples (0.206 ± 0.007 µg/g) and refrigerated

samples (0.112 ± 0.006 µg/g). These results therefore show that HS might have a negative impact on the oxidative stability of the product, when compared to refrigerated samples, by favouring chemical reactions or microbiological activity that promote lipid oxidation. For instance, HP may induce cell disruption favouring the contact between prooxidants and PUFA causing the increase of the TBARS values, mainly when the products are exposed to atmospheric pressure. Nevertheless, it is worth to mention that this value was lower than that observed for RT stored samples, showing the ability of this new preservation methodology to increase the stability of food commodities, when compared to those stored at the same temperature and 0.1 MPa.

Table 16 - pH and TBARS values expressed in µg MDA/g (mean ± SD) of minced pork meat previously stored under refrigeration, RT and HS conditions both before and after storage for 4 days at 4 ºC and 0.1 MPa. Lower case letters (a-b) and upper case letters (A-C) represent significant differences (P<0.05) between storage times for the same samples and significant differences between samples at the same storage time, respectively.

Condition	pH		TBARS	
	24 hours	Post HS	24 hours	Post HS
4 ºC	6.06±0.04 aA	6.05±0.06 aB	0.014±0.004 aA	0.112±0.006 bA
RT	6.01±0.06 bA	5.76±0.12 aA	0.178±0.022 aB	0.320±0.029 bC
HS	6.35±0.06 bB	6.22±0.04 aC	0.035±0.002 aA	0.206±0.007 bB

In general the pH decay and increase in TBARS values for the samples stored under the different conditions (refrigeration, RT at 0.1 MPa and RT at 100 MPa) might be related to the advanced spoilage observed in the samples. An informal sensorial analysis showed that all the samples presented a putrefactive odour that was more intense in the samples previously stored at RT and 0.1 MPa followed by those stored at RT and 100 MPa and those stored at 0.1 MPa and 4 ºC.

In summary, these results point that the spoilage inhibition achieved by storing the samples under HS conditions is lost along the post storage period at 0.1 MPa and 4 ºC and that this novel preservation methodology may trigger undesired changes in food commodities when placed at atmospheric pressure. Nevertheless more studies are required in order to confirm this hypothesis since these samples were analyzed in an advanced deterioration state.

Conclusions

Nowadays, meat products are preserved under refrigeration conditions to which are associated high energetic costs since temperature control is required. As such, this study was focused on the evaluation of hyperbaric storage (HS) at room temperature (RT) as a feasible alternative to refrigeration in sliced cooked ham preservation at lab scale and minced pork meat at a larger scale.

Microbial growth inhibition, in the case of sliced cooked ham, was achieved by using pressures around 50 MPa, at 30 ºC, yielding microbial loads similar to refrigeration (around 3.6 Log CFU/g of sample for TAM) while ENT an YM remained below the detection limit for all the storage conditions. The increase of storage pressure to 100 and 150 MPa leaded to microbial loads reduction when compared to the initial samples. The physicochemical parameters (pH, water holding capacity (WHC), lipid oxidation and colour) of sliced cooked ham stored under HS conditions were also assessed and it was verified that after 4 and 8 hours of storage no significant differences were observed when compared to the initial samples. Similarly, the HS of minced pork meat at 100 MPa and RT for 24 hours led to the decrease of the microbial counts whereas under refrigeration and RT at 0.1 MPa increased. In general the physicochemical parameters were preserved under HS conditions, similarly to refrigeration while in the samples stored at RT and 0.1 MPa significant changes were observed. *Per se*, this is remarkable since it shows that meat products can be preserved at RT by using pressure as a storage condition, maintaining or even reducing the microbial counts (this may present an additional advantage by increasing the products shelf life). The most noteworthy fact is that this is possibly achieved by requiring lower energetic costs when compared to refrigeration, since energy is only required during the compression/decompression processes and no temperature control is required.

Despite of these advantages, further studies are needed regarding the HS affects on other food matrixes such as on their texture, especially when longer storage periods are applied. In order to really understand how this novel preservation methodology can be applied as an alternative to refrigeration, studies of pathogenic microbial growth under these conditions are also required since this is the most important element in relation to food preservation. Studies regarding the metabolism of microorganisms might also be of

great interest in order to determine their capacity to produce metabolites that may have detrimental effects on sensorial quality but also on food safety such is the case of biogenic amines, important in the case of meat products.

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Appendices

A. Laboratory scale equipment experiments

Table 17 - TAM counts (expressed in Log CFU/g) of sliced cooked ham both before and after storage for 4 and 8 hours at the different pressures and temperature conditions.

Temperature	Pressure	Time (h)		
(ºC)	(MPa)	0	4	8
4 ºC	0.1	3.66±0.10	3.66±0.04	3.53±0.14
25 ºC	0.1	3.66±0.10	5.54±0.07	5.68±0.29
	100	3.66±0.10	2.64±0.23	2.34±0.09
30 ºC	0.1	3.66±0.10	5.36±0.06	7.37±0.01
	25	3.66±0.10	5.03±0.40	-
	50	3.66±0.10	4.05±0.06	-
	100	3.66±0.10	2.63±0.19	2.40±0.25
	150	3.66±0.10	2.70±0.26	-
37 ºC	0.1	3.66±0.10	4.62±0.56	-
	100	3.66±0.10	<2.0	-

Table 18 - LAB counts (expressed in Log CFU/g) of sliced cooked ham both before and after storage for 4 and 8 hours at the different pressures and temperature conditions.

Temperature	Pressure	Time (h)		
(ºC)	(MPa)	0	4	8
4 ºC	0.1	3.66±0.10	3.66±0.03	3.55±0.03
25 ºC	0.1	3.66±0.10	5.47±0.03	5.78±0.21
	100	3.66±0.10	2.64±0.25	2.22±0.06
30 ºC	0.1	3.66±0.10	5.34±0.08	7.37±0.11
	25	3.66±0.10	4.99±0.33	-
	50	3.66±0.10	3.94±0.09	-
	100	3.66±0.10	2.47±0.10	2.41±0.22
	150	3.66±0.10	2.58±0.26	-
37 ºC	0.1	3.66±0.10	4.65±0.59	-
	100	3.66±0.10	<2.0	-

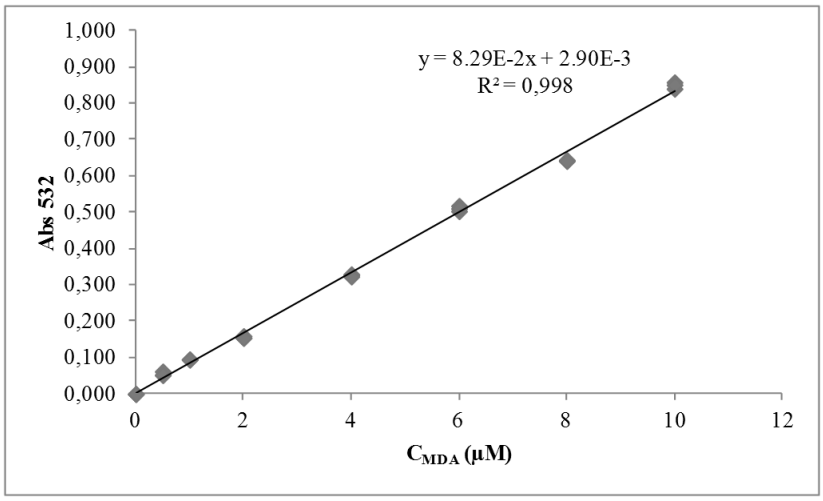

Figure 12- Standard curve of malondialdehyde (MDA) content by TBARS method.

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B. Industrial scale equipment experiments

Table 19 - TAM, ENT and YM counts expressed in Log CFU/g (mean ± SD) of minced pork meat both before and after storage for 12 and 24 hours at the different storage conditions.

	Condition	Time (h)		
		0	12	24
TAM	4 °C	5.24±0.07	6.02±0.05	>6.00
	RT	5.24±0.07	>6.00	>6.00
	HS	5.24±0.07	5.06±0.05	4.83±0.06
ENT	4 °C	2.65±0.14	3.63±0.14	3.92±0.31
	RT	2.65±0.14	4.52±0.18	>5.00
	HS	2.65±0.14	2.62±0.13	<2.00
YM	4 °C	3.90±0.30	3.92±0.12	3.46±0.15
	RT	3.90±0.30	3.82±0.26	3.75±0.12
	HS	3.90±0.30	2.96±0.14	2.85±0.25

Table 20 - TAM, ENT and YM counts expressed in Log CFU/g (mean ± SD) of minced pork meat previously stored under refrigeration, RT and HS conditions both before and after storage for 4 days at 4 °C and 0.1 MPa.

	Condition	Time (h)	
		24	Post HS
TAM	4 °C	>6.00	7.95±0.20
	RT	>6.00	8.02±0.03
	HS	4.83±0.06	>7.00
ENT	4 °C	3.92±0.31	>6.00
	RT	>5.00	>6.00
	HS	<2.00	>5.00
YM	4 °C	3.46±0.15	5.22±0.50
	RT	3.75±0.12	4.92±0.18
	HS	2.85±0.25	5.59±0.35