Universidade de Aveiro Departamento de Química
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Mauro Diogo Batista dos Santos

Avaliação do armazenamento hiperbárico como novo método de preservação para carne crua comparativamente à refrigeração

Evaluation of hyperbaric storage as a novel food preservation methodology for raw meat versus refrigeration

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica da Doutora Ivonne Delgadillo, Professora associada com agregação do Departamento de Química da Universidade de Aveiro e Doutor Jorge A. Saraiva, Investigador auxiliar do Departamento de Química da Universidade de Aveiro

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o júri

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palavras-chave

Armazenamento hiperbárico, refrigeração, carne de suíno e bovino fresca, avaliação microbiológica e físico-química, atividade enzimática, oxidação lipídica, ácidos gordos, compostos voláteis, textura

resumo

Ultimamente, tem-se observado um número crescente de estudos relacionados com o armazenamento hiperbárico (AH) devido à possibilidade deste se tornar uma alternativa ou um complemento à refrigeração (RF) pelos promissores resultados na conservação de alimentos.
Este trabalho teve como objetivo o estudo do AH até 60 dias na conservação de carne fresca de suíno e bovino (picada e em pedaços), à temperatura ambiente (TA) e a uma temperatura inferior a esta, de forma a ser usado como uma alternativa ou um completo, respetivamente, à RF, sendo posteriormente os resultados comparados com os obtidos na RF convencional.
Ao longo do trabalho foi possível observar a viabilidade do AH no controlo do crescimento de microrganismos à TA variável a partir de 75 MPa e a 10 °C a partir de 50 MPa, ambos até 60 dias de armazenamento. Foi também verificado que, no geral, não só o crescimento de microrganismos foi inibido, como também estes foram inativados ao longo do tempo. Em relação à *Escherichia coli* e *Listeria innocua* inoculadas, o AH foi capaz não só de inibir o seu crescimento como também de as inativar ao longo do armazenamento.
Vários parâmetros físico-químicos foram estudados, não sendo afetados na generalidade pelo AH ou sendo afetados de maneira similar como na RF. Por exemplo, o pH foi mais bem preservado em AH do que em RF, assim como as diferenças de cor foram similares entre AH e RF. Foi também observado que para níveis de pressão mais elevados, principalmente 100 MPa à TA variável, se verificou uma tendência para valores mais baixos e mais altos de *moisture* e *drip loss*, respetivamente, ao longo do armazenamento.
Os resultados das substâncias reativas ao ácido tiobarbitúrico (TBARS) demonstraram que o AH pode potenciar o aumento dos produtos secundários de oxidação lipídica, no entanto quando utilizado a baixas temperaturas, neste caso 60 MPa/10 °C, os resultados mostraram ser semelhantes aos obtidos em RF. Os perfis de ácidos gordos e compostos orgânicos voláteis foram também estudados sendo observado que apesar de 75 MPa/25 °C preservar melhor esses perfis que a RF, 60 MPa/10 °C demonstrou ser a melhor condição de conservação uma vez que os valores iniciais foram mais bem mantidos.
Quando as amostras foram analisadas relativamente à atividade enzimática, no geral, a carne de suíno em pedaços revelou uma diminuição da atividade enzimática como a RF, enquanto que a carne de bovino picada apresentou maiores atividade enzimáticas em AH do que em RF, não sendo observadas diferenças entre as duas condições de AH.
Apesar de se verificarem diferenças mínimas na textura da carne cozinha após conservação por AH e RF para o mesmo tempo de conservação, 60 MPa/10 °C demonstrou ser igual ou melhor na conservação das características iniciais da amostra. As imagens de microscopia eletrónica de varrimento revelaram que 75 MPa/25 °C pode levar a uma aparência mais suave das fibras musculares e uma organização menos individualizada.

keywords

Hyperbaric storage, refrigeration, raw pork and bovine meat, microbial and physicochemical evaluation, enzymatic activity, lipid oxidation, fatty acids, volatile compounds, texture

abstract

Lately, an increasing number of hyperbaric storage (HS) studies has been observed, since HS could become an alternative or a complement to refrigeration (RF) due to the promising results for food preservation.
This work aimed to study the HS methodology up to 60 days, for raw fresh meat preservation, pork and bovine (minced and in pieces), at a room like temperature and at a temperature below it in order to be used as an alternative to RF or a complement to it, respectively, being for that the results compared to the traditional RF.
Throughout the work it was possible to observe the HS feasibility for microbial growth control at RT from 75 MPa and at 10 °C from 50 MPa, both for 60 days of storage. Moreover, it was verified that generally not only microbial growth was inhibited but also microorganisms could be inactivated over time.
Regarding inoculated *Escherichia coli* and *Listeria innocua*, HS was also capable to inhibit its growth, and additionally led to its inactivation over storage.
Several physicochemical parameters were studied, being generally not affected by HS or affected in a similar way as samples stored at RF. For instance, pH was better maintained at HS conditions than in RF, as well as colour differences were similar between HS and RF. It was also observed for the higher pressure levels used, mainly 100 MPa at variable RT a tendency of lower and higher moisture and drip loss values, respectively, over storage.
The thiobarbituric acid reactive substances (TBARS) results demonstrated that HS could potentiate the increase of secondary lipid oxidation products, however when HS was coupled to low temperatures, in this case 60 MPa/10 °C, the results obtained were very similar to the ones obtained in RF.
The fatty acid and volatile organic compounds profiles were also studied being observed that although 75 MPa/25 °C preserved better the initial profiles than RF, 60 MPa/10 °C revealed to be the best storage condition since the initial values could be better maintained than in RF. Moreover, when samples were analysed regarding enzymes activity, generally pork meat in pieces revealed a decrease of enzymes activity alike RF over storage, while minced bovine meat presented higher activity values for HS conditions than for RF, not being observed differences between both HS conditions.
Although minor differences were observed on texture of cooked meat preserved by HS and RF for the same storage period, 60 MPa/10 °C demonstrated an equal to better meat preservation characteristics compared to the initial sample. The scanning electron microscopy images revealed that 75 MPa/25 °C could lead to smother muscular fibres and a less individualized organization.

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List of abbreviations

AP	Atmospheric Pressure
AU	Absorbance Units
a_w	Water Activity
BM	Bovine Minced
BP	Bovine in Pieces
CFU	Colony Forming Unit
COL	Coliforms
ENT	*Enterobacteriaceae*
HPP	High Pressure Processing
HS	Hyperbaric Storage
LAB	Lactic Acid Bacteria
MDA	Malondialdehyde
MP	Myofibrillar Protein
MUFAs	Monounsaturated Fatty Acids
PM	Pork Minced
PP	Pork in Pieces
PUFAs	Polyunsaturated Fatty Acids
PV	Peroxides Value
RF	Refrigeration
RT	Room Temperature
SEM	Scanning Electron Microscopy
SFAs	Saturated Fatty Acids
SP	Sarcoplasmic Protein
TAM	Total Aerobic Mesophiles
TAP	Total Aerobic Psychrophiles
TBARS	Thiobarbituric Acid Reactive Substances
VOCs	Volatile Organic Compounds
WHC	Water Holding Capacity
YM	Yeasts and Moulds

List of publications

Research papers published and related to the work performed for the PhD thesis

1. **Mauro D. Santos**, Liliana G. Fidalgo, Carlos A. Pinto, Ricardo V. Duarte, Álvaro T. Lemos, Ivonne Delgadillo, and Jorge A. Saraiva (2019). Hyperbaric storage at room like temperatures as a possible alternative to refrigeration: evolution and recent advances. *Critical Reviews in Food Science and Nutrition* (still under revision after major revision).
2. **Mauro D. Santos**, Ivonne Delgadillo, Jorge A. Saraiva (2019). Extended preservation of raw beef and pork raw meat by hyperbaric storage at room temperature. *International Journal of Food science and technology* (accepted).
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1. **Mauro D. Santos**, Ivonne Delgadillo, Jorge A. Saraiva (2018). Hyperbaric storage of fresh raw meat as an alternative/complement to refrigeration. *14º Encontro de Química dos Alimentos*, November 6-9, Viana do Castelo, CO02.

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1. Carlos Pinto, Silvia A. Moreira, Liliana G. Fidalgo, **Mauro D. Santos**, Rui P. Queirós, Ivonne Delgadillo, Jorge A. Saraiva (2015). The first study comparing the shelf-life of a highly perishable food preserved by hyperbaric storage at room temperature and refrigeration, International Nonthermal Processing Workshop, November 12-13, Greece.

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3. Ana M. Salgueiro, Dmitry V. Evtyugin, **Mauro D. Santos**, Jorge Saraiva, Filipe Almeida (2016). Ultra-high pressure modified cellulosic fibres with antimicrobial properties. EWLP - European Workshop on Lignocellulosics and Pulp, June 28 - July 1, France.

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Chapter 1 – Hyperbaric Storage, a literature review

1.1. Hyperbaric Storage – a new food preservation storage methodology

This chapter is an updated version of the review paper written during the PhD thesis and under revision as "Mauro D. Santos, Liliana G. Fidalgo, Carlos A. Pinto, Ricardo V. Duarte, Álvaro T. Lemos, Ivonne Delgadillo, and Jorge A. Saraiva (2019). Hyperbaric storage at room like temperatures as a possible alternative to refrigeration: evolution and recent advances, in *Critical Reviews in Food Science and Nutrition*" (still under revision after major revision).

Nowadays, High Pressure Processing (HPP) is being widely applied in different food industries as a non-thermal pasteurization procedure (450-600 MPa, over 1-10 min, at ≈8-14 °C), allowing to obtain pathogenic-free (vegetative forms) food products with minimal impact on their nutritional and sensorial features, contrarily to thermal treatments that can induce changes on foods characteristics, including non-enzymatic browning, protein denaturation, loss of vitamins, among others [1]. Moreover, HPP is also applied, for instance, in the texturization and removal of meat from shellfish/crustaceans, and for bacterial spores inactivation when applied at high temperatures (pressure-assisted thermal sterilization, PATS) [2, 3]. Enzymes inactivation [4] and macromolecules modification [5, 6] have also been accomplished through high pressure technology.

Recently and based on high pressure technology, a new food preservation technique known as Hyperbaric Storage (HS) has emerged. In 1968, the "(un)fortunately" accident with the research submarine Alvin was the starting point, since when it was recovered after being sunk over 10 months at 1540 m (≈15 MPa) and ≈4 °C, some foods were found in consumable conditions (including apples, sandwiches, and bouillon) [7]. This event opened the possibility to preserve food products under pressure.

Since then, several studies have been performed, such as the evaluation of organic matter degradation at ≈50 MPa/≈3 °C by Jannasch, Eimhjellen, Wirsen and Farmanfarmaian [7], or the shelf-life extension due to microbial growth inhibition and enzymatic activity constraining on foods stored under pressure (≥20 MPa) at temperatures between -3 and 0 °C

[8]. However, it must be noted that these two experiments required temperature control, what could lead to substantial energetic costs, similar to the conventional refrigeration (RF).

Although several HS experiments had been carried out at refrigerated temperatures, recently, some HS studies have been performed at room temperature (RT), or above it, making this methodology environmentally friendlier than the conventional RF process, with lower energy consumption, since it is only required at the compression and decompression phases, and no additional temperature control is needed along storage [9]. Thus, according to the last published studies, HS can be defined as a food preservation methodology that consists on food storage under pressure, mainly between 25 and 220 MPa, at RT or above it, as an alternative/improvement to RF [10-12]. Hyperbaric storage has become a novel conceptual food preservation methodology by microbial growth inhibition, where an additional microbial inactivation can also be achieved after storage at higher pressure levels, remaining the physicochemical parameters, in general, unchanged over that period [13-15].

In fact, the promising results of HS as a food preservation methodology relies on the effect of pressure on biological structures, enzymes and other compounds. Hydrostatic pressure is reported to trigger several effects on vegetative microorganisms, such as the abolishment of biological and structural functions (e.g. motility, cell division, DNA replication/growth, and viability, when applied a pressure level from 10, 20-50, 50, and 200 MPa, respectively, for *Escherichia coli*), as reported by Oger and Jebbar [16]. Moreover, the effect of low pressures (20-200 MPa) is reported for several *Bacillus* spp. and *Clostridium* spp endospores, mainly in buffer solutions and culture media, usually above RT and up to 24h. For instance, pressures up to 200 MPa seems to trigger a nutrient-like physiological germination, in which endospores release dipicolinic acid from the core that activates the cortex lytic enzymes, with consequent cortex degradation [17, 18]. Thus, pressure acts as an hurdle that does not allow further outgrowth of the endospores and the development of a vegetative microorganism [19]. Two patents regarding HS concept were already published: (1) "Method of pressure preservation of food products" US5593714, 1997 [20] and (2) "Hydraulic pressure sterilization and preservation of foodstuff and feedstuff" US6033701, 2000 [21], where is claimed that food products could be preserved at RT from a few hours to more than a month using a pressure range up to 250 MPa. However, these patents expired due to failure to pay maintenance fee.

Although food storage under pressure have been studied at pressures ranging from 0.1 to 220 MPa, this review paper will focus on HS studies performed at a pressure level above 25 MPa, mainly at RT and above it, since recent studies have demonstrated its feasibility as an alternative to RF for different food products (e.g., meat, fish, dairy, ready-to-eat meals, fruit juices) confirmed by several microbial, physicochemical, biochemical and sensorial analyses, as it was already pointed a few years ago by Segovia-Bravo, Guignon, Bermejo-Prada, Sanz and Otero [10,] Fernandes, Moreira, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [22], among others.

The results obtained in the several published studies allowed to conclude that HS presents a great potential to substitute RF since an equal to better microbial quality of the food products can be attained, being the physicochemical parameters variations generally minor than the ones observed in RF. Moreover, in this review paper it was also reported the HS feasibility for possible shelf-life extensions of several food products, from fruit juices until ready-to-eat foods, including fresh fish, meat and dairy products by a better microbial inhibition or inactivation.

Despite the economic evaluation of HS revealed to be less competitive than the conventional RF processes, until now, it can be surpassed by the reduced energetic costs, reduced carbon foot-print, as well by the arose of new equipment manufacturers that could lower the price of industrial units specifically designed for HS (since the current industrial equipment are designed to support pressures up to 600 MPa, 3 to 6-fold higher than those employed on HS). Thus, it is expected for the next years a wide research regarding HS as a new food preservation, its impacts on specific microorganisms (e.g., spores and pathogenic), foods constituents as well as the implementation on food industries and/or consumers' homes.

1.2. Food storage under pressure up to ≈1.0 MPa

It must be noted that although HS experiments can be, in general, described as studies applying a pressure level considerable higher than 1.0 MPa (usually between 25 and 220 MPa), using for that a pressurization fluid (for instance, water), there are another kinds of experiments regarding food storage under pressure using milder pressure levels of 0.1-1.0 MPa, reached by the flow of compressed gases. This latter case has been mostly used for fruit and vegetables preservation, such as mushrooms, in which ≈3.6 MPa at 20 °C with an

atmosphere composed by O_2, N_2 and CO_2 allowed to obtain a lower moisture loss, a lower browning extent and no larval flies development up to 16.4 days when compared to control samples [23].

Moreover, Baba, Ito, Ikeda and Manago [24] verified that 0.5 MPa for 10 days at 4 °C prevented discoloration of mume fruit and reduced its chilling injuries such as skin pitting and browning, as well 0.025 MPa was effective in inhibiting chilling injuries on sweet basil leaves and prevented yellowing and fungal growth on rocket-salad leaves over two months of storage.

Sweet cherries and table grapes were also preserved by this kind of technology, in which a decrease of brown/total rots and grey/blue moulds in sweet cherries was observed at 0.15 MPa and 20 °C up to 24 hours [25].

1.3. Hyperbaric storage performed at low temperatures

It is known that pressure decreases the water-freezing point, making possible to store foods at sub-zero temperatures without the textural changes associated to the freezing/thawing process [26]. Given this fact, food storage under pressure at sub-zero temperatures was carried out successfully [27], as summarized in Table 1.1.

Table 1.1. Hyperbaric storage studies at sub-zero temperatures.

Product	Conditions	Storage period (up to)	Outcomes	Reference
Cod fish fillets	22.8 MPa at –3 ºC	36 days	Preserved at least up to 36 days by microbial inhibition and enzymatic activity constraining (peroxidase and trypsin). Similar to better organoleptic quality when compared to frozen samples at 0.1 MPa	Charm, Longmaid and Carver [8]
Beef	200 MPa at –20 ºC	#	Microbial load reduction by inactivation of several microorganisms (e.g., COL, ENT, LAB, yeasts)	Deuchi and Hayashi [28]
Strawberry and tomatoes	50-200 MPa at –5 to –20 ºC	#	Well preserved for a longer time period, presenting fresh flavor and typical color. Catalase, β-amylase, cathepsin and lactate dehydrogenase activities were reduced by pressure	Deuchi and Hayashi [27]

Chicken and carp	170 MPa at –8 and –15 ºC	50 days	Meat texture preservation over 50 days without significant protein denaturation. Inhibitory effect by pressure on the enzymatic degradation of nucleic acid-related substances	Ooide, Kameyama, Iwata, Uchio, Karino and Kanyama [29]

#Data not available

In 1977, a study performed by Charm, Longmaid and Carver [8] proved that fish and meat products (cod fish, pollock, beef and chicken) could be preserved under pressure (≥20 MPa) at temperatures between -3 and 0 °C by microbial growth inhibition and enzymatic activity constraining (peroxidase and trypsin), showing that cod fillets stored at 22.8 MPa/-3 °C during 36 days appeared to have similar, or even better quality (organoleptically tested by an expert panel) than those stored at atmospheric pressure (AP) and -20 and -3 °C, respectively.

Later on, Deuchi and Hayashi [27] also demonstrated that catalase, β-amylase, cathepsin and lactate dehydrogenase activities were only reduced at 200 MPa/-20 °C, contrarily to frozen storage at AP. These authors also observed that strawberry and tomato colour, as well as its fresh flavour were maintained over weeks under pressures of 50-200 MPa and sub-zero temperatures (-5 to -20 °C) (Deuchi and Hayashi [27], cited by Fernandes, Moreira, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [22]). Additionally, the feasibility of this methodology for microbial load reduction was also revealed for coliforms (COL), *Enterobacteriaceae* (ENT), psychrotrophs, *enterococci*, lactic acid bacteria (LAB), and yeasts on ground beef preserved at 200 MPa/-20 °C [28]. For chicken and carp muscles, the meat texture was preserved without significant protein denaturation over 50 days (170 MPa and -8 or -15 °C), and an inhibitory effect by pressure on the enzymatic degradation of nucleic acid-related substances was observed [29]. As it was previously mentioned, the "(un)fortunate" accident, that occurred with the sinking of the research submarine Alvin in 1968, opened the possibility to store food products under pressure using low temperatures of ≈4 °C (water temperature in that place at a depth of 1540m). As far the authors are aware, all studies regarding the use of HS at refrigerated temperatures are cited in Table 1.2**.**

Table 1.2. Hyperbaric storage studies performed at refrigerated temperatures.

Product	Conditions	Storage period (up to)	Outcomes	Reference
Apples, bouillon and sandwiches	15 MPa at 3-4 ºC	10 months	Well preserved food was recovered from a sunk research submarine after 10 months at deep sea	Jannasch, Eimhjellen, Wirsen and Farmanfarmaian [7]
Rice, wheat and soy beans	3.5 MPa at 1 ºC	1 year	Lower changes in seed moisture, fatty acids and reducing sugars for 1 year under pressure than at 0.1 MPa	Mitsuda, Kawai and Yamamoto [30]
Dressed cod	24.12 MPa at 1 ºC	21 days	Preserved during 21 days and classified as having 8.2 days, while samples stored at 0.1 MPa were unacceptable	Charm, Longmaid and Carver [8]
Pollock		12 days	Preserved over 12 days with higher quality (evaluated as having 6.7 days) than those at 0.1 MPa	
Hake loins	50 MPa at 4 ºC	7 days	Microbial proliferation was hindered and the total volatile basic nitrogen remained stable, contrarily to RF. Increased shear resistance and higher whiteness for samples kept at HS. Moderated differences between samples kept at HS and RF were detected by the expert panel	Otero, Pérez-Mateos and López-Caballero [31]
Mackerel	50 MPa at 5 °C	12 days	No microbial growth was observed at HS conditions. H_2S-producing organisms and sulphite-reducing bacteria were reduced under the detection limits. It was not detected lipid oxidation degradation and most of fish-quality indicators were better preserved at HS conditions.	Otero, Pérez-Mateos, Holgado, Márquez-Ruiz and López-Caballero [32]
Atlantic salmon fillets	40-60 MPa at 5-15 °C	50 days	Microbial inactivation was observed at 60 MPa/10 ºC after 50 days of HS. Total volatile base-nitrogen limit was exceeded after 30 days at the latter condition (contrarily to the 6 days of the traditional RF storage). An increase of formaldehyde and dimethylamine-nitrogen content was detected after 6 days. HS led to an increase of the secondary lipid oxidation products although to a lower extent compared to RF.	Fidalgo, Castro, Trigo, Aubourg, Delgadillo and Saraiva [33]
Atlantic salmon fillets	60 MPa at 10 °C	50 days	When preserved under pressure, an increase and a decrease of acid phosphatase and cathepsin B were observed, respectively. Calpains activity presented a decrease just after 3 days at HS conditions, as well the myofibrillar fragmentation index revealed a decrease at short storage periods.	Fidalgo, Delgadillo and Saraiva [34]

Jannasch, Eimhjellen, Wirsen and Farmanfarmaian [7] studied the impact of pressure on organic matter degradation over storage at a depth of 5300 m (≈53 MPa) and ≈3 °C. In this study, the decomposition rate of several carbon sources (acetate, mannitol and amino acids) marked radioactively with $_{14}C$ were 8–700 times slower under pressure than at the same temperature at AP. On the other hand, the same authors verified that the incubation of several carbon sources (e.g., starch, galactose, albumin) with mixed microbial populations and pure cultures at the same conditions did not allow the microbial growth, contrarily to samples at the same temperature and AP [7]. Moreover, when some food products, as rice, wheat and soybeans were kept at a depth of 30 m in a fresh water lake for one year, biochemical changes on seed moisture, fatty acids, vitamin B_{12} and reducing sugars were minor than those stored at AP [30].

Afterwards, Charm, Longmaid and Carver [8] verified that a pressure increase (up to 41.3 MPa) at a constant temperature (tested at four temperature levels, between ≈-3 and ≈23 °C) led to a decrease on enzymes activity, i.e., peroxidase activity was reduce 25-30% under 41.3 MPa/≈4 °C, when compared to AP. In the same study, trypsin activity did not present the same behaviour, since an increase and a decrease were detected when pressure increased (to 41.3 MPa) at temperatures near ≈23 °C or below ≈4 °C, respectively. Thus, leading to a general conclusion by the authors that at these tested conditions (between ≈-3 and ≈23 °C, and 0.1 and 41.3 MPa), trypsin should have a critical temperature value below which pressure reduces the reaction rate and above it, it increases the reaction rate [8]. More temperature and pressure values would have needed to be studied to increase the accuracy of the temperature/pressure value below which the reaction rate decreases and above it increases.

Charm, Longmaid and Carver [8] also studied the effect of ≈24 MPa/1 °C over 12 days in cod fish fillets, demonstrating that under pressure the microbial load nearly did not change (total bacterial count of ≈4.5 log units), contrarily to samples stored at AP. Additionally, an expert panel evaluated the characteristics of fish stored under pressure, indicating that pollock with 12 days was classified as having 6.7 days, and codfish with 21 days as having 8.2.

More recently, Otero, Pérez-Mateos and López-Caballero [31] studied the shelf-life extension of hake loins in about 7 days using HS at 50 MPa/5 °C, where it was observed a better performance when compared to the traditional RF. After 7 days under HS at low

temperature it was possible to perceive a slight reduction of total aerobic mesophiles (TAM) and ENT counts (4.51±0.34 and <1 log colony forming unit, CFU/g, respectively) when compared to the initial ones (4.76±0.43 and 1.87±0.34 log CFU/g, respectively), while samples under RF presented higher values (7.70±0.21 and 6.48±0.24 log CFU/g, respectively) at the 7th day of storage. Regarding physicochemical parameters, although without statistical differences in pH between samples preserved by HS and RF, the total volatile basic-nitrogen content of refrigerated samples increased (38.65±4.52 mg/100 g), contrarily to HS samples (9.96±1.12 mg/100 g), which showed values close to the initial one (11.08±1.02 mg/100 g). On the other hand, although water content, water holding capacity (WHC), shear resistance and whiteness of samples preserved by HS were reported to be statistically different from the initial ones, after cooking, weight losses were less than half for control samples, as well as whiteness differences disappeared, leading to a sensorial analysis where only moderate differences were found between cooked samples [31].

Similar findings were found by Otero, Pérez-Mateos, Holgado, Márquez-Ruiz and López-Caballero [32] for mackerel (*Scomber scombrus*, L.), a fish fattier than hake, where 50 MPa/5 °C did not allow the increase of the microbial growth over 12 days, and avoided lipid degradation of the samples. In this study a better preservation of the most fish-quality indicators (e.g., pH, drip loss, WHC) was also observed for HS when compared to RF [32].

Atlantic salmon was also subjected to HS preservation studies (40–60 MPa) at low temperatures (5–15 °C) [33]. In this study performed by Fidalgo, Castro, Trigo, Aubourg, Delgadillo and Saraiva [33], the microbial growth was better controlled (microbial inactivation observed at the 50th day of storage when applied 60 MPa/10 °C), being also detected that contrarily to RF, the total volatile base-nitrogen limit was surpassed only after 30 days (38.65±3.49 mg N/100 g muscle) instead of 6 days at AP/10 °C (44.24±4.59 mg N/100 g muscle). It was verified that HS allowed to obtain similar to lower contents of formaldehyde, trimethylamine-nitrogen and dimethylamine-nitrogen than on samples stored at RF up to 6 days, being observed an increase of the secondary lipid oxidation products throughout storage for HS samples (from 0.36±0.04 to 2.65±0.68 μg malondialdehyde, MDA/g muscle at the 50th day) [33].

The same research group has extended the HS impact studies on Atlantic salmon analysing it effects on enzymes activities and proteins [34]. The authors were capable to detect after 10 days at 60 MPa and 10 °C an increase of acid phosphatase value (1.5-fold), a

decrease of 48% of the residual activity of cathepsin B when compared to AP/5 ºC, and a decrease of calpains activity just after 3 days, being cathepsin D generally not affected by pressure at this temperature [34]. Furthermore, myofibrillar fragmentation index presented a decrease of 45% when Atlantic salmon was stored at 60 MPa/10 °C similarly to when preserved at AP/10 ºC (decreasing 34%) [34].

Additionally, a study performed by Lemos, Ribeiro, Fidalgo, Delgadillo and Saraiva [35] proved the HS feasibility on extending watermelon juice shelf-life (up to at least 58 days) by combining pressure with lower temperatures (up to 10 °C), pointing out that at lower pressures, temperature is a limiting factor on the microbial stability along storage. In this work, a pressure level of 50 MPa and 10 °C allowed a slower microbial growth throughout storage when compared to HS (50 MPa) at 15 and ≈25 °C, wherein TAM and TAP (total aerobic psychrophiles) grew above 6.0 log CFU/mL after 7 days of storage at HS/15 °C, and after 3 days at HS/≈25 °C [35]. The authors also carried out HS experiments at 75 and 62.5 MPa (both at 15 °C), where it was observed a slower microbial load reduction on the latter for ENT and YM and similar microbial counts were obtained in both pressure conditions on the last days of storage (21st and 58th day) for TAM and TAP [35]. Regarding physicochemical parameters, the behaviour detected for colour and pH was similar to other HS studies [15, 36-38], since pH remained stable along HS conditions (50, 62.5 and 75 MPa, and 15 °C) when compared to control samples, as well as total colour variation, ΔE were from less to equally affected by HS (50, 62.5 and 75 MPa, and 15 °C) when compared to AP storage [35]. Briefly, this work proved the HS feasibility at low temperature to control microbial and physicochemical degradation of watermelon juice, promoting the shelf-life extension up to, at least, 58 days compared with only about 3 days by RF, which represent a huge potential of HS to future replacement of RF, either on food industries or even at our houses.

1.4. Hyperbaric storage at and above room temperature (HS/RT)

From 2012, HS application at and above RT has become a new trend, and several studies have been performed since then, being pointed in all of them possible energy savings and lower carbon footprint when compared to RF [9, 22]. It should be noted that several studies described on this review [12, 13, 39, 40] were carried out using the current existing high pressure equipment, capable to reach 600 MPa (industrial scale), or even more as 900 MPa (in some laboratorial equipment), in sec to min, and for short periods of time. HS does not require so

powerful equipment since a pressure level range between 25 and 200 MPa and lower pressurization rates are capable to preserve food products with an equal to better performance than RF [9].

1.4.1. Fruit juices

Segovia-Bravo, Guignon, Bermejo-Prada, Sanz and Otero [10] tested the HS concept (25/100/220 MPa) at controlled RT (20 °C) for 15 days using strawberry juice as a case-study. In this work, it was recognised the HS feasibility at RT as a novel food preservation technique for strawberry juice, since not only a microbial growth inhibition was observed (for TAM and yeasts and moulds, YM), but also a microbial growth inactivation was detected by the reduction of the initial microbial loads (from >2 log units to levels below of 10 CFU/mL for TAM and below 100 CFU/mL for YM), being these results better than the ones obtained when RF was applied to the same sample where TAM and YM increased more than 3 log units after 15 days of storage. Some evidences also showed that HS is effective on viscosity and colour losses attenuation when compared to control samples (AP/20 °C), since a viscosity reduction between 79.2 and 63.7% was observed for HS samples being these values lower than in control sample (83.6%), as well lower ΔE values were obtained for HS (4.5±0.7 *vs.* 1.3±0.1 for control sample and HS, respectively). An additional storage at refrigerated temperature after a HS period (called "post-HS" experiment) was also evaluated, and from that, the stability of the microbial load, viscosity and colour were confirmed over those days [10].

At this point, it must be highlighted the usefulness of a post-HS study as well the existence of these studies in some published works, e.g., Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11,] Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13]. From those, it was possible to analyse the behaviour of the samples when stored at refrigerated conditions after having been preserved by HS over days to weeks, leading to several conclusions on the microorganism inhibition/inactivation and their ability to proliferate at RF conditions after HS. This could be of great importance for a HS application at a practical/industrial level since the microbial load in post-HS studies is lower than on samples stored only at RF conditions (without any HS period), due to microbial inactivation by HS, leading to higher microbial shelf-lives. All HS studies regarding fruit juices are briefly compiled in Table 1.3.

Table 1.3. Published studies on the last few years regarding hyperbaric storage of fruit juices.

Fruit juice	Conditions	Storage period (up to)	Main outcomes	Reference
Watermelon juice	100 MPa at 18-21 ºC	60 hours	Microbial growth inhibition and microorganisms inactivation at the tested conditions. Further shelf-life extension at RF conditions after a HS period	Fidalgo, Santos, Queirós, Inácio, Mota, Lopes, Gonçalves, Neto and Saraiva [15]
	25-150 MPa at 20-37 ºC	8 hours	Microbial growth inhibition at 75 MPa and inactivation at 100 and 150 MPa. No significant changes on the physicochemical parameters were observed	Santos, Queirós, Fidalgo, Inácio, Lopes, Mota, Sousa, Delgadillo and Saraiva [36]
	100 MPa at 18-21 ºC	7 days	Shelf-life extension when compared to the juice kept at 4 ºC and 0.1 MPa	Pinto, Moreira, Fidalgo, Santos, Delgadillo and Saraiva [38]
	50-75 MPa at 10-25 ºC	58 days	Microbial slowdown at 50 MPa/10 ºC and microbial inactivation at 62.5 and 75 MPa. Additionally, 62.5 MPa/25 ºC promoted a shelf-life extension up to at least 58 days. Colour parameters and pH less affected by HS than AP (both 4 and 15 ºC) storage.	Lemos, Ribeiro, Fidalgo, Delgadillo and Saraiva [35]
	50-100 MPa at 18-23 ºC	10 days	At 50 MPa was verified a microbial growth similar to RF, while at 75/100 MPa were observed microbial load reductions on endogenous and inoculated microorganisms, resulting in a shelf-life extension compared to RF. Physicochemical parameters remained stable at 75 MPa when compared to the initial raw juice.	Pinto, Moreira, Fidalgo, Santos, Vidal, Delgadillo and Saraiva [37]
Melon juice	25-150 MPa at 20-37 ºC	8 hours	Microbial growth inhibition achieved at 50/75 MPa and microorganisms inactivation at 100/150 MPa. The overall physicochemical characteristics remained unchanged	Queiros, Santos, Fidalgo, Mota, Lopes, Inacio, Delgadillo and Saraiva [41]
Strawberry juice	25-220 MPa at 20 ºC	15 days	Microorganisms inactivation at HS over 15 days, e.g., YM and TAM below the detection limits at the tested conditions	Segovia-Bravo, Guignon, Bermejo-Prada, Sanz and Otero [10]
	50/200 MPa at 20 ºC		Pressure avoided spoilage of samples stored at 20 ºC for 15 days and kept the volatile profile of the strawberry juice similar to the initial samples	Bermejo-Prada, Vega, Pérez-Mateos and Otero [42]
			Neither pectin methylesterase catalytic activity was affected by pressure on strawberry extract, nor pectin methylesterase inactivation was found up to 200 MPa	Bermejo-Prada, Segovia-Bravo, Guignon and Otero [43]

			Significant peroxidase inactivation on longer storage periods (5, 7 and 15 days) and lower percent of polymeric colour at the 5th, 7th and 10th days at 200 MPa, compared to samples stored at 0.1 MPa	Bermejo-Prada and Otero [44]
	25-200 MPa at 20 ºC		At 25 MPa the microbial growth was retarded, HS at 50 MPa yielded microbial load reductions. Higher pressures resulted in higher microbial loads reductions	Bermejo-Prada, López-Caballero and Otero [45]
	25 MPa at 20 ºC		Reduced energetic costs and lower carbon footprint than RF (4 ºC), despite the higher total storage costs for HS. No significant differences were detected by the expert panel on samples at HS and RF	Bermejo-Prada, Colmant, Otero and Guignon [9]
Carrot juice	25-100 MPa at 18-23 ºC	60 days	Inoculated *Bacillus subtilis* endospores inactivation along storage (≈6 log CFU/mL) at 50 and 100 MPa. Endospore germination triggered at 25 MPa, resulting in juice spoilage	Pinto, Santos, Fidalgo, Delgadillo and Saraiva [46]
Apple juice	25-100 MPa at 18-23 ºC	30 days	Inoculated *Alicyclobacillus acidoterrestris* endospores inactivation below the detection limit at 50 and 100 MPa after 24 and 48 hours, respectively. Slight endospore inactivation at 25 MPa along storage	Pinto, Martins, Santos, Fidalgo, Delgadillo and Saraiva [47]

Further studies performed by the same research group regarding HS/RT effect on strawberry juice quality parameters allowed to assign to pressure some colour degradation, where a lower percent of polymeric colour and a significant peroxidase inactivation (15%) in samples stored at 200 MPa were detected when compared to RF (and AP) samples [44]. The same authors highlighted that pressure did not significantly affect total phenolic and total monomeric anthocyanin contents during storage and only storage time had a significant effect on them. Nonetheless, although colour differences were instrumentally perceptible, they were very slight to be easily perceived by the naked eye since differences between ΔE* values did not exceed 1 (the threshold value frequently assumed as a basis for a colour perceptible difference) [44]. Bermejo-Prada, Vega, Pérez-Mateos and Otero [42] also studied the effect of this preservation methodology on the volatile profile of strawberry juice and found that samples preserved by HS were more similar to the juice at day 0 than to the samples stored under RF. Moreover, HS was also efficient to avoid changes in all key aroma compounds detected on strawberry juice [42].

Furthermore, pectin methylesterase activity and serum viscosity of strawberry juice stored under pressure were also evaluated [43]. The pectin methylesterase activity decreased

(≈55%) throughout 15 days under different storage conditions (AP included), not being detected a HS effect (up to 200 MPa and 20 °C for 15 days) on pectin methylesterase. Nonetheless, the methanol release was also quantified to check the enzymatic behaviour of pectin methylesterase under pressure, and its release was twice higher at 200 MPa and 20 °C than at RF and 50 MPa, what might be related with the enhanced-activity of other endogenous pectinases (others than pectin methylesterase) that could facilitate the access of pectin methylesterase to the methyl ester bonds of pectin, thus affecting the characteristics of pectin and serum viscosity. In this study of Bermejo-Prada, Segovia-Bravo, Guignon and Otero [43], contrarily to the first one published [10] regarding viscosity decay on strawberry juice (where lower values of viscosity reduction were observed for HS samples than in control samples), it was found that HS enhanced it occurring in a greater manner in the first days: 42.5%, 55.5%, and 74.5%, for AP, 50 and 200 MPa, respectively, at first day of storage.

Since strawberry juice is an acidic food product and, in consequence, already presents some intrinsic microbial barriers at RT and particularly under RF, experiments regarding watermelon juice, a highly perishable food product, with low acidity and high water activity (a_w) were carried out by a different research group. These authors, Fidalgo, Santos, Queirós, Inácio, Mota, Lopes, Gonçalves, Neto and Saraiva [15,] Santos, Queirós, Fidalgo, Inácio, Lopes, Mota, Sousa, Delgadillo and Saraiva [36] had also tried to prove for watermelon juice the HS feasibility at temperatures up to 37 °C, controlled and uncontrolled, since the previous studies performed with strawberry juice were carried out only at controlled RT.

In Fidalgo, Santos, Queirós, Inácio, Mota, Lopes, Gonçalves, Neto and Saraiva [15] and Santos, Queirós, Fidalgo, Inácio, Lopes, Mota, Sousa, Delgadillo and Saraiva [36] studies, watermelon juice was preserved by HS (25-150 MPa/20-37 °C) from 8 up to 60 hours, being observed, for TAM, ENT and YM, an equal to better growth inhibition than in RF. In summary, 75 MPa presented an inhibitory effect on microbial growth over 8 hours, and an additional inactivation effect was verified for storages at 100 and 150 MPa with the reduction of the initial microbial loads below to the detection limit (1.00 log CFU/mL) for ENT and YM, and from 4.43 ± 0.04 to 3.31 ± 0.04 and 2.99 ± 0.07 log CFU/mL, respectively, for TAM (25 °C) [36]. When performed at 30 °C/100 MPa up to 60 hours, it was verified that after the initial microbial load decrease (≈1/≈2/≈1 log units for TAM/ENT/YM, respectively, reaching ≈3 log units for TAM and <1.00 log CFU/mL for ENT and YM) over the first 8 hours, the values remained unchanged until the end of storage [15]. The physicochemical

analyses performed in these two studies (pH, titratable acidity, total soluble solids, browning degree and cloudiness) did not show a clear variation trend with pressure and no considerable differences among storage conditions were verified [15, 36].

A post-HS experiment for watermelon juice (7 days of storage at 5 °C, after a HS period of 60 hours at 100 MPa/≈21 °C) confirmed a similar behaviour previously observed for strawberry juice, where the microbial loads were found at a level of 2.27 ± 0.38 and 3.57 ± 0.86 log CFU/mL for TAM and YM, respectively, and <1.00 log CFU/mL for ENT at the 7th day, becoming these results clearly better than the ones observed on juice preserved only under RF (≥4, ≈3, ≈3 log units, for TAM, ENT and YM, respectively, in the first 2 days of RF) [15].

Similar microbial and physicochemical results were observed for a HS (25-150 MPa and 25/30/37 °C, over 8 hours) of melon juice in the publication of Queiros, Santos, Fidalgo, Mota, Lopes, Inacio, Delgadillo and Saraiva [41], in which 50/75 MPa resulted in similar or lower microbial counts while at 100/150 MPa an additional inactivation effect was observed (for TAM, ENT and YM). The authors also concluded that for the pressure levels and temperatures studied, the microbial load reduction was temperature independent, being higher as the storage pressure increased, since a linear behaviour was observed with slopes of -0.011 and -0.020 log CFU/mL/MPa (R^2 = 0.968 and 0.985) for TAM and YM, respectively.

The studies regarding watermelon juice were furtherly expanded, with a very recent publication [38] showing the possibility of shelf-life extension (compared to RF) for this highly perishable food product using HS at 100 MPa and ≈21 °C. These HS conditions allowed to preserve watermelon juice up to at least 7 days, being obtained lower microbial loads (≈2 log CFU/mL for TAM and TAP, and below the detection limit, 1.00 log CFU/mL, for YM) when compared to samples stored at AP (≈21 °C and RF) that presented values above the acceptable limit (>6.00 log CFU/mL) for the same microorganisms. The physicochemical parameters evaluated after storage (pH, total soluble solids, browning degree, cloudiness and colour) presented levels very close to those found initially for this sample [38].

Moreover, Pinto, Moreira, Fidalgo, Santos, Vidal, Delgadillo and Saraiva [37] tested three pressure levels for watermelon juice storage (50, 75, and 100 MPa) and compared to AP/RT (0.1 MPa/18–23 °C) and AP/RF (0.1 MPa/4 °C) samples inoculated with two specific microorganisms, *Listeria innocua* (ATCC 33090) and *E. coli* (ATCC 25992), being also

determined along storage the enzymatic activities of polyphenol oxidase, peroxidase, and pectin methylesterase. In this study, the overall microbial analyses performed at the natural juice microflora were in accordance to previous studies [15, 36]. Regarding inoculated samples, AP/RT and AP/RF resulted in microbial loads increments to values above 6.00 log CFU/mL for both microorganisms, whilst 50 MPa condition reduced ($p < 0.05$) *E. coli* loads in about 1.00 log CFU/mL after 3 days, and to below the detection limit at the 6th day of storage and onwards, contrarily to *L. innocua*, whose counts increased to above 6.00 log CFU/mL after 10 days of storage, similarly to juice stored at AP/RF. Nonetheless, on HS/RT at 75 MPa, *E. coli* and *L. innocua* were reduced ($p < 0.05$) to below the detection limit at the 10th day, indicating that this pressure was effective on *E. coli* and *L. innocua* inactivation over storage, as occurred for HS/RT at 100 MPa [37]. Concerning enzymatic activities, polyphenol oxidase activity during HS was less affected along storage when compared to AP/RF, showing a less pronounced activity reduction [37]. For peroxidase, HS/RT at 50 and 75 MPa led to an activity decrease ($p < 0.05$) similar to AP/RF, presenting residual activities of 40.6% (50 MPa) and 54.6% (75 MPa) at the end of storage. Lastly, the pressure level increase tended to decrease pectin methylesterase activity, since after 10 days of HS/RT at 100 MPa it was verified a residual activity of 42.8%. Pinto, Moreira, Fidalgo, Santos, Vidal, Delgadillo and Saraiva [37] also reported on the same study that all physicochemical parameters studied remained stable at 75 MPa when compared to the initial raw juice, except for browning degree that increased 1.72-fold, whilst at 100 MPa were observed higher colour variations attributed to a lycopene content decrease (25%), as well as reductions on peroxidase residual activity (16.8%) after 10 days, while both polyphenol oxidase and pectin methylesterase residual activities were similar to RF.

Very recently the germination and outgrowth control of endospores by HS/RT (18-23 °C) was accessed by Pinto, Santos, Fidalgo, Delgadillo and Saraiva [46], who reported the possibility of controlling *B. subtilis* endospore germination and outgrowth in carrot juice (pH 6.00). The results showed that, at pressures of 50 and 100 MPa there were endospore loads inactivation to below the quantification limit after 60 days of storage, in contrast with conventional RF, whose loads increased ≈1 log unit. At 25 MPa, the endospore germination and outgrowth was quickly triggered, leading to juice spoilage [46].

In another study Pinto, Martins, Santos, Fidalgo, Delgadillo and Saraiva [47] performed with an atypical case of an endospore able to germinate and outgrowth under very acidic

conditions, *Alicyclobacillus acidoterrestris* in commercial apple juice (pH 3.50), it was observed that pressure levels of 50 and 100 MPa were able to inactivate *A. acidoterrestris* ascospores to below the detection limit (10 CFU/mL) right after 48 and 24 hours of storage, respectively. At 25 MPa, an endospore load reduction was also observed but at lower rates than at 50 and 100 MPa [47].

1.4.2. Dairy products and ready-to-eat meals

The studies performed by Duarte, Moreira, Fernandes, Fidalgo, Santos, Queirós, Santos, Delgadillo and Saraiva [48] and Moreira, Fernandes, Duarte, Santos, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [49] aimed to study a highly perishable dairy food (a traditional Portuguese whey cheese, *requeijão*) and a ready-to-eat carrot soup at HS conditions, as presented in Table 1.4.

Table 1.4. Hyperbaric storage studies at and above room temperature regarding dairy and ready-to-eat meals.

Dairy product/ Ready-to-eat meal	Conditions	Storage period (up to)	Main outcomes	Reference
Requeijão (Portuguese whey cheese)	100/150 MPa at 25-37 ºC	8 hours	Microbial load reduction after HS. Pressure retained the colour, pH and a_w of the whey cheese. Lipid oxidation levels stable when compared to RF	Duarte, Moreira, Fernandes, Fidalgo, Santos, Queirós, Santos, Delgadillo and Saraiva [48]
	100 MPa at ≈17 and 21 ºC	10 days	Microbial inactivation observed for all microorganisms in the 3rd day, with physicochemical parameters unaffected under HS comparatively to RF.	Duarte, Moreira, Fernandes, Santos, Inácio, Alves, Bessa and Saraiva [14]
Carrot soup	100/150 MPa at 25 and 30 ºC	8 hours	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa after HS. General physicochemical parameter similar to RF.	Moreira, Fernandes, Duarte, Santos, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [49]
Caldo verde and *bacalhau com natas*	50-150 MPa at ≈21 ºC	12 hours	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa. No significant changes on the physicochemical parameters evaluated.	Moreira, Duarte, Fernandes, Alves, Bessa, Delgadillo and Saraiva [40]

In Duarte, Moreira, Fernandes, Fidalgo, Santos, Queirós, Santos, Delgadillo and Saraiva [48] work, *requeijão* samples were preserved over 4 and 8 hours at 100 and 150 MPa in a temperature range from 25 to 37 °C, wherein the authors observed that 4 hours at 100 MPa yielded the maintenance of the microbial counts similarly to RF and the initial load (≈3

log CFU/g at all tested temperatures for TAM, ENT, and LAB), whereas 150 MPa during 8 hours allowed a microbial load reduction to undetectable levels, except for TAM (reduction of ≈1 log unit). Generally, HS retained whey cheese colour, pH and a_w parameters, however for lipid oxidation, it was verified a similar behaviour to RF storage (for instance, lipid oxidation values slightly increased from 0.022 ± 0.004 to 0.035 ± 0.006 and 0.037 ± 0.002 mg MDA/g for 150 MPa/25 °C and RF, respectively) [48].

A second study was conducted by the same authors for longer storage periods, up to 10 days, using the same food product (*requeijão*) under 100 MPa and variable RT. As predicted, YM and LAB counts were inactivated to values below the detection limit under HS, just in the first 12 hours. For TAM and ENT, HS was capable to avoid microbial growth in the first 24 hours, while after 3 days of storage it was observed a microbial inactivation to values below the detection limit, which were maintained up to 10 days [14]. Similar results were found by Moreira, Fernandes, Duarte, Santos, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [49], in which a ready-to-eat carrot soup was kept at 100 and 150 MPa, over 4 and 8 hours and at 25 and 30 °C. In this study, the authors concluded that, globally, despite the microbial growth inhibition, the microbial inactivation effect observed was more evident when soup was stored at 150 MPa over 8 hours, being TAM less susceptible to HS, which confirmed previous studies [36, 48]. Regarding physicochemical analyses (pH, titratable acidity, reducing sugars, and colour) of samples preserved by HS a similar performance to RF was observed [49].

A possible application of this methodology using a current available industrial high pressure equipment (in this case an equipment with 55 litters capacity, Hiperbaric model 55, Burgos, Spain) was already performed by Moreira, Duarte, Fernandes, Alves, Bessa, Delgadillo and Saraiva [40], wherein two ready-to-eat meals (*bacalhau com natas* and *caldo verde* soup, traditional Portuguese ready-to-eat meals) were stored under pressure over 12 hours at 50, 100 and 150 MPa, at variable RT (≈21 °C). In this experiment (Table 1.4), the authors observed a microbial growth inhibition at 100 MPa for all microorganisms studied and an additional inactivation effect at 150 MPa resulted in values below the detection limit for ENT and YM, leading to an equal to better storage performance when compared to RF, without detectable changes on the evaluated physicochemical parameters (pH, titratable acidity, colour and fatty acid content) [40].

1.4.3. Raw and processed meat and fish products

As far as the authors are aware, there are only few studies regarding HS at RT of fresh fish and meat (Table 1.5).

Table 1.5. Hyperbaric storage studies at and above room temperature concerning raw and processed meat and fish products.

Meat/fish product	Conditions	Storage period (up to)	Main outcomes	Reference
Tilapia fillets	203 MPa at 25 ºC	12 hours	Microorganisms inactivation about 2.0 log CFU/g at HS, presenting an improved freshness when compared to samples stored at 0.1 MPa	Ko and Hsu [50]
Sea cucumber guts	60 MPa at 30 ºC	24 hours	Reduction of the psychotrophic counts of about 0.9 log CFU/g	Okazaki, Shigeta and Aoyama [51]
Sliced cooked ham	25-150 MPa at 23-37 ºC	8 hours	HS was efficient to inhibit microbial growth at pressures above 50 MPa for similar levels of RF. Microbial inactivation at 100 and 150 MPa	Fernandes, Moreira, Duarte, Santos, Queirós, Fidalgo, Santos, Delgadillo and Saraiva [39]
Raw bovine meat	50-150 MPa at ≈21 ºC	12 hours	At 50 MPa it was faced a similar microbial development inhibition when compared to the refrigerated samples, while at 100 and 150 MPa it was verified an additional microbial inactivation effect	Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13]
	100 MPa at ≈21 ºC	10 days	Shelf-life extension by HS over RF and no significant differences were found on the quality parameters of the meat	
Atlantic salmon fillets	50/60 MPa at 25-37 ºC	10 days	HS at 50 MPa promoted the increase of the microbial load. When the pressure was increased to 60 MPa a microbial growth slowdown was observed, increasing the microbial shelf-life up to at least 6 days. HS at 75 MPa caused a reduction of about 3.5 log units of initial microbial counts, leading to an increase of the microbial shelf-life of at least 25 days, compared to RF (3 days). Additionally, no changes in colour was detected during the storage period.	Fidalgo, Lemos, Delgadillo and Saraiva [12]
	75 MPa at 25 ºC	25 days		

Raw pork minced meat	100 MPa at ≈20 ºC	24 hours	HS led to lower microbial loads at the end of the storage. D value of 57.8 hours for TAM. Fatty acid composition was not affected by storage conditions probably due to short storage period.	Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11]
Atlantic Salmon	50-75 MPa at 25 and 37 ºC	50 days	A decrease of the acid phosphatase content was observed after 10 days to values similar to RF. Calpains seemed only pressure sensitives at 25 ºC since at 37 ºC the values obtained were similar to samples at AP at that temperature. The myofibrillar fragmentation index revealed an increase just after 3 days at 75 MPa.	Fidalgo, Delgadillo and Saraiva [34]

In Ko and Hsu [50] work, it is reported that raw tilapia fillets stored at ≈101 and ≈203 MPa and 25 °C over 12 hours presented a microbial growth inhibition at ≈101 MPa and an inactivation at ≈203 MPa with reductions from 4.7 to ≈2.0 log CFU/g. It was also observed by the freshness quality index (K-value) that a higher freshness was obtained in samples stored under pressure [50]. In fact, the values of 51, 44, 33, and 28% that were obtained for ≈51, ≈101, ≈203, and ≈304 MPa, respectively, over 12 hours demonstrated that K-value increased slowly at higher pressures, compared to that obtained at AP over 12 hours (92%) (K-value above 60% indicates putrefaction) [50].

Recently, Fidalgo, Lemos, Delgadillo and Saraiva [12] evaluated the quality parameters of Atlantic salmon over 10 days when stored under pressure at RT (25 and 37 °C), at different storage conditions (50, 60 and 75 MPa at 25 °C, and 75 MPa at 37 °C). The authors reported that 60 MPa allowed a microbial growth slowdown promoting a possible shelf-life extension up to 6 days (at least). Moreover, 75 MPa/25 °C led to a reduction of ≈3.5 log units, fact that could increase this product shelf-life at least up to 25 days (when compared to only 3 days of shelf-life at RF). Additionally, the physicochemical analyses performed were similar to the ones obtained on previous studies and no changes on colour, a_w and pH of salmon fillets were detected over storage, possibly due to microbial load reduction. Nonetheless, HS/RT enhanced an increase on the primary and secondary lipid oxidation products compared to RF (more pronounced on higher storage pressure), while for AP/RT the tertiary lipid oxidation had increased.

Further studies regarding the HS impact on Atlantic salmon performed by Fidalgo, Delgadillo and Saraiva [34] revealed a decrease of the acid phosphatase content for 50 MPa/25

°C and 75 MPa at 25 and 37 ºC after 10 days to values similar to RF. Furthermore, cathepsin B presented a similar behaviour when HS was performed at 25 ºC when compared to samples stored at AP, being only observed its activity decrease (to values lower than 6%) when HS temperature increased to 37 ºC [34]. Moreover, calpains revealed a decrease of its activity being pressure sensitives at 25 ºC (residual activities of about 12%, 7% and 31% obtained in 50, 60 and 75 MPa storage conditions, respectively) while at 37 ºC no pressure effect was observed [34]. In what concerns the myofibrillar fragmentation index, while 60 MPa/25 °C led to a decrease of this value, 75 MPa/25 °C and 75 MPa/37 °C translated to an increase of 1.5 and 2.7-fold, respectively after 3 days [34].

Moreover, Okazaki, Shigeta and Aoyama [51] found out while developing an autolysis process for sea cucumber guts, that psychrotrophic bacteria counts were reduced of about ≈0.9 log CFU/g after being under pressure at 60 MPa/30 °C during 24h, although mesophilic bacteria count presented an increase of ≈1.2 log units under these conditions.

Another work performed by Fernandes, Moreira, Duarte, Santos, Queirós, Fidalgo, Santos, Delgadillo and Saraiva [39], which aimed the use of HS at RT (25-150 MPa and 25-37 °C) along 4 and 8 hours on sliced cooked ham, yielded similar results to the ones cited above. Here, 50 MPa/30 °C has led to a microbial growth inhibition, resulting in TAM and LAB loads similar to RF, being observed that when the storage pressure increased to 100-150 MPa, it resulted in an additional microbial inactivation. In fact, it was observed a linear inactivation trend of −0.028 and −0.030 log CFU/g/MPa for TAM and LAB, respectively at 100 MPa/30 °C [39]. Globally, in this study, the quality attributes (e.g., colour, WHC, pH, lipid oxidation - TBARS) of sliced cooked ham were similarly affected by HS and RF.

The HS feasibility was also proved for raw bovine meat by Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13], during 12 hours at 50, 100 and 150 MPa and RT (≈21 °C). The microbial results obtained agreed with the aforementioned studies, since it was observed for 12 hours of storage at 50 MPa a similar microbial growth inhibition when compared to RF, and at 100 and 150 MPa an additional microbial inactivation effect. For instance, TAM decreased from 2.86 ± 0.08 log CFU/g to 2.06 ± 0.03 log CFU/g and <2.0 log CFU/g for 100 and 150 MPa, respectively, being verified a similar behaviour for TAP. Although, the initial counts of ENT, YM and COL were below 2.0 log CFU/g, these microorganisms showed some susceptibility under HS, being gradually more affected by the increase of pressure, with counts below the detection limit (<1.0 log CFU/g) under HS.

Moreover, these authors found log-linear curves to describe the microbial inactivation of TAM and TAP during 12 hours of HS of fresh beefs, as a function of pressure, being noticed a similar susceptibility for both microorganisms, -0.013 log CFU/MPa/g as the specific inactivation rate. A post-HS during 6 days was also evaluated for raw bovine meat, where it was verified that microorganisms were able to grow under RF after a HS period (150 MPa, RT, 12 hours), indicating that pressure inhibited/inactivated the microbial growth during HS but microorganisms were still able to proliferate after it under RF and AP [13]. In a second experiment, the same authors, studied for a longer storage period (10 days at 50 MPa/RT) raw bovine beef. The outcomes hint for a possible raw bovine meat shelf-life extension by HS when compared to samples stored at RF conditions. HS was effective in TAM and TAP loads maintenance with values below 6.0 log CFU/g up to the 7th day of storage, contrarily to samples stored at AP that presented values of >6.0 log CFU/g right after 3 days at RT and after 7 days at RF temperatures. As regard to the physicochemical analyses performed in this study (pH, colour, fatty acid determination), the authors did not find significant differences in pH for RF and HS samples (10 days) relatively to the initial value, as well as for colour parameters wherein no significant differences in L^*, a^* and b^* were observed between samples stored under RF and by HS up to 10 days [13]. In what concerns to fatty acid content, the monounsaturated fatty acids (MUFA) and the several polyunsaturated fatty acids (PUFA) class proportions were not significantly affected by HS, whereas saturated fatty acid (SFA) presented some significant differences, although no consistent pattern relating HS and fatty acid composition arose from the results obtained, thus indicating no particular detrimental effect caused by HS [13].

Further studies regarding raw meat storage under pressure were performed by Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11], in this case pork minced meat was stored up to 24 hours at 100 MPa and variable RT. In this work, the authors observed that lower counts were detected on the samples with the increase of storage time, obtaining for instance a D value of 57.8 hours for TAM (58 hours required to reduce 90% the initial counts) [11]. Moreover, the fatty acid composition of pork minced meat revealed that storage conditions did not affect this parameter probably due to the short storage period, being observed that palmitic (C16:0), stearic (C18:0), oleic (C18:1c9), and linoleic (C18:2n-6) acids were the most abundant, presenting values around 24%, 10%, 36%, and 17% of total fatty acids, respectively [11].

1.5. Hyperbaric storage – Industrial viability and environmental impact

The conventional cold storage processes (both freezing and RF) are very well established at both domestic and industrial environments. In fact, the food cold-chain market is thought to be worth more than 167.4$ billion United-States dollars (USD) and it is forecasted to be worth more than 271.3$ billion USD by 2022, with an estimated annual growth of 7.0% [52], showing that these processes are widely consolidated. Nevertheless, the cold-chain industry is responsible for considerable emissions of greenhouse effect gases, such as the chlorofluorocarbons (also known as CFCs), as well by elevated energetic spends due to the almost constant power supply to keep these units operating, which results on significant emissions of CO_2 to the atmosphere.

On the perspective of a HS acceptance on both domestic and industrial contexts to mitigate the environmental impacts of RF, it is of upmost importance to evaluate the economic and environmental impacts of HS to ensure if it is economically advantageous and an environmental friendlier alternative to the traditional RF processes. For so, the next sections will focus an economic and environmental evaluation of HS.

1.5.1. Storage costs estimation

The potential energetic savings related to a HS application as a new food preservation methodology was referred by several authors, such as Fernandes, Moreira, Fidalgo, Santos, Queirós, Delgadillo and Saraiva [22], Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13], Fidalgo, Santos, Queirós, Inácio, Mota, Lopes, Gonçalves, Neto and Saraiva [15], Segovia-Bravo, Guignon, Bermejo-Prada, Sanz and Otero [10], among others, with inherent economic and environmental gains, stating that energy would be only needed to compress and decompress the pressure vessel, since when the desire pressure is achieved, energy would not be needed to keep it along storage, and thus, virtually, no energetic costs. Though, until now, this statement was only investigated by Bermejo-Prada, Colmant, Otero and Guignon [9], which estimated that the energetic costs inherent to HS of 800 Kg of strawberry juice at 25 MPa and 20 °C for 15 days was 0.001 €/Kg against 0.026 €/Kg of RF.

Although a great reduction of the energetic costs is associated to this new food preservation methodology, the equipment price can overlap that potential, mainly due to the

costs of the pressure vessel, intensifiers and hydraulic pumps, which are considerably higher than the conventional RF facilities, which resulted, as estimated by Bermejo-Prada, Colmant, Otero and Guignon [9], in a total storage cost for HS of about 0.291 €/Kg of strawberry juice, against 0.081 €/Kg for conventional RF. These costs include equipment maintenance and amortization (a measure of the initial investment depreciation), as well the inherent energetic costs (this last parameter seems to be the best of HS against RF). In addition, this study estimated HS/RT costs considering a completely loaded vessel (maximum mass of 2,000 kg) so it could be moved to a warehouse with a forklift, thus, heavier pressure vessels are to be moved with more expensive and complex structures and equipment that are, by induction, more expensive than a forklift, which can increase even more HS costs. Moreover, as the storage pressure increases, the pressure vessel thickness required to keep it for long periods of time also increases, thus increasing the storage costs. As mentioned, despite the HPP equipment high initial cost, it did not stop its implementation in the food industry as a non-thermal pasteurization method, and as result, a decreasing trend in equipment costs was observed from 1996 until now. Innovations related to the HP technology, such as HS, might lead to the arising of new manufacturers, which could also lower the price of these units [3], even if specifically designed for HS, that would require less resistant units since the pressure levels employed on HS are considerably lower than in HPP.

1.5.2. Carbon foot-print assessment

According to Gilbert [53], RF is the third major source of CO_2 of all food industry (with 490 megatons of CO_2 released to the atmosphere in 2008), being even estimated that 35 to 50% of the energetic consumptions in super and hypermarkets is due to RF and freezing facilities, contributing for approximately 1% of the CO_2 emissions worldwide [54]. Thus, environmentally friendlier food preservation methodologies need to be considered, in order to reduce the carbon footprint related with RF, being HS a possible solution for this issue. Besides CO_2, RF facilities are also responsible for considerable emissions of greenhouse effect gases, which are used as refrigerant on these facilities, belonging to a class of compounds known as CFCs and hydrochlorofluorcarbons, which are responsible for ozone degradation [54].

The carbon footprint associated with HS of 1 Kg of strawberry juice for 15 days was assessed by Bermejo-Prada, Colmant, Otero and Guignon [9] and compared with RF storage.

The outcomes revealed that RF had a 26-fold higher carbon footprint when compared to HS (0.1085 Kg CO_2/Kg of strawberry juice against 0.0042 Kg CO_2/Kg of strawberry juice, respectively). In what concerns to RF, the two main sources of CO_2 were the energetic consumption and the refrigerant leakage, while for HS the main source of CO_2 emission was attributed to the hyperbaric chamber material, with an estimated emission of 0.0041 Kg CO_2/Kg of strawberry juice, while the CO_2 released by the energetic consumption was negligible ($3x10^{-5}$ Kg CO_2/Kg of strawberry juice), proving that HS is considerably less pollutant than the conventional RF processes.

From the social point of view, Bermejo-Prada, Colmant, Otero and Guignon [9] concluded that HS/RT could also be preferred over RF, since the pressure vessels could be shipped to foreign geographies where electricity is less available, thus providing safer food products, while contributing for a more sustainable food-chain industry.

Chapter 2 – Meat quality and its importance in the human diet

Briefly, meat could be defined as the flesh of a dozen of the 3000 mammalian species used as food, being most of the times included in this definition organs such as liver, kidney, brains and other edible tissues [55]. Moreover, it should be noted that although the bulk of the meat consumed by European countries relies on sheep, cattle, pigs, rabbit, hare, poultry, horse, goat and deer, outside Europe other animals are ate, such as seal and polar bear by Inuit, or giraffe, rhinoceros, hippopotamus and elephants by certain tribes of Central Africa, or even kangaroo by the Australian aborigines and dogs and cats in Southeast Asia [55].

Meat is a rich nutrient matrix, presenting approximately 75% of water, 20% of protein, 1–10% of fat, and 1% of glycogen [56]. It is recognized as the first-choice source of animal protein all over the world (Figure 2.1), and to exemplify it, consumption values of 36.6 and 101 kg of meat capita^{-1} were estimated for Canada at 2008 and USA at 2007, respectively [57]. Furthermore, it is predicted the increase of meat consumption more than 158% for beef and 137% for pork meat between 2010 and 2050, mainly due to population growth [58, 59].

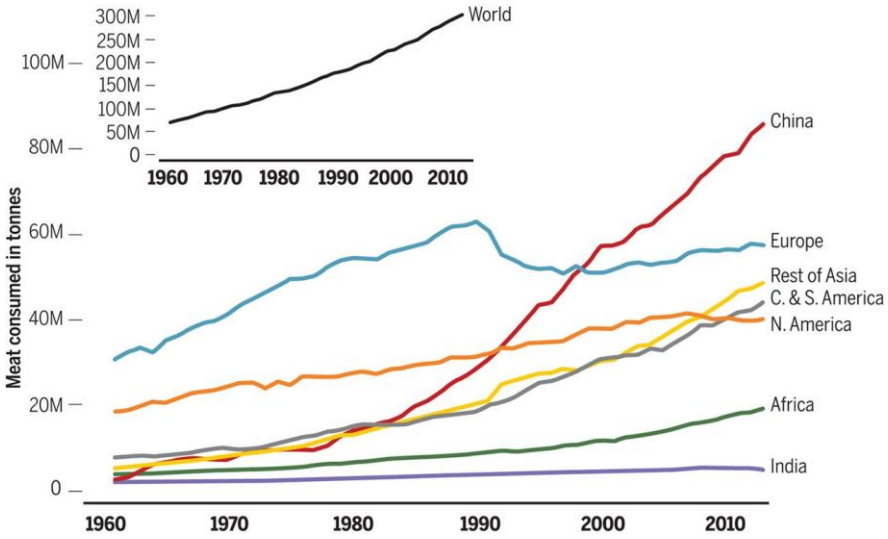


Figure 2.1. Graphical representation of the worldwide meat consumption (in million metric tons). Image from Godfray, Aveyard, Garnett, Hall, Key, Lorimer, Pierrehumbert, Scarborough, Springmann, and Jebb [59] work.

On the other hand, it should be highlighted the huge amount of meat and meat products spoiled worldwide every day. At 1995 in the USA, it was estimated a waste of ≈3.5 billion kg of poultry and meat at the consumer, retailer and foodservice levels [60]. Generally,

a food product is handled an average of 33 times before being in the hands of the consumer, and therefore food losses can occur at different stages due to several reasons, for instance during transportation or storage at the supermarket or even at consumers home due to inadequate packaging, improper temperatures or simply the passage of time [60]. Even before that, meat production involves several operations and the inadequate operational techniques and facilities will also cause for instance unnecessary suffering and injuries to animals which lead to loss of meat, reduced meat quality and spoilage of meat [57].

Since different technical operations are involved in the slaughtering process (such as stunning, bleeding, skinning, among others) and several particularities have an important role on the final meat quality (for instance, the stress induced on the animals during the slaughtering process), in this section some aspects of meat production, properties/characteristics, quality and safety will be addressed.

2.1.The conversion of muscle to meat

Usually, carcasses of sheep, cattle and pigs represent the animal's body after removal of blood, the head, feet, hides, digestive tract, intestines, bladder, heart, trachea, lungs, kidney, spleen, liver and adhering fatty tissue; and only 50, 55 and 75% of the live weight of sheep, cattle and pigs, respectively remains on the carcass [55]. Moreover, it is known that the weight of the muscular tissue depends on the fatty tissue content being the latter related to several factors, such as, age, breed, and plane of nutrition of the animal [55].

Since these animals' musculature comprises ≈300 anatomically distinct units, it is expected that muscles will differ superficially and intrinsically, revealing different sizes, shape, attachments (to bone, cartilage or ligaments), in blood and nerve supply, in their association with other tissues, and in their action [55].

Briefly, the skeletal muscle consists of ≈90% muscle fibres and ≈10% of connective and fat tissues, being the connective tissue divided into the endomysium, perimysium and the epimysium which surrounds each muscle fibre, bundles of muscle fibres, and the muscle as a whole, respectively [56]. Fibres are recognized as long, narrow, multinucleated cells, and surrounding each fibre and underneath the endomysium is present a sheath called sarcolemma [55]. The myofibrils are surrounded by a fluid phase, the sarcoplasm where can be found the organelles, which include mitochondria, lysosomes, peroxisomes, being the muscle cell nuclei generally found just below the sarcolemma [55]. Therefore, when a

longitudinal cross section of myofibrils is observed by electron microscopy, alternating dark (A bands) and light areas (I bands) are observed, and each I band is divided into two portions by a Z-line, being called of sarcomere the distance between two adjacent Z-lines [56] (Figure 2.2).

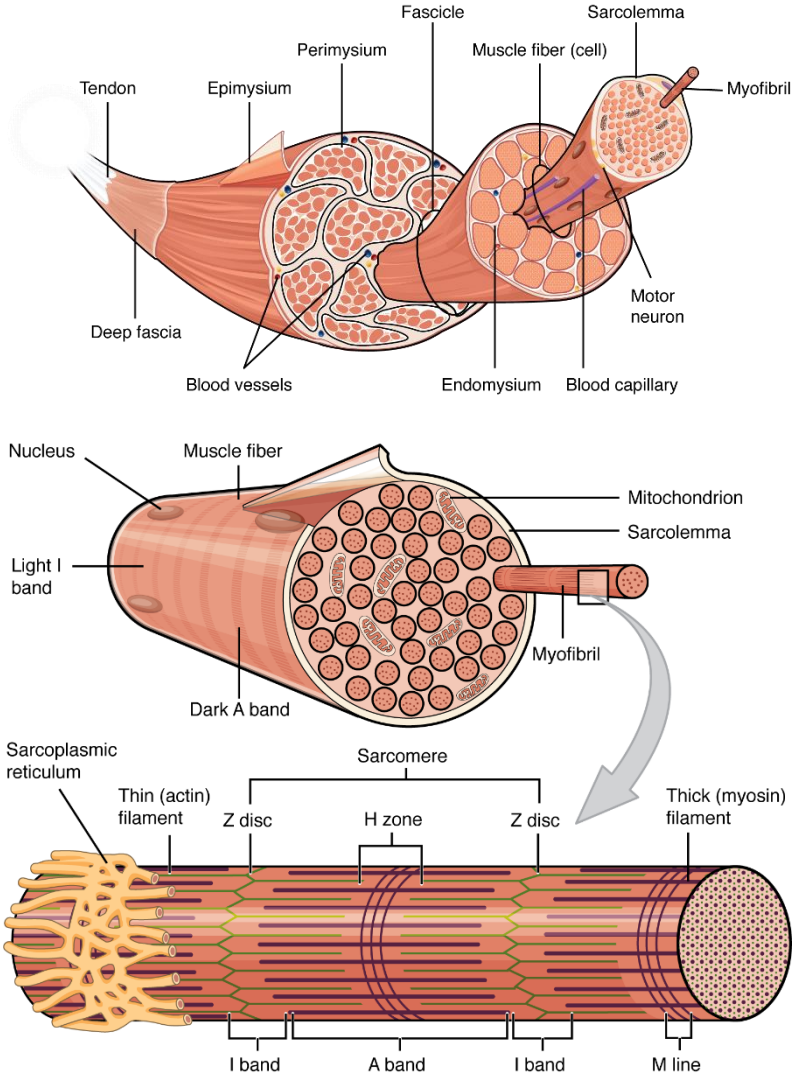


Figure 2.2. Representation of the skeletal muscle organization. Image from Lindsay M. Biga, Sierra Dawson, Amy Harwell, Robin Hopkins, Joel Kaufmann, Mike LeMaster, Philip Matern, Katie Morrison-Graham, Devon Quick and Runyeon [61]

The conversion of muscle to meat could be described as a three-step process, starting with a pre-rigor phase in which muscle remains excitable and might correspond to the duration of survival of the nervous system; secondly the rigor phase being this period where

energy compounds are exhausted and muscle elasticity decreases reaching the maximum toughness; and thirdly the tenderization phase where meat texture results primarily from the weakening of the myofibrillar structure [62]. Some scientists and recent advances also suggest a possible new step (a fourth one) before the rigor mortis that will correspond to the setting of cellular death and apoptosis [63]. A sequence of events could be described during rigor mortis, starting with the loss of ability to synthesize ATP, causing the combination of actin and myosin to form actomyosin leading to a stiffening of the muscle, the reduction of the oxidation-reduction potential due to oxygen privation, the slow development of rancidity due to loss of vitamins and antioxidants, the reduction of the temperature of the carcass and solidification of fat due to nervous/hormonal regulations loss, and conversion of glycogen to lactic acid reducing pH (from 7.2 to 5.7) leading to proteins denaturation [64].

In a general manner, after slaughter, meat is stored at cold temperatures (4 ºC) from 2 up to 30 days depending on species, processing methods and packaging, for instance, beef presents one of the longest storage periods, one to two weeks for carcasses to one month when packed under vacuum in pieces [56]. After slaughter and during the first hours of storage, the water content distribution in the muscle tissue reveals some changes since the packing density of thick filaments per unit area increases and the cross sectional area of myofibrils decreases, leading to a filament spacing decrease, being this event correlated to the pH decrease [65]. Consequently, it is reported a movement of water from the myofibrillar into the inter-myofibrillar space, and then into the extracellular space during post-mortem changes [65].

Thus, the final quality of the meat will depend in fact of several factors, for instance of the WHC parameter, since it is influenced by the rate and extent of post-mortem pH decrease [56]. It is known that a higher rate combined with high muscle temperature causes denaturation of muscle proteins, reduces WHC, increases exudation, and cooking loss of meat in pigs and poultry [56]. Notwithstanding, other procedures have also an important impact on the final quality of meat, such as, the pre-slaughter fasting, pre-slaughter handling (pre-slaughter stress adversely affect meat quality), stunning method (e.g., electrically stunned pigs have a more rapid pH decline early post-mortem and an inferior WHC compared with meat from CO_2 stunned pigs), the slaughter procedure (e.g., chilling rate, electrical stimulation, pelvic suspension) [66].

Furthermore, in the conversion of muscle to meat it is well accepted by the scientific community that the improvement of meat tenderness results from the softening of the myofibrillar structures by endogenous peptidases [62]. The tenderization process is recognized to be mainly enzymatic, being the cathepsins, calpains, and proteasomes the most studied proteolytic systems [63]. Calpains and cathepsins are recognized as two proteolytic systems, acting synergistically, capable of hydrolyzing myofibrillar proteins during post-mortem storage [67-69].

2.2. Raw meat spoilage

Raw fresh meat is highly perishable due to microbial growth, as well by the chemical and enzymatic activities. Furthermore, post-rigor meat present higher contents of nonproteinaceous nitrogen compounds, peptides, proteins, and carbohydrates with a pH of ≈5.7 and aw >0.97 presenting optimum conditions for microorganisms contamination and growth [64].

There are many factors affecting the shelf-life of meat, and could be divided into intrinsic and extrinsic factors [57]. The former group of factors consists of type of animal, breed, feed regime, animal's age, initial microflora, chemical properties (peroxide value, pH, acidity, redox potential), availability of oxygen, processing conditions/control, hygiene; and the latter consists of quality management systems, temperature control, packing system and storage types [57].

The stress of the animal before slaughter is one important parameter for the final meat quality and spoilage since it is related to the final meat pH. The pre-slaughter stress could lead to a glycogen reduction content in the muscles and as a consequence lower amounts of acid lactic are produced (by anaerobic glycolytic pathway responsible for the glycogen breakdown and lactic acid production) leading to a lower pH fall and "dark, firm, and dry" meat [64, 70, 71]. This type of meat retains higher amounts of water, and present a higher pH which leads to a straightforward microbial growth causing off-odours and off-flavours [64].

2.2.1. Microbial spoilage

The intestinal tract and skin of the animal are recognized as the main sources of the endogenous microflora found on meat since the inner tissue of healthy living meat animals is practically sterile [72].

In a general manner, several microorganisms can be found on meat, such as *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Brochothrix*, *Carnobacterium*, *Escherichia*, *Enterobacter*, *Enterococcus*, *Flavobacterium*, *Hafnia*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Moraxella*, *Proteus*, *Pseudomonas*, *Sarcina*, *Serratia*, *Shewanella*, and *Streptococcus* as well as YM [57, 64].

Regarding bacteria, *Enterococcus* spp. is the most observed one, as an example, Hayes, English, Carter, Proescholdt, Lee, Wagner and White [73] have detected it in 99% of several meat samples (chicken, turkey, pork and beef) in the Iowa state. In this study, pork/beef samples presented a total of 54%/17% of *Enterococcus faecalis*, 38%/65% of *Enterococcus faecium*, 3.4%/14% *Enterococcus hirae*, 2.4%/2% of *Enterococcus durans*, respectively, among others in minor content (*Enterococcus casseliflavus*, *Enterococcus gallinarum*) [73]. In what concerns pathogenic microorganisms, the microorganisms most frequently observed are *Salmonella enteric* strains, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *L. monocytogenes*, *E. coli*, *Staphylococcus*, *Streptococcus*, *Clostridium*, and *Salmonella*. [64].

Moreover, when meat is preserved at low temperatures several bacterial (*Acinetobacter*, *Moraxella*, *Pseudomonas*, *Aeromonas*, *Alcaligenes*, and *Micrococcus*), mould (*Alternaria*, *Cladosporium*, *Geotrichum*, *Mucor*, *Monilia*, *Penicillium*, *Sporotrichum*, and *Thamnidium*), and yeast (*Candida*, *Torulopsis*, *Debaryomyces*, and *Rhodotorula*) genera could be easily found [64]. Over storage at 5 °C, psychrotrophic aerobes and facultative anaerobes are the main cause of microbial spoilage, for instance, *Pseudomonas* spp. presents a short generation time using firstly glucose and secondly amino acids to growth, as well other psychrotrophs (e.g., *Aeromonas*, *Shewanella putrefaciens*, *Acinetobacter*) use free amino acids and related simple nitrogenous compounds to growth after simple carbohydrates exhaustion, producing methyl sulphides, esters, and acids [64].

In Table 2.1 are described some examples of spoilage compounds developed by microorganisms in meat. The presence of putrid and sulphury odours were related to

hydrogen sulphide, methylsulphide and dimethylsulphide production by microorganisms, as well cadaverine and putrescine have been studied as meat spoilage indicators [57].

Table 2.1. Examples of spoilage bacteria and their spoilage compounds, adapted from Dave and Ghaly [57].

Spoilage bacteria	Spoilage compounds
Pseudomonas spp. and *Enterobacteriaceae*	Cysteine, cystine, methionine, hydrogen sulphide, methylsulphide and dimethylsulphide
Pseudomonas fluorescens	Methylamine, dimethylamine and trimethylamine ethyl esters
Brochothrix thermosphucta	Acetoin and acetic acid
Photobacterium phosphoreum	Trimethylamine, hypoxanthine
Vibrionacaea	Trimethylamine, hydrogen sulphide
Aerobic spoilers	Ammonia, acetic, butyric and propionic acid

2.2.2. Lipid oxidation

In a general manner, lipid oxidation in foods relies on a reaction chain that starts with peroxides formation (primary products), that when are exposed to further oxidation lead to aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers production (secondary products) presenting undesirable sensorial and biological effects [74].

In the conversion of muscle to meat, autoxidation of lipids and the production of free radicals occur since blood circulation and metabolic processes have stopped [75]. Briefly, lipid oxidation could be defined as the reaction of oxygen with double bounds of fatty acids and involves three stages: initiation, propagation and termination [76].

Oxidation starts by the removal of a hydrogen from a methylene carbon in a fatty acid, being easier with the increase of the number of double bonds (PUFA are highly susceptible to oxidation) [77]. The initiation could be catalysed by HO* or iron-oxygen complexes, and the fatty acyl radical (R*) will react with O_2 forming a peroxyl radical (ROO*) [77]. The ROO* will preferentially oxidize other unsaturated fatty acids and propagate the chain reaction since it is more highly oxidized than the fatty acyl radical or the fatty acid itself, thus leading to lipid hydroperoxides (ROOH) [77]. Thus, hydroperoxides are at the same time oxidation products and substrates for further reaction with Fe^{2+} and Cu^{+} to yield ROO* and alkoxyl radicals (RO*) that can initiate further reactions [77]. Moreover, RO* can also undergo β-scission and degrade to alkyl radicals (R'CH*$_2$) and a range of aldehydes (R"CHO), in which (R'CH*$_2$) can initiate other reactions leading to ethane and pentane

development, while aldehydes can react to yield Maillard-type complexes [77]. Several factors could influence the oxidative stability of lipids, such as, the exposure to light/heat, presence of oxygen, degree of unsaturation in fatty acids, pro-oxidant and antioxidant compounds [57].

It must be noted that meat lipid hydrolysis can be of enzymatic and non-enzymatic origin. Lipases, esterases and phospholipase are responsible for these events in the enzymatic lipid hydrolysis, where lipases split the glycerides forming free fatty acids which are responsible for common off-flavour, frequently referred as rancidity [78]. This process involves 3 phases, starting with a cleavage of triacylglycerol, an acyl migration and ending by a cleavage of 1-monoacyl-sn-glycerol [57]. On the other hand, the non-enzymatic hydrolysis has as origin heme proteins such as hemoglobin, myoglobin and cytochrome which are susceptible to oxidation and produce hydroperoxides [79].

2.2.3. Autolytic enzymatic spoilage

As already described in section number 2.1, enzymes have an important role on the ageing of meat, and as catalysts of chemical reactions could lead to meat deterioration. Briefly, the major endogenous proteinases responsible for post-mortem proteolytic degradation of myofibrillar proteins are calpain and cathepsin systems.

The acid proteases cathepsins, that are located in the lysosomes, could be liberated to the cytoplasm and intracellular spaces after lysosomal disruption/cell dead due to pH decrease, leading to muscle ageing [68]. From all cathepsins, cathepsin B, L, H and D are the most involved in the muscle ageing, and in the case of mammalian meat B, D and L are the most important due to the meat pH (≈6.5 decreasing to 5.7-5.4 after 24h of storage after death) and cathepsins optimal pH [68, 80, 81].

Moreover, calpains activity is known to be calcium dependent, and subclassified into μ-calpain and m-calpain depending on their sensitivity to calcium ions [68]. Calpains have a large and a small subunit, and although the active site is present in the large subunit, the presence of the smallest one is fundamental for activity. Calpains are known to alter the z-line density, a modification often observed post-mortem [63]. Calcium plays a key role in the activation mechanism of calpains leading to dissociation and/or autoproteolysis even in the presence of an alternative substrate, and calpastatin is considered the endogenous specific inhibitor of the calpains [68].

Lately, few studies have supported the potential role of the 20S proteasome in tenderization process as does calpains, showing that m- and z-lines are significant damaged leading to myofibrils degradation [62, 82].

2.3. Raw meat preservation

From early civilizations up to present times, refrigerated temperatures have been used to preserve perishable food products. Raw meat is one of these food products requiring strict control over preservation to guarantee its quality and safety.

Firstly, it is important to underline that the term "raw fresh meat" includes not only recently processed animals, but also vacuum-packed meat or meat packed in controlled-atmospheric gases [83]. Moreover, and as it has been described in this literature revision, several factors affect microbial safety and meat shelf-life, such as, type of meat and how it was prepared, the preservation temperature, atmospheric oxygen, endogenous enzymes, moisture (dehydration), light, and microorganisms [83]. Though meat deterioration can be caused only by oxidation, microorganisms are the most important element on meat spoilage [84].

The use of low temperatures for meat storage could be divided into above (chilling) or below (freezing) the freezing point of meat, although the preservation of meat 1–2 °C below the initial freezing point (superchilling or partial freezing) has been successfully used for several food products [85].

By using chilling temperatures, the temperature is reduced, enhancing carcass drying and reducing microbial growth [83]. Moreover, the chilling method influences the time required to fully refrigerate the deeper tissue of carcasses and the shelf-life of the product, for instance, forced-convection air chilling coupled with fans is much more efficient leading to lower evaporation from the surface [83].

Generally, the freezing process leads to higher shelf-life periods, preventing at the same time microbial growth and chemical changes. Regarding freezing process, its rate will depend on several factors, such as the temperature and freezing method of the apparatus, the meat and its thermal properties (specific heat and thermal conductivity), the size of the pieces and how they are packed (when applicable) [83]. In this methodology, the time spent up to the complete freezing of meat influences meat quality since a fast freezing leads to small ice crystals uniformly distributed inside and outside the cells, while a slow freezing lead to large

extracellular ice crystals and structural changes in meat with the increase of drip loss values upon thawing [86].

Although refrigeration temperatures have been of high importance for raw meat preservation over centuries, there are several other methods currently used to control microbial growth and chemical changes on meat. The use of modified atmosphere packaging, vacuum packaging, or the addition of chemical preservatives are some of them, where, for instance, carbon dioxide and ozone could be used to prevent microbial growth in the surface of carcasses at chilling temperatures over storage, or even chemicals addition (as sodium lactate, nitrites, sulphites, ascorbic acid, benzoic acid, among others) could be used to enhance flavour, the shelf-life period and safety [57, 83]. Notwithstanding, the consumers demand for natural and safer food products lead to a deeper scientific research and the development of new procedures/technologies to improve raw meat quality and safety, for instance, by substituting chemicals for natural compounds, or using HPP (to inactivate microorganisms/pathogens), or applying active/antimicrobial packaging to prevent microorganisms growth [83].

Since several of these methodologies, and mainly the use of low temperatures renders only to microbial inhibition, leading to food safety issues as well short shelf-life periods, it is of great importance to validate the HS feasibility as a new food preservation methodology for raw meat as it has been demonstrating several capabilities such as not only the microbial growth inhibition on foods but also microorganisms inactivation.

Chapter 3 – Scope and outline

This PhD thesis is based on previous research work carried out at the Chemistry Department in University of Aveiro. From 2012 a new research area emerged, being conducted several studies regarding food preservation under pressure. Among these studies, the preservation of highly perishable fruit juice was carried out successfully over days to weeks at RT or even above it. The promising results led to a need of further and deeper studies involving other food products in to order to attest the real HS feasibility as a new food preservation methodology, and at the same time, studying its impact on foods characteristics.

Globally, the aim of this PhD thesis was to evaluate HS for raw bovine and pork meat preservation, both in pieces and minced, at variable RT and at lower temperature, as an alternative and a complement, respectively, to RF. The HS effect on microbial load, physicochemical, enzymes, and texture parameters was studied and compared with the same samples when stored under RF (at AP).

The work performed during the PhD thesis is outlined in Figure 3.1.

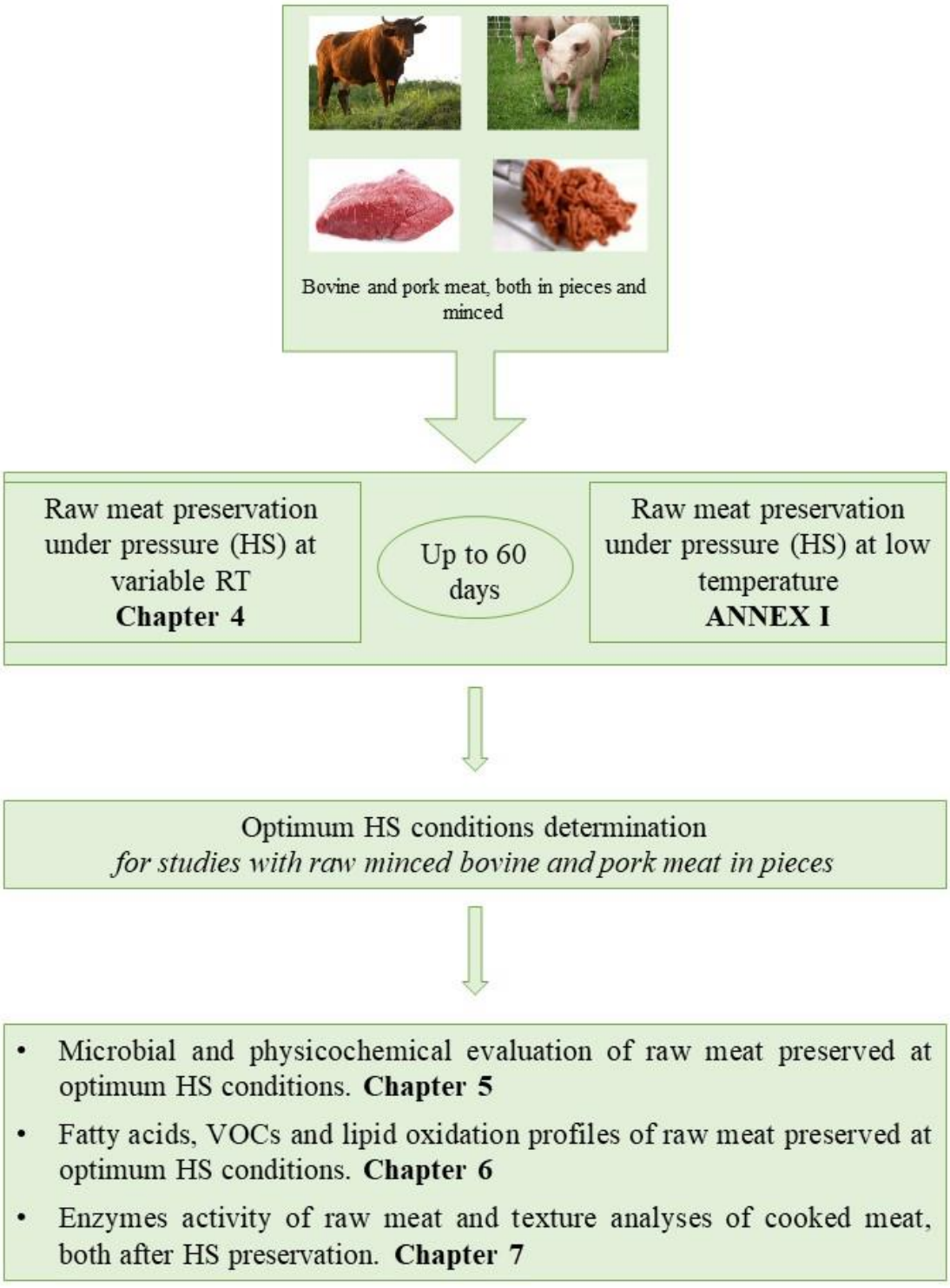


Figure 3.1. Schematic representation of the work performed during the PhD period.

Chapter 4 – Raw bovine and pork meat, minced and in pieces, preserved by hyperbaric storage at variable room temperature

This chapter is based on the paper written during the PhD thesis and published as "Mauro D. Santos, Ivonne Delgadillo, Jorge A. Saraiva (2020). Extended preservation of raw beef and pork raw meat by hyperbaric storage at room temperature, in *International Journal of Food science and technology*" (accepted).

4.1. Overview

Since 2012, an increasing number of studies regarding HS as a new food preservation methodology have been published [9]. Among them, strawberry juice [10, 44], watermelon juice [35, 36], melon juice [41], ready-to-eat meals [40], whey cheese [14], sliced cooked ham [39], raw bovine meat [13], raw pork meat [11], hake loins [31] were successfully stored under pressure at different storage conditions (pressure level/temperature) over different periods of time.

This food preservation methodology relies on food storage under pressure (mainly between 50 and 150 MPa) at variable uncontrolled RT (18-25 ºC) as a possible alternative to RF or at cold temperatures (5-15 ºC) as a complement to RF, because HS can not only inhibit microorganisms (e.g., TAM, ENT, YM), but also inactivate them making it possible to extend food products shelf-lives when compared to RF [22]. Moreover, the overall physicochemical analyses (e.g., pH, total soluble solids, cloudiness, browning degree, colour, enzymes, among others) in these studies allowed conclude that generally, there were no considerable differences between samples stored under pressure and RF, the main exceptions not usually being not relevant for consumers [12, 37, 49]. Although sensorial analyses still lacking regarding food products stored by HS, a study performed by Otero, Pérez-Mateos and López-Caballero [31] on hake loins allowed to conclude that even though perceptible, the differences between samples previous to any storage time and stored under pressure seem not to be large since only moderate evidence of apparent differences were found (mainly in texture characteristics).

When performed at variable RT, HS is also recognised as environmentally friendly since energy (in small amounts) is only required during the compression and decompression phases and is not needed during the storage period. Thus, a lower carbon footprint and lower

energy costs are achieved by this preservation methodology when compared to the conventional RF [9].

Red meat products have an important role in worldwide nutrition due to key micronutrients and protein content with large increases in global consumption being predicted its consumption (>158% for beef and >137% for pork meat between 2010 and 2050), mainly due to population growth [58]. Furthermore, besides the use of cold temperatures for raw meat preservation and microbial control, several chemicals have been applied for the same objective (such as nitrites, sodium chloride, sulphites, phosphates, among others), being its use each time less accepted by consumers' [87].

To date, as far the authors are aware, only two studies regarding fresh meat stored under pressure have been performed. In the work of Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13], samples of raw bovine (in pieces) were stored at 50, 100 and 150 MPa and RT (without temperature control) for 12h and in a second experiment for 10 days at 50 MPa. At the end, the authors observed a microbial inhibition effect at 50 MPa, similar to RF, and an inactivation effect at 100 and 150 MPa over 12h of storage. Nonetheless, 10 days of storage at 50 MPa indicated a shelf-life extension since ENT, COL and YM presented lower loads when compared to RF, although TAM and psychrophilic bacteria have reached values ≥6 log CFU/g, similarly to samples stored at RF. Moreover, in this study, physicochemical parameters (pH, colour and fatty acid profile) showed no considerable detrimental effects compared to the initial and refrigerated samples.

Similar findings were reported by Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] on 3 different batches of minced pork when stored under pressure at 100 MPa up to 24h. In this study, HS could not only inhibit microbial growth but also inactivate microorganisms during storage, being verified by a pH increase (of ≈0.4) on HS samples during storage. Regarding lipid oxidation, although one of the 3 tested batches presented an increase of 25% on TBARS values in the last 12h of HS, the two other batches in the study exhibited no statistically significant differences between HS and RF. Regarding fatty acid content and colour parameters, in general, HS had no effect on fatty acids probably due to the short storage time studied (10 days), and only affected on the b^* colour parameter [11].

These studies indicate the need to determine the minimum pressure level required for raw meat preservation. Information is also needed on the effect of HS on long term storage

and the maximum shelf-life extension potentially achievable by this new food preservation methodology. So, the present study aimed to study the effect of HS on raw bovine and pork, both in pieces and minced, at different pressure levels, i.e., 50, 75 and 100 MPa at variable RT up to 60 days, and to compare the results with samples stored at AP/RT and AP/4 ºC (RF). Microbiological and physicochemical analyses will be used to evaluate the feasibility of HS as a novel preservation methodology for these products.

4.2. Materials and Methods

Sample preparation and storage experiments. Fresh raw bovine and pork, *Longissimus thoracis et lumborum*, were purchased in a local butcher shop, both minced and in pieces (*ca*. 2x1x1 cm). bovine in pieces (BP), minced bovine (BM), pork in pieces (PP) and minced pork (PM) samples were divided into small portions of ≈10 g under aseptic conditions and packaged into low permeability polyamide-polyethylene bags (PA/PE-90, Albipack – Packaging Solutions, Águeda, Portugal). The air inside each bag was removed carefully to leave as little air as possible before the heat-sealing process.

For the HS experiments a high-pressure equipment was used (SFP FPG13900, Stansted Fluid Power Ltd., Harlow, UK) with three pressure vessels of 37 mm inner diameter and 52 cm height (with a 0.4 L usable volume due to the volume occupied by the bottom and top covers), using a mixture of propylene glycol and water (40:60 v/v) as the pressurizing fluid. In this study, three different HS conditions (one condition per pressure vessel), 50, 75 and 100 MPa were tested at variable uncontrolled RT (18±1 - 23±1 ºC) up to 60 days. HS results were assessed by comparison with samples stored at AP/RT and AP/4 ºC (RF) for the same storage time, in the dark and surrounded by glycol and water (40:60 vol/vol) to mimic the same storage conditions, except for pressure. Storage experiments were carried out in triplicate (for each storage day/meat sample/storage condition).

Microbiological Analyses. In order to evaluate the HS impact on microbial counts, all samples were analysed for Total Aerobic Mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic Acid Bacteria (LAB) and Yeasts and Moulds (YM). To do so, 10 g of each sample were aseptically homogenized for 2 min in a Stomacher bag with Ringers solution (Merck, Darmstadt, Germany), with decimal dilutions prepared and triplicates of all dilutions were plated on the appropriate media. TAM counts were determined in Plate Count agar

(Liofilchem, Roseto degli Abruzzi, Italy), by incubation at 30 ± 1 °C for 72 hours. LAB loads were quantified in Man Rogosa and Sharp agar (Merck, Darmstadt, Germany) , by incubation at anaerobic conditions and 30 ± 1 °C for 120 hours. ENT were determined in Violet Red Bile Dextrose agar (Merck, Darmstadt, Germany), being incubated at 37 ± 1 °C for 24 hours. YM were enumerated on Rose-Bengal Chloramphenicol agar medium (Liofilchem, Roseto degli Abruzzi, Italy), by incubation at 25 ± 1 °C for 120 hours. At the end, petri dishes containing 15–300 colony forming units (CFU) were selected and the results expressed as log CFU units per g of meat (log CFU/g). The following analytical higher and lower microbial limits were used for presenting the results, based on the serial dilutions used: 7.00-1.00, 5.00-1.00, 7.00-1.00 and 6.00-1.00 log CFU/g of meat for TAM, ENT, LAB and YM, respectively.

Physicochemical analyses. The pH value was measured at RT, in 6 random points of the full sized samples, with a pH/temperature penetration meter (Testo 205, Testo, Inc., Sparta, NJ, USA). Moisture content analyses were performed thermogravimetrically, drying samples at 105±2 ºC until constant weight and values presented as percentages.

Drip loss was calculated and obtained by the adapted method of Marcinkowska-Lesiak, Zdanowska-Sąsiadek, Stelmasiak, Damaziak, Michalczuk, Poławska, Wyrwisz and Wierzbicka [88] according to the eq. 4.1:

$$DL = \frac{(m_1 - m_2)}{m_1} \times 100\% \qquad \text{Eq. 4.1}$$

where DL is an indicator of drip loss in percentage, m_1 the weight of raw material before storage and m_2 the weight of raw material after storage in g.

Colour analyses were performed using a colorimeter (Konica Minolta CM 2300d, Osaka, Japan) and the CIE*Lab* parameters determined using the original software (SpectraMagic™ NXSoftware, Konica Minolta, Ramsey, NJ, USA), according to the regulations of the International Commission on Illumination: red/green colour (a^*), yellow/blue colour (b^*) and luminosity (L^*) parameters. For these analyses, ≈10 g of each sample was placed in a small dish and the colour measured in six random spots. The colour difference (ΔE) was calculated (eq. 4.2) according to Freitas, Pereira, Santos, Alves, Bessa,

Delgadillo and Saraiva [13], where L^*_0, a^*_0 and b^*_0 are the parameters values at the beginning of the study, prior to any storage time.

$$\Delta E = \lfloor (L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2 \rfloor^{1/2} \quad \text{Eq. 4.2}$$

Sarcoplasmic (SP) and myofibrillar protein (MP) solubility was determined by the method of Warner, Kauffman and Greaser [89] with few modifications. For that, 1 g of each sample was homogenised in 10 mL of 0.025 M potassium phosphate buffer pH 7.2 (Panreac, Barcelona, Spain) to obtain sarcoplasmic proteins solubility, and 1 g of each sample was added to 20 mL of 1.1 M KI (Sigma-Aldrich, Missouri, USA)/0.1 M potassium phosphate pH 7.2 (Panreac, Barcelona, Spain) for total protein solubility quantification. Briefly, samples were minced, homogenised using an Ultraturrax always at cold temperatures to minimise protein denaturation. Samples were then centrifuged (1500 g at 4 °C for 20 min) and the supernatants decanted. The difference between total and SP solubility allowed the calculation of the MP solubility. Protein concentrations were determined by the Biuret method using bovine serum albumin (Sigma-Aldrich, Missouri, USA) as standard [89]. Results were calculated and presented as the relative values of MP and SP.

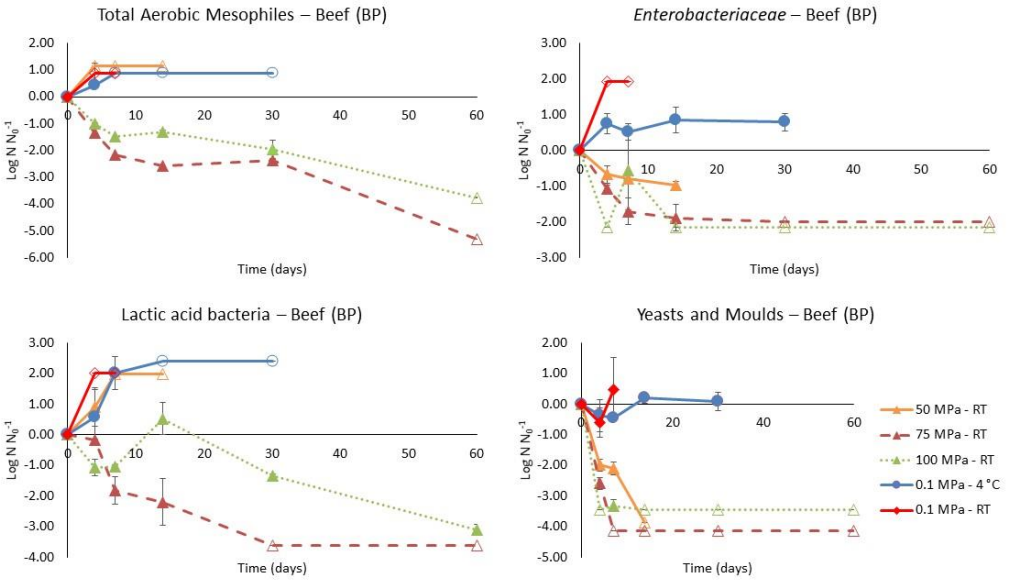
Statistical analysis. Storage experiments were analyzed for microbial and physicochemical parameters in triplicate. Statistical data analysis was performed using Minitab 17 (Minitab Inc., State College, PA, USA) and ANOVA followed by a Tukey's HSD Test, at a 5% level of significance to identify differences between storage conditions and over storage time at each storage condition.

4.3. Results and Discussion

Microbiological analyses. For the microbial analyses, TAM, ENT, LAB and YM were quantified up to 60 days of storage depending on the microbial growth behaviour and the considered maximum and minimum limits (between 7.00-1.00, 5.00-1.00, 7.00-1.00 and 6.00-1.00 log CFU/g of meat for TAM, ENT, LAB and YM, respectively). Generally, the initial microbial values found were between 5.68-6.30, 3.15-4.04, 3.08-5.36, and 2.95-5.33 for TAM, ENT, LAB and YM, respectively.

The results suggested that for all samples (bovine and pork, both minced and in pieces), 50 MPa was insufficient to control the microbial load leading to counts above the considered limits for TAM and LAB after 3 days of storage ($p < 0.05$), although allowing microbial reductions in some cases around 1-2 and 2-4 log CFU/g for ENT and YM, respectively, on the 15th day (Figure 4.1, Figure 4.2, Figure 4.3, and Figure 4.4). In contrast, the microbial behaviour at 75 and 100 MPa storage presented an overall reduction of all microorganisms for all samples with time ($p < 0.05$), these results being better than those obtained on samples stored at AP/4 ºC and AP/RT. For instance, as shown in Figure 4.1 and Figure 4.2 microbial reductions higher than ≈3, ≈2, ≈2, and ≈3 log units were observed on TAM, ENT, LAB and YM, respectively, for 75 and 100 MPa storage conditions.

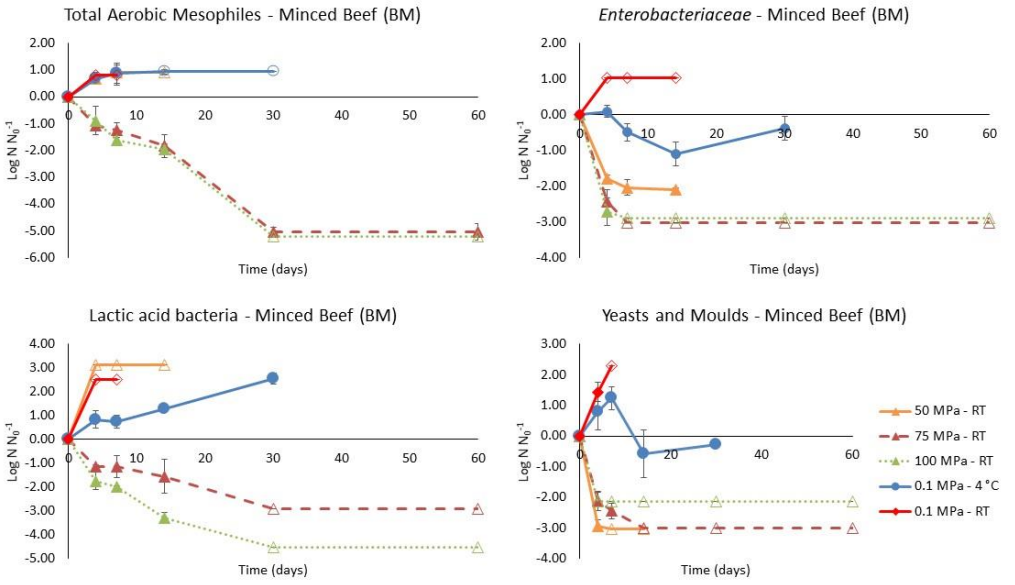
In contrast, samples stored at AP/4 ºC, presented an increase of the microbial load ($p < 0.05$) for TAM and LAB reaching values above the considered limit in some cases, while ENT and YM revealed similar values ($p > 0.05$) over the 30 days of storage (with a few exceptions for ENT on BP and PP samples, and YM on PM samples that increased slightly). On the other hand, as it was expected, AP/RT did not allow the preservation of the samples, values being found above the considered limits just after 3 days of storage in most of the samples/microorganism analysed.



BP		Storage day 0	3	7	14	30	60
TAM	50 MPa - RT	b	aA*	aA*	aA*	#	#
	75 MPa - RT	a	bC	cD	dD	eB	fB*
	100 MPa - RT	a	bC	cC	bcC	dB	eA*
	0.1 MPa – 4 ºC	c	bB	aB*	aB*	aA*	#
	0.1 MPa - RT	b	aA*	aB*	#	#	#
ENT	50 MPa - RT	a	bC	bcBC	cB	#	#
	75 MPa - RT	a	bD	cC	cC	cB*	cA*
	100 MPa - RT	a	bE*	aBC	bC*	bB*	bB*
	0.1 MPa – 4 ºC	b	aB	abAB	aA	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
LAB	50 MPa - RT	c	bAB	aA*	aA*	#	#
	75 MPa - RT	a	aBC	bB	bC	cC*	cB*
	100 MPa - RT	a	bC	bB	aB	bA	cA
	0.1 MPa – 4 ºC	b	bB	aA	cC*	Cb*	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
YM	50 MPa - RT	a	bB	bB	cC*	#	#
	75 MPa - RT	a	bBC	cC*	cD*	cC*	cB*
	100 MPa - RT	a	bC*	bBC	bB*	bB*	bA*
	0.1 MPa – 4 ºC	a	aA	aA	aA	aA	#
	0.1 MPa - RT	a	aA	aA	#	#	#

not applicable

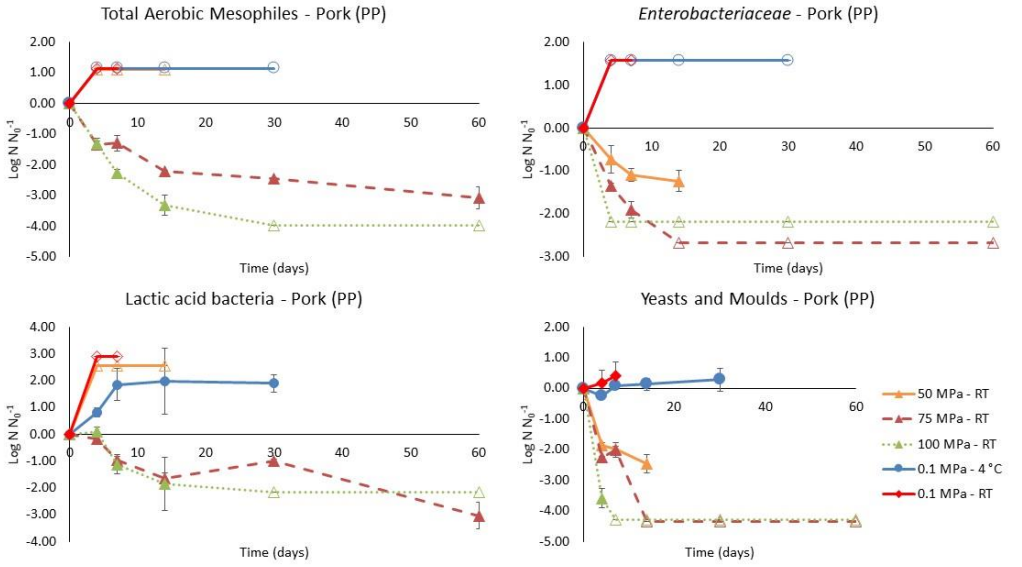
Figure 4.1. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on bovine in pieces (BP) expressed in Log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) and atmospheric pressure/variable room temperature (0.1 MPa - RT). Different lower case letters (a-f) and different upper case letters (A-E) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.



		Storage day					
BM		0	3	7	14	30	60
TAM	50 MPa - RT	c	bA	aA*	aA*	#	#
	75 MPa - RT	a	bB	bcB	cB	dB	dA
	100 MPa - RT	a	bB	cB	cB	dB*	dA*
	0.1 MPa – 4 ºC	b	aA	aA	aA*	aA*	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
ENT	50 MPa - RT	a	bC	bcC	cB	#	#
	75 MPa - RT	a	bD	cD*	cC*	cB*	cA*
	100 MPa - RT	a	bD	bD*	bC*	bB*	bA*
	0.1 MPa – 4 ºC	a	aB	abB	bA	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
LAB	50 MPa - RT	b	aA*	aA*	aA*	#	#
	75 MPa - RT	a	bC	bC	bC	cB*	cA*
	100 MPa - RT	a	bC	bD	cD	dC*	dB*
	0.1 MPa – 4 ºC	c	bB	bB	bB	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
YM	50 MPa - RT	a	bB	bD*	bB*	#	#
	75 MPa - RT	a	bB	bC	cB*	cC*	cB*
	100 MPa - RT	a	bB*	bC*	bB*	bB*	bA*
	0.1 MPa – 4 ºC	abc	abA	aB	cA	bcA	#
	0.1 MPa - RT	c	bA	aA*	#	#	#

not applicable

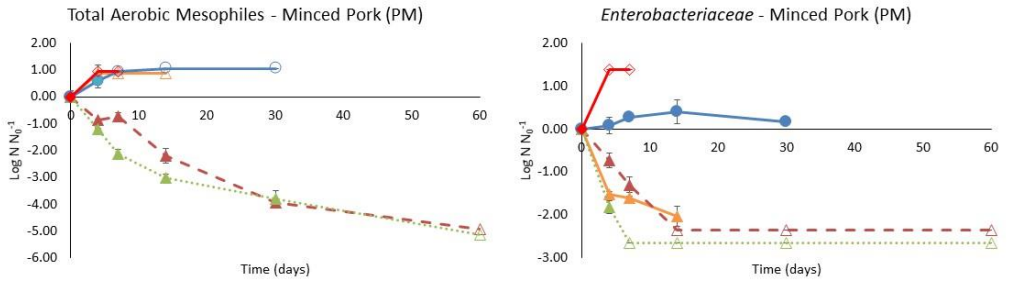
Figure 4.2. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on minced bovine (BM) expressed in Log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) and atmospheric pressure/variable room temperature (0.1 MPa - RT). Different lower case letters (a-d) and different upper case letters (A-D) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.



PP		Storage day 0	3	7	14	30	60
TAM	50 MPa - RT	b	aA*	aA*	aA*	#	#
	75 MPa - RT	a	bB	bB	cB	cB	dA
	100 MPa - RT	a	bB	cC	dC	eC*	eB*
	0.1 MPa – 4 °C	b	aA*	aA*	aA*	aA*	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
ENT	50 MPa - RT	a	bB	bB	bB	#	#
	75 MPa - RT	a	bC	cC	dD*	dC*	dB*
	100 MPa - RT	a	bD*	bC*	bC*	bB*	bA*
	0.1 MPa – 4 °C	b	aA*	aA*	aA*	aA*	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
LAB	50 MPa - RT	b	aB*	aAB*	aA*	#	#
	75 MPa - RT	a	aD	bC	cB	bB	dB
	100 MPa - RT	ab	aD	bcC	cB	cC*	cA*
	0.1 MPa – 4 °C	b	abC	aB	aA	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
YM	50 MPa - RT	a	bB	bB	cB	#	#
	75 MPa - RT	a	bB	bB	cC*	cB*	cB*
	100 MPa - RT	a	bC	cC*	cC*	cB*	cA*
	0.1 MPa – 4 °C	ab	bA	abA	abA	aA	#
	0.1 MPa - RT	a	aA	aA	#	#	#

not applicable

Figure 4.3. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on pork in pieces (PP) expressed in Log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) and atmospheric pressure/variable room temperature (0.1 MPa - RT). Different lower case letters (a-e) and different upper case letters (A-D) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.



PM		Storage day 0	3	7	14	30	60
TAM	50 MPa - RT	b	aAB*	aA*	aA*	#	#
	75 MPa - RT	a	bC	bB	cB	dB	eA*
	100 MPa - RT	a	bC	cC	dC	eB	fB*
	0.1 MPa – 4 ºC	c	bB	aA*	aA*	aA*	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
ENT	50 MPa - RT	a	bD	bD	cB	#	#
	75 MPa - RT	a	bC	cC	dBC*	dB*	dA*
	100 MPa - RT	a	bD	cE*	cC*	cC*	cB*
	0.1 MPa – 4 ºC	a	aB	aB	aA	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#
LAB	50 MPa - RT	b	aA*	aA*	aA*	#	#
	75 MPa - RT	a	bE	cD*	cD*	cB*	cB*
	100 MPa - RT	a	bD	cD*	cC*	cB*	cA*
	0.1 MPa – 4 ºC	c	cC	bC	aB	aA	#
	0.1 MPa - RT	b	aB*	aB*	#	#	#
YM	50 MPa - RT	a	bC	bC*	bB*	#	#
	75 MPa - RT	a	bD	bcD	cD*	cC*	cB*
	100 MPa - RT	a	bE*	bCD*	bC*	bB*	bA*
	0.1 MPa – 4 ºC	c	cB	bB	aA	aA	#
	0.1 MPa - RT	b	aA*	aA*	#	#	#

Figure 4.4. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on minced pork (PM) expressed in log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) and atmospheric pressure/variable room temperature (0.1 MPa - RT). Different lower case letters (a-f) and different upper case letters (A-E) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.

In general, 100 MPa led to the faster microbial load reduction with values below 1.00 log CFU/g being observed just after 3 and 7 days of storage for some microorganisms. However, it must be noted that the differences between 75 and 100 MPa storage conditions are minor after 60 days of storage, with 75 MPa achieving similar results to 100 MPa in the microbial load reduction, with values <1.00 log CFU/g just after 7 and 15 days of storage (depending on microorganism/sample).

The few published studies regarding meat products stored under pressure reported similar results for microbial behaviour, but for shorter storage periods and different storage conditions. For instance, Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13]

performed HS studies on sliced fresh top round beef and found that at variable RT, 50 MPa allowed a microbial effect similar to RF and 100 and 150 MPa led to a microbial inactivation (of TAM, ENT, YM, coliform and psychrophilic bacteria) over 12h. Later, these authors extended the storage period up to 10 days at 50 MPa (variable RT) and reported results pointing to a possible microbial shelf-life extension considering coliform bacteria, ENT and YM with the reduction of these microbial loads (although TAM and psychrophilic bacteria presented values above 6 log CFU/g). Interesting results were also obtained by Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] at 100 MPa and variable RT with raw minced pork meat, since microbial inactivation of TAM, ENT and YM were obtained over 24h. On the other hand, in the study of Fernandes, Moreira, Duarte, Santos, Queirós, Fidalgo, Santos, Delgadillo and Saraiva [39], sliced cooked ham was stored under pressure at RT and exhibited microbial growth inhibition at 50 MPa/30 ºC for TAM and LAB, while microbial inactivation was achieved at 100/25, 100/30, 100/37 and 150 MPa /30 ºC for TAM and LAB (it should be highlighted that this study regards a cooked meat product and a storage period of only 8h).

Thus, from a microbiological point of view, the present work reinforces and increases the data already published by Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] and Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] that HS at a pressure level of only 75 MPa is capable not only of inhibiting microbial growth at variable RT but also of inactivating microorganisms over 60 days for these products, and looks a better alternative than RF to extend the microbial shelf-life of raw meat.

Physicochemical analyses. Throughout AP/4 ºC storage it was observed that the pH decreased ($p < 0.05$) up to the 30th day (for instance, 6.39±0.05 to 5.42±0.03, and 6.13±0.03 to 5.87±0.03 for PP and PM respectively), except for BM where no significant differences ($p > 0.05$) were verified during storage (Table 4.1 and Table 4.2).

PM under HS maintained pH values over 60 days of storage at the 3 different pressure levels tested (Table 4.2). The same was not observed in PP samples since significant differences ($p < 0.05$) were observed with time where pH decreased, but much more slowly than with AP/4 ºC and AP/RT (reaching values of 5.64±0.03-5.93±0.03 for HS compared with 5.42±0.03 and 5.36±0.05 for AP/4 ºC and AP/RT, respectively).

Table 4.1. Results (values presented as mean ± SE) of pH, moisture content (%), drip loss (%), and total color differences (ΔE) obtained for bovine in pieces (BP), and minced bovine (BM), when stored up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), 0.1 MPa at 4 ºC and 0.1 MPa at RT. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		pH		**Moisture Content (%)**		**Drip loss (%)**		**Colour ΔE**	
Storage Condition	**Storage Day**	BP	BM	BP	BM	BP	BM	BP	BM
50 MPa - RT	**0**	5.47±0.03 bc	5.86±0.05 b	55.325±1.193 bc	54.695±2.051 b	#	#	#	#
	3	5.57±0.04 aAB	5.95±0.03 abA	54.984±0.748 cA	56.560±0.352 abA	0.82±0.57 cBC	1.50±0.35 bAB	2.8±0.23 aA	3.81±0.13 aAB
	7	5.51±0.02 abB	5.94±0.05 abA	56.395±1.46 abcA	57.341±1.207 abA	1.22±0.14 bcBC	1.59±0.49 bAB	4.25±0.36 aAB	4.67±0.25 aB
	14	5.47±0.01 bcB	5.92±0.08 abA	57.936±0.984 abA	57.522±1.153 abA	2.12±0.33 abA	2.89±0.67 abA	5.78±2.17 aA	2.4±0.99 aB
	30	5.31±0.06 dB	5.97±0.05 abB	58.472±0.283 aA	58.518±1.133 aA	1.98±0.51 abA	2.99±0.11 abC	5.58±1.85 aA	3.68±1.2 aA
	60	5.38±0.02 cdB	6.03±0.06 aAB	57.174±0.981 abcA	56.678±0.713 abA	2.31±0.21 aA	3.26±1.01 aAB	6.03±0.94 aA	4.42±2.55 aA
75 MPa - RT	**0**	5.47±0.03 d	5.86±0.05 a	55.325±1.193 a	54.695±2.051 ab	#	#	#	#
	3	5.57±0.03 cdAB	5.97±0.07 aA	55.063±1.143 aA	54.021±1.72 abA	1.12±0.21 bcBC	1.50±0.45 bAB	2.3±1.11 aA	2.53±0.31 bB
	7	5.63±0.05 cA	5.93±0.02 aAB	56.863±0.374 aA	56.633±3.134 aA	0.89±0.41 cBC	1.71±0.17 bAB	2.29±0.65 aBC	3.84±0.08 abBC
	14	5.66±0.02 bcA	5.91±0.03 aB	55.863±2.313 aAB	53.176±1.621 abB	2.12±0.51 aA	2.13±0.75 bA	4.31±3.53 aA	2.94±0.94 bB
	30	5.77±0.04 abA	5.94±0.05 aB	54.593±1.573 aB	49.209±2.921 bB	1.69±0.22 abcA	4.37±0.51 aB	3.36±0.65 aA	5.58±1.73 aA
	60	5.85±0.09 aA	5.97±0.02 aB	55.849±1.222 aA	55.629±1.869 aA	2.01±0.17 abAB	2.19±0.12 bB	4.67±2.81 aA	4.52±0.61 abA
100 MPa - RT	**0**	5.47±0.03 c	5.86±0.05 b	55.325±1.193 a	54.695±2.051 a	#	#	#	#
	3	5.66±0.06 bA	5.92±0.03 bA	55.864±0.847 aA	54.611±0.555 aA	1.31±0.26 aB	1.92±0.21 dAB	4.63±2.26 aA	6.64±1.35 aA
	7	5.72±0.04 abA	5.97±0.04 bA	51.473±1.522 bB	52.563±3.959 aA	1.73±0.44 aB	2.59±0.61 cdA	3.43±1.10 aBC	7.16±0.53 aA
	14	5.72±0.02 abA	6.12±0.06 aB	50.266±1.241 bC	50.158±1.571 aB	1.33±0.24 aAB	3.33±0.35 bcA	5.08±1.38 aA	6.3±1.24 aA
	30	5.75±0.05 abA	6.12±0.04 aA	52.588±2.021 abB	52.750±3.249 aAB	1.53±0.41 aA	5.56±0.25 aA	6.05±4.08 aA	6.66±2.53 aA
	60	5.84±0.08 aA	6.09±0.02 aA	51.549±0.934 bB	41.701±0.371 bB	1.38±0.36 aB	4.37±0.78 abA	6.89±1.21 aA	6.67±0.72 aA
0.1 MPa - 4 ºC	**0**	5.47±0.03 ab	5.86±0.05 a	55.325±1.193 ab	54.695±2.051 a	#	#	#	#
	3	5.53±0.02 aB	5.79±0.03 aB	54.284±0.631 abA	43.505±2.501 bB	0.36±0.12 aC	0.29±0.03 bB	1.62±1.22 bA	3.03±2.56 aAB
	7	5.43±0.05 abcB	5.90±0.06 aAB	55.853±0.841 aA	55.015±1.462 aA	0.51±0.34 aC	0.89±0.37 aB	1.76±0.34 bC	2.67±0.84 aC
	14	5.37±0.05 bcC	5.82±0.04 aB	53.584±0.914 bBC	53.539±0.947 aB	0.47±0.21 aB	0.55±0.03 abB	2.71±1.66 bA	2.35±0.02 aB

	30	5.34±0.04 cB	5.79±0.03 aC	55.853±0.135 aAB	54.798±0.985 aAB	0.55±0.28 aB	0.68±0.03 abD	5.85±1.16 aA	5.91±1.54 aA
0.1 MPa - RT	**0**	5.47±0.03 a	5.86±0.05 a	55.325±1.193 a	54.695±2.051 a	#	#	#	#
	3	5.37±0.04 aC	5.89±0.05 aAB	50.582±0.937 bB	45.354±5.745 bB	3.58±0.25 aA	4.14±2.96 aA	5.26±1.95 aA	3.87±0.85 bAB
	7	5.23±0.05 bC	5.81±0.03 aB	49.548±1.424 bB	57.086±0.924 aA	2.89±0.36 aA	1.87±0.87 aAB	6.17±1.39 aA	6.78±0.46 aA

not applicable

Table 4.2. Results (values presented as mean ± SE) of pH, moisture content (%), drip loss (%), and total color differences (ΔE) obtained for pieces of pork (PP), and pork minced meat (PM) when stored up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), 0.1 MPa at 4 ºC and 0.1 MPa at RT. Different lower case letters (a-c) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		pH		**Moisture Content**		**Drip loss (%)**		**Colour (ΔE)**	
Storage Condition	**Storage Day**	PP	PM	PP	PM	PP	PM	PP	PM
50 MPa - RT	**0**	6.39±0.05 a	6.13±0.03 a	59.124±1.512 a	58.659±1.587 a	#	#	#	#
	3	5.69±0.03 bC	6.09±0.04 aB	60.142±2.143 aA	56.392±1.703 aA	1.21±0.21 aA	1.50±0.52 abB	4.56±1.69 aA	3.83±0.69 aA
	7	5.59±0.07 bC	6.11±0.03 aAB	58.184±1.912 aA	58.276±3.425 aA	1.32±0.12 aA	1.02±0.11 bA	3.58±0.29 aB	2.14±1.22 aB
	14	6.00±0.1 bA	6.08±0.05 aAB	59.472±1.435 aA	57.874±1.321 aA	1.15±0.11 aA	1.70±0.12 abA	2.10±0.08 aB	3.76±2.01 aA
	30	5.57±0.07 bB	6.07±0.08 aA	58.253±1.391 aA	57.114±2.191 aA	0.98±0.25 aA	1.51±0.11 abB	3.13±0.84 aB	2.98±0.45 aA
	60	5.64±0.03 bC	6.04±0.04 aA	58.588±2.018 aA	58.306±1.685 aA	1.38±0.17 aA	2.07±0.19 aAB	2.74±1.21 aA	3.31±1.24 aA
75 MPa - RT	**0**	6.39±0.05 a	6.13±0.03 a	59.124±1.512 a	58.659±1.587 a	#	#	#	#
	3	5.88±0.05 bcB	6.1±0.02 aB	57.941±1.749 aAB	53.823±1.171 abAB	1.21±0.10 aA	1.55±0.09 aB	3.42±1.80 aA	3.67±1.26 aA
	7	5.81±0.03 cAB	6.11±0.08 aAB	57.341±1.231 aA	58.928±0.266 aA	1.14±0.29 aA	1.78±0.59 aA	2.79±0.13 aB	6.08±1.56 aAB
	14	5.64±0.01 dAB	6.14±0.04 aA	58.012±3.138 aAB	55.131±0.872 aA	0.93±0.16 aA	1.81±0.90 aA	5.49±0.39 aAB	5.43±3.54 aA
	30	5.92±0.07 bcA	6.08±0.06 aA	56.859±1.384 aAB	53.273±4.251 abAB	1.21±0.17 aA	2.32±0.41 aA	4.90±2.06 aAB	3.19±2.09 aA
	60	5.93±0.03 bA	6.11±0.08 aA	57.835±1.382 aA	48.085±1.939 bC	1.32±0.10 aA	2.69±0.63 aA	3.63±2.07 aA	4.45±2.20 aA

100 MPa - RT	**0**	6.39±0.05 a	6.13±0.03 abc	59.124±1.512 a	58.659±1.587 a	#	#	#	#
	3	6.22±0.08 bA	6.22±0.05 aA	55.284±1.731 aABC	47.415±2.740 bBC	1.58±0.12 aA	2.52±0.48 aA	3.03±0.29 cA	5.77±4.66 aA
	7	5.86±0.09 cA	6.17±0.03 abA	54.951±2.194 aAB	54.955±3.743 abA	1.10±0.14 bA	1.25±0.64 aA	3.78±0.06 bcB	4.59±2.71 aB
	14	5.87±0.03 cA	5.97±0.09 cAB	55.837±0.841 aAB	55.231±1.253 aA	1.22±0.23 abA	1.20±0.51 aA	6.01±1.60 abA	4.82±1.02 aA
	30	5.83±0.05 cA	6.05±0.07 bcA	54.742±1.581 aAB	54.309±0.626 abAB	1.02±0.21 bA	2.29±0.03 aA	6.54±0.89 aA	4.44±1.05 aA
	60	5.80±0.03 cB	6.13±0.06 abcA	56.741±1.983 aA	53.841±1.437 abB	1.34±0.1 abA	1.22±0.74 aB	6.29±1.11 abA	3.99±1.81 aA
0.1 MPa - 4 ºC	**0**	6.39±0.05 a	6.13±0.03 a	59.124±1.512 a	58.659±1.587 a	#	#	#	#
	3	5.94±0.08 bB	6.07±0.02 aB	53.513±1.851 bBC	42.804±3.071 cC	0.47±0.19 bB	0.93±0.10 abB	3.70±0.94 aA	3.58±0.35 aA
	7	5.66±0.08 cBC	6.05±0.04 abAB	54.194±2.531 bAB	53.278±2.709 abA	0.53±0.14 abB	0.78±0.09 bA	4.23±1.24 aB	2.99±0.07 aB
	14	5.38±0.04 dB	5.95±0.07 bcB	54.355±0.984 abB	52.589±1.244 bA	0.87±0.09 abA	1.16±0.15 aA	3.68±2.24 aAB	4.85±0.86 aA
	30	5.42±0.03 dB	5.87±0.03 cB	53.853±1.731 bB	50.074±1.157 bB	0.90±0.18 aA	0.98±0.18 abB	3.67±0.77 aAB	3.70±1.24 aA
0.1 MPa - RT	**0**	6.39±0.05 a	6.13±0.03 a	59.124±1.512 a	58.659±1.587 a	#	#	#	#
	3	5.39±0.04 bD	6.06±0.05 abB	50.831±2.194 bC	47.624±3.285 bBC	1.31±0.21 aA	1.66±0.35 aAB	6.31±1.53 aA	5.55±1.45 aA
	7	5.36±0.05 bD	6.02±0.03 bB	49.853±2.573 bB	52.503±3.646 abA	1.14±0.18 aA	1.24±0.51 aA	7.00±1.55 aA	17.18±9.15 aA

not applicable.

When HS was compared to RF at each storage day it was possible to conclude that for all samples, RF presented an overall lower pH value at the end of storage (30th day), and between HS conditions at the 60th day.

Similar tendencies were detected on BP and BM with a few exceptions, where for instance pH presented in some HS conditions a small initial increase ($p < 0.05$) then remained at a similar value up to the 60th day. The lowest pH values were obtained in 50 MPa storage, except for PM where no significant differences ($p > 0.05$) were detected.

The review of Doulgeraki, Ercolini, Villani and Nychas [90] suggests reasons why the initial microbial load/storage conditions have an important role in the pH variations observed on meat samples over storage, and Drosinos and Board [91] demonstrated how some pH variations on meat products over time can be explained by the metabolic activity of microorganisms capable, for instance, of producing organic acids.

The total colour variation (ΔE) results suggested that colour differences of meat samples (generally ranged from ≈2 to ≈7) did not change during storage at each condition since no significant differences ($p > 0.05$) were found in most of the cases (except for PP at 100 MPa and BP at AP/4 ºC where lower ΔE values were found at the end) (Table 4.1 and Table 4.2). Similarly, no significant differences ($p > 0.05$) were observed for the different storage conditions at each storage day when HS was compared with AP/4 ºC.

This analysis was affected by natural sample heterogeneity, as showed by considerable variations between replicates previous to any storage day/condition in L^* (PP – 50.57±1.46, PM – 52.43±1.34, BP – 39.48±1.99, BM – 48.14±0.94), a^* (PP – 10.31±4.09, PM – 13.19±2.82, BP – 16.01±1.29, BM -16.21±1.56) and b^* (PP – 13.92±1.72, PM – 16.05±0.78, BP – 13.27±2.33, BM – 17.54±1.69).

The pressure effect on meat colour is well documented [2, 92], showing that pressure treatments above 150 MPa over min (even at cold temperatures) cause colour changes similar to those in cooked meat, probably due to globin denaturation and/or heme displacement or release, and oxidation of ferrous myoglobin to ferric metmyoglobin [93]. Nonetheless, these facts are mainly associated with pressure treatments > 200 MPa, levels higher than those applied in HS. Concerning lower pressure levels as used in HS, and its impact on colour variations, Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] obtained lower ΔE values ranging between ≈1.5 and ≈2.3, but this work studied only a storage period of 12h.

Concerning moisture content, PM and PP generally presented no significant differences ($p > 0.05$) over time at each storage condition, with AP/4 ºC exhibiting higher differences ($p < 0.05$) where a decrease tendency was observed (Table 4.2). A similar behaviour was verified for BM and BP where no significant differences ($p > 0.05$) were detected except for 100 MPa where lower values were found at the end of storage (Table 4.1), particularly for BM. The comparison between conditions at each storage day for all samples analysed was also performed, allowing concluding that overall 75 and 100 MPa storage conditions led to values similar to the ones found in samples stored at AP/4 ºC, and that the increase in pressure level, mainly in 100 MPa storage led to lower moisture contents (particularly, in BM samples).

Meanwhile, generally, no significant differences ($p > 0.05$) were detected in drip loss during storage time at each condition in all samples, except for BP at 50 MPa and BM at 50 and 100 MPa, where drip loss increased. When a comparison between storage conditions was made, all samples at HS presented equal to higher drip loss values compared with AP/4 ºC, mainly on bovine samples. Physicochemical changes such as WHC and drip loss are correlated, for instance, to oxidation processes [88]. Simonin, Duranton and de Lamballerie [94] have shown that pressures higher than 300 MPa for a few min during storage can initiate the oxidation of lipids and proteins. Thus, in the present study, this deterioration could occur during long storage under pressure (although less than 300 MPa) in the presence of oxygen inside the packages which were not vacuum packed.

Protein denaturation can be evaluated by changes in protein solubility, since insoluble protein aggregates can no longer be extracted [95]. Regarding protein solubility, values between 173.25 and 188.33 mg/g of meat for pork, and 163.21 and 202.59 mg/g of meat for bovine, were obtained as total protein content before storage. The overall results showed a SP value decrease ($p < 0.05$), with values decreasing with time under the HS conditions. On the other hand, MP as was calculated by the difference between total protein and SP, increased during storage (Table 4.3 and Table 4.4).

Table 4.3. Results (relative values presented as mean ± SE) of sarcoplasmic protein (SP) and myofibrillar protein (MP) obtained for bovine in pieces (BP), and minced bovine (BM) when stored up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), 0.1 MPa at 4 ºC and 0.1 MPa at RT.. Different lower case letters (a-d) and different upper case letters (A-D) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		BP			**BM**		
Storage Condition	**Storage Day**	SP	MP	SE	SP	MP	SE
50 MPa - RT	**0**	0.403 b	0.597 a	0.019	0.424 a	0.576 c	0.026
	3	0.449 aA	0.551 bC	0.001	0.398 abcA	0.602 abcD	0.008
	7	0.400 bAB	0.600 aBC	0.001	0.380 bcB	0.620 abC	0.018
	14	0.442 aA	0.558 bB	0.009	0.433 aA	0.567 cB	0.030
	30	0.388 bA	0.612 aB	0.011	0.352 abA	0.648 bcB	0.022
	60	0.375 bA	0.625 aC	0.001	0.407 cA	0.593 a A	0.043
75 MPa - RT	**0**	0.403 a	0.597 b	0.019	0.424 a	0.576 c	0.026
	3	0.398 aB	0.602 bB	0.014	0.306 bcBC	0.694 abBC	0.001
	7	0.352 bC	0.648 aA	0.011	0.413 aA	0.587 cD	0.009
	14	0.378 abB	0.622 abA	0.006	0.289 cB	0.711 aA	0.035
	30	0.368 abAB	0.632 abAB	0.016	0.373 abAB	0.627 bcAB	0.025
	60	0.340bC	0.660 aA	0.006	0.317 bcA	0.683 abA	0.026
100 MPa - RT	**0**	0.403 ab	0.597 bc	0.019	0.424 a	0.576 c	0.026
	3	0.427 aAB	0.573 cBC	0.011	0.301 bcC	0.699 abB	0.003
	7	0.402 abC	0.598 bcC	0.007	0.287 bcC	0.713 abB	0.002
	14	0.377 bcB	0.623 abA	0.001	0.308 bcB	0.692 abA	0.017
	30	0.355 cB	0.645 aA	0.001	0.320 bB	0.680 bA	0.003
	60	0.356 cB	0.644 aB	0.005	0.266 cA	0.734 aA	0.007
0.1 MPa - 4 ºC	**0**	0.403 a	0.597 b	0.019	0.424 a	0.576 d	0.026
	3	0.401 aB	0.599 bB	0.001	0.273 cC	0.727 bA	0.002
	7	0.352 bA	0.648 aA	0.004	0.213 dD	0.787 aA	0.014
	14	0.385 aB	0.615 bA	0.007	0.439 aA	0.561 dB	0.014
	30	0.341 bB	0.659 aA	0.002	0.337 bAB	0.663 cAB	0.013
0.1 MPa - RT	**0**	0.403 a	0.597 b	0.019	0.424 a	0.576 b	0.026
	3	0.354 bC	0.646 aA	0.009	0.315 bB	0.685 aC	0.001
	7	0.378 abB	0.622 abB	0.008	0.334 bB	0.666 aC	0.009

Table 4.4. Results (relative values presented as mean ± SE) of sarcoplasmic protein (SP) and myofibrillar protein (MP) obtained for pieces of pork (PP), and minced pork (PM) when stored up to 60 days at different storage conditions: 50 MPa, 75 MPa, 100 MPa at variable room temperature (RT), 0.1 MPa at 4 ºC and 0.1 MPa at RT. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		PP			**PM**		
Storage Condition	**Storage Day**	SP	MP	SE	SP	MP	SE
50 MPa - RT	**0**	0.391 bc	0.609 b	0.013	0.377 a	0.623 c	0.016
	3	0.460 aA	0.540 cC	0.004	0.370 abA	0.630 bcC	0.005
	7	0.410 bA	0.590 bB	0.001	0.372 abAB	0.628 bcAB	0.004
	14	0.396 bcB	0.604 abA	0.006	0.347 bcA	0.653 abB	0.006
	30	0.378 cBC	0.622 aAB	0.006	0.350 abcA	0.650 abcC	0.003
	60	0.389 bcA	0.611 abB	0.003	0.323 cA	0.677 aB	0.008
75 MPa - RT	**0**	0.391 ab	0.609 bc	0.013	0.377 a	0.623 c	0.016
	3	0.422 aAB	0.578 cBC	0.021	0.302 cC	0.698 aA	0.004
	7	0.369 bcB	0.631 abA	0.003	0.317 bcB	0.683 abA	0.010
	14	0.404 aB	0.596 cA	0.000	0.345 abA	0.655 bcB	0.010
	30	0.361bcC	0.639 abA	0.001	0.333 bcB	0.667 abB	0.009
	60	0.347 cB	0.653 aA	0.001	0.326 bcA	0.674 abB	0.003
100 MPa - RT	**0**	0.391bc	0.609 bc	0.013	0.377 a	0.623 b	0.016
	3	0.285 dC	0.715 aA	0.013	0.316 bBC	0.684 aAB	0.017
	7	0.436 aA	0.564 dB	0.018	0.322 bB	0.678 aA	0.003
	14	0.428 abB	0.572 cdA	0.015	0.301 bB	0.699 aA	0.002
	30	0.384 bcB	0.616 bcB	0.006	0.314 bC	0.686 aA	0.002
	60	0.366 cAB	0.634 bAB	0.013	0.302 bB	0.698 aA	0.005
0.1 MPa - 4 ºC	**0**	0.391 bc	0.609 ab	0.013	0.377 a	0.623 a	0.016
	3	0.375 cB	0.625 aB	0.008	0.379 aA	0.621 aC	0.015
	7	0.426 bA	0.574 bB	0.014	0.362 aB	0.638 aA	0.038
	14	0.709 aA	0.291 cB	0.013	0.321 aB	0.679 aA	0.004
	30	0.424 bA	0.576 bC	0.010	0.336 aAB	0.664 aBC	0.005
0.1 MPa - RT	**0**	0.391 a	0.609 a	0.013	0.377 b	0.623 a	0.016
	3	0.402 aB	0.598 aB	0.027	0.343 bAB	0.657 aBC	0.012
	7	0.411 aA	0.589 aB	0.015	0.432 aA	0.568 bB	0.016

These results were somewhat similar to those for AP/4 ºC, but although some variation was observed, PP and PM showed no significant differences ($p > 0.05$) at the 30th day when compared with the initial value. After a comparison between HS conditions it was observed that generally, when significant differences ($p < 0.05$) were detected, the increase of pressure level led to lower SP and higher MP values. The decrease of SP in HS samples could be

related to the fact that this fraction is water-soluble while MP is recognized as the salt-soluble protein fraction [96], thus SP was affected to a major extent by its solubility as well as by the pressure level. The same SP decrease/pressure level dependence behaviours were observed by Marcos, Kerry and Mullen [95] when meat was high pressure processed from 200 MPa up to 600 MPa over 20 min, notwithstanding that in the present work lower pressure levels were used however over a longer time period.

4.4.Conclusion

This new food preservation methodology (HS) has been demonstrated to be capable of not only inhibiting the microbial load but also of inactivating microorganisms on raw pork and bovine, both minced and in pieces, mainly at 75 and 100 MPa and variable RT up to 60 days where no energy was required during storage. Under these conditions, significant microbial reductions of TAM, ENT, LAB and YM were achieved leading to a longer shelf-life for these products compared with the traditional RF process.

Regarding physicochemical analyses performed, in general, the pH, colour, and moisture content of bovine and pork showed no significant changes between the HS and AP/4º C treatments (with few exceptions). Regarding drip loss, the values obtained for HS tended to be similar to higher compared with samples stored under AP/4 ºC, while for protein solubility, the SP content decreased for all samples with the results indicating a pressure level dependency, where the increase of pressure level led to a greater reduction in protein solubility.

Although HS appeared to be an alternative to RF for raw meat products further analyses should be performed (relating to enzymes, lipid oxidation, texture, and sensory attributes to better understand the impact of this preservation technology in these products.

Chapter 5 – Raw minced bovine and pork meat in pieces preserved by hyperbaric storage at room temperature and below it

This chapter is based on the paper written during the PhD thesis and published as "Mauro D. Santos, Rodolfo Castro, Ivonne Delgadillo, Jorge A. Saraiva (2019). Improvement of the refrigerated preservation technology by hyperbaric storage for raw fresh meat, in *Journal of the Science of Food and Agriculture*, 100 (3), 969-977 (DOI: 10.1002/jsfa.10083)".

5.1. Overview

Hyperbaric storage, a new food preservation methodology has been mainly studied at RT (≈18-25 ºC) in order to be used as an alternative to the conventional RF [22]. Since RF is responsible for a high energy consumption and high carbon-footprint, for instance 35-50% of the total energy consumption in super and hypermarkets is due to RF contributing to 1% of the total CO_2 worldwide [54], it is of upmost importance the development of new and environmentally friendlier food preservation methodologies.

The unfortunate accident occurred in 1968 with the research submarine Alvin was the starting point for HS since although sunk over 10 months at 1540 m (≈15 MPa) and 4 ºC, some foods were found in consumable conditions when it was recovered [7]. Since then, and mainly after 2012, several food products have been successful preserved under pressure at controlled or variable RT, including fruit juices [10, 15, 41], ready-to-eat meals [40, 49], sliced cooked ham [39], raw meat [11, 13] and raw fish [12, 32].

Nowadays, HS could be defined as a food preservation methodology that relies on food storage under pressure, usually between 50 and 220 MPa (or even below) at variable uncontrolled RT as an alternative to RF. However some works have been pointing some advantages for the food product when HS is used at cold temperatures, such as the work of Otero, Pérez-Mateos, Holgado, Márquez-Ruiz and López-Caballero [32], Otero, Pérez-Mateos and López-Caballero [31]. During HS not only is possible to inhibit the microbial growth but also to inactivate microorganisms (e.g., TAM, ENT, and YM) while the overall physicochemical parameters (e.g., pH, total soluble solids, cloudiness, browning degree, colour, enzymes, among others) are maintained or changed in the same or lower proportion than in samples stored at RF [15, 22, 37].

Notwithstanding, when HS is applied at variable RT there is a reduction of the energy costs and a lower carbon foot-print associated over storage since energy is only required in the compression and decompression phases [9]. The use of HS at cold temperatures leads to higher energy costs and higher carbon foot-print than when applied at variable RT, but still in a lower extent than RF (0.1 MPa) since higher temperatures are used, for instance ≥10 ºC [9].

In the published studies regarding HS at cold temperatures, it was already possible to observe that Cape hake loins stored at 50 MPa and 5 ºC for 7 days presented values of microbial counts and total volatile basic-nitrogen equal to lower than the initial ones [31]. Similar results were also observed for Atlantic mackerel fillets, a fattier fish than Cape hake loins studied by Otero, Pérez-Mateos and López-Caballero [31], where no microbial growth was detected over 12 days at 50 MPa and 5 °C [32]. Moreover, Otero, Pérez-Mateos, Holgado, Márquez-Ruiz and López-Caballero [32] observed no significant lipid degradation in HS contrarily to samples stored at RF, as well several fish-quality indicators (e.g., pH, TVB-N, drip loss, water-holding capacity, among others) were better maintained at HS when compared to RF [32].

As stated before, there are two published studies regarding HS of raw meat, both at variable RT, being mainly only up to 24 hours of storage. In fact, Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] and Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] preserved raw bovine meat (12 hours) and pork minced meat (24 hours), respectively, and microorganisms inactivation was observed from 100 MPa. Moreover, the authors concluded that the overall physicochemical parameters (such as pH, colour and fatty acid profile) did not present detrimental effects comparatively to the initial/RF samples with some exceptions, as b^* parameter in colour analyses in Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] work [11, 13].

Since there are no studies, as far as the authors are aware, comparing HS at RT and HS at cold temperatures, as well no studies regarding lipid oxidation of raw meat over long storage periods under pressure were found, this work aimed to study the HS impact on bovine minced meat (BM) and pork meat in pieces (PP) at 75 MPa/25 ºC and 60 MPa/ 10ºC up to 60 days. For that, microbiological (including pathogenic microorganism surrogates

inoculation) and physicochemical analyses (such as, secondary lipid oxidation) were performed to perceive possible differences between HS at 25 ºC and at 10 ºC.

5.2. Material and Methods

Sample preparation and storage experiments. Raw PP (ca. 2x1x1 cm) and BM, *Longissimus thoracis et lumborum*, were purchased in a local butcher shop, divided into small portions (10-12 g) under aseptic conditions and packaged into low permeability PA/PE (90, Plásticos Macar – Indústria de Plásticos Lda., Palmeira, Portugal). Before the heat-sealing process, the maximum amount of air was manually removed.

Taking into consideration previous studies where 75 MPa and 100 MPa, both at variable RT were capable to guarantee the overall quality of fresh raw meat, in this study, two HS conditions were tested, 60 MPa/10 ºC and 75 MPa/25 ºC, both up to 60 days. For that two high-pressure equipment were used, the SFP FPG13900 (Stansted Fluid Power Ltd., Essex, UK) with a pressure vessel of 37 mm inner diameter and 52 cm height (0.4 L volume) for the 60 MPa/10 ºC condition, and the FPG7100 (Stansted Fluid Power Ltd., Essex, UK) with a pressure vessel of 100 mm diameter and 250 mm height a (2 L volume) for the 75 MPa/25 ºC condition, both using a mixture of propylene glycol and water (40:60 vol/vol) as pressurizing fluid. HS feasibility was assessed by comparison to samples stored at 4 ºC and AP for the same time period (control samples), in the dark and surrounded by the same fluid to mimic the same storage conditions. Storage experiments were carried out in triplicate (for each storage day/meat sample/storage condition).

For microbiological analyses purposes, before any storage period, samples (10 g) were incubated with *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090 suspensions in saline water on a final concentration of 3.21±0.12 and 3.89±0.04 log CFU/g, respectively for PP and 3.82±0.07 and 3.69±0.09 log CFU/g, respectively for BM.

Microbiological Analyses. The microbiological analyses were performed according to the previous studies of Fernandes, Moreira, Duarte, Santos, Queirós, Fidalgo, Santos, Delgadillo and Saraiva [39] and Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13]. Briefly, all samples were analysed for Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB) and Yeasts and Moulds (YM), being used for that 10 g of each sample. After 2 min of homogenization in a STOMACHER 400

(Seward Laboratory Systems Inc., Florida, USA) using Stomacher bags with Ringers solution, decimal dilutions were done and triplicates of all dilutions were plated/incubated in Plate Count agar 30 ± 1 °C over 72 hours for TAM, Man Rogosa and Sharp agar 30 ± 1 °C during 120 hours at anaerobic conditions for LAB, Violet Red Bile Dextrose agar 37 ± 1 °C over 24 hours for ENT, and Rose-Bengal Chloramphenicol agar 25 ± 1 °C during 120 hours for YM. The inoculated samples with *E. coli* and *L. innocua* were plated/incubated in Chromocult Coliform agar/36±2 ºC over 21±3 hours and Listeria Palcam Agar plus Listeria Palcam supplement/36±2 ºC for 48±2 hours, respectively. At the end, petri dishes containing 15–300 CFU were considered and the results expressed as log CFU units per g of meat (log CFU/g).The following higher and lower microbial limits were considered on results presentation: 7.00-1.00, 5.00-1.00, 7.00-1.00, 6.00-1.00, 6.00-1.00 and 6.00-1.00 log CFU/g of meat for TAM, ENT, LAB, YM, *E. coli* and *L. innocua* respectively.

Physicochemical analyses. The pH of the samples stored under pressure was measured and compared to the control samples in 6 random points with a pH/temperature penetration meter Testo 205 (Testo, Inc., New Jersey, USA).

Moisture content and drip loss were analysed by drying samples at 105 ºC until constant weight in the former, values presented in percentage, and in the latter (drip loss), the formula (eq. 4.1) was used. DL is an indicator of drip loss in percentage, m_1 the weight of raw material before storage and m_2 the weight of raw material after storage in g [88].

$$DL = \frac{(m_1 - m_2)}{m_1} \times 100\% \qquad \text{Eq. 4.1}$$

A Konica Minolta CM 2300d colorimeter (Konica Minolta, Japan) was used in colour analyses and the CIE*Lab* parameters determined using the original software (SpectraMagic™ NXSoftware, Konica Minolta, USA), according to the regulations of the International Commission on Illumination: red/green colour (a^*), yellow/blue colour (b^*) and luminosity (L^*) parameters. The colour was measured using the reflectance mode with an aperture diameter of 11 mm, observer angle of 10° and D65 illuminant, in six random spots when placed in a small dish. The colour difference (ΔE) was calculated according to eq. 4.2.

$$\Delta E = \lfloor (L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2 \rfloor^{1/2} \qquad \text{Eq. 4.2}$$

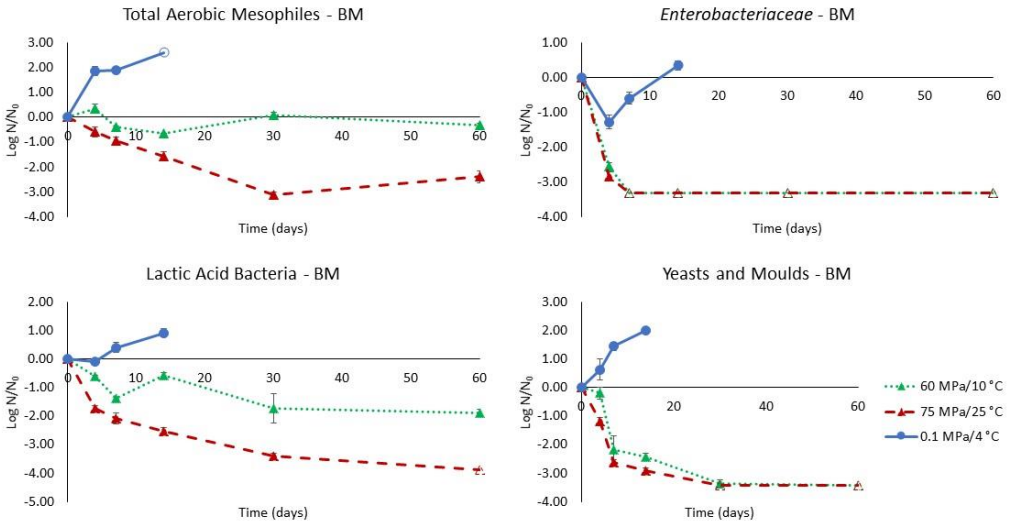
Lipid oxidation was evaluated by quantification of secondary lipid oxidation products using the thiobarbituric acid reactive substances (TBARS) assay, which was performed as described by Vyncke [97] with slight modifications.

Briefly, trichloroacetic acid (25 mL, 7.5%) was added to each sample (5 g) and homogenized over 1 min (MICCRA D-9 Homogenizer, MICCRA GmbH, Deutschland). The resulted suspension was filtered and centrifuged at 3600 g at 4 °C for 20 min (Laboratory Centrifuge 3 K30, Sigma, Germany). The extracted samples (5 mL) were added to 5 mL of 2-thiobarbituric acid (0.02 M in acetic acid 99%) and the mixture immersed in a boiling water bath for 40 min being, at the end, cooled down by cold water. The absorbance was measured at 538 nm (Microplate Spectrophotometer Multiskan GO, Thermo Scientific, USA) and a standard curve was prepared using 1,1,3,3-tetramethoxypropane as standard at concentrations ranging from 0 to 4 μM. TBARS results were expressed as μg MDA/g sample.

Statistical analysis. All microbial and physicochemical analyses were performed in triplicate and samples stored in triplicate for each storage condition/storage day of analysis. Statistical data analysis was performed using the one-way analysis of variance ANOVA, followed by a Tukey's HSD Test, at a 5% level of significance to identify differences between storage conditions and throughout storage time at each condition.

5.3. Results and discussion

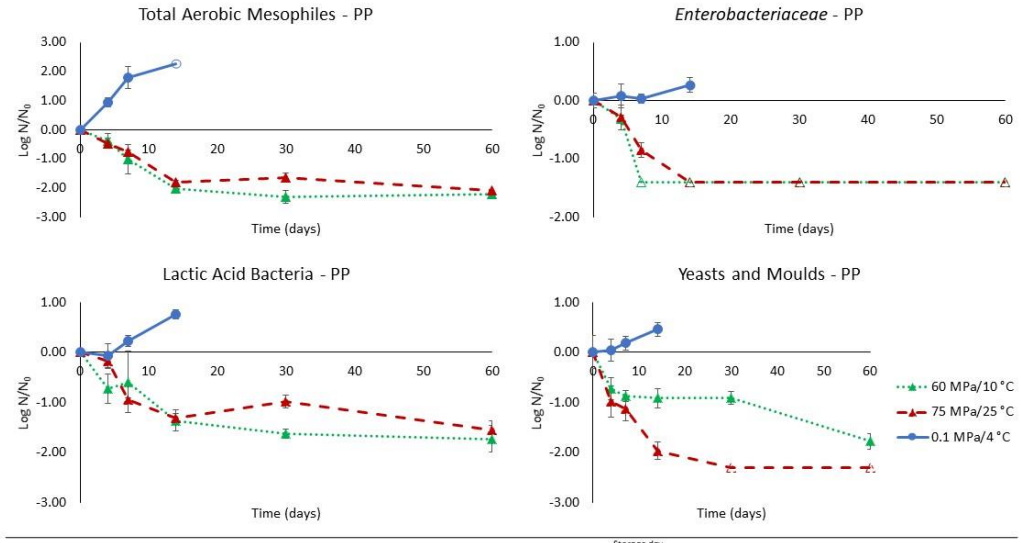
Microbiological analyses. All samples were analysed regarding endogenous TAM, ENT, LAB and YM, being quantified initial microbial loads of 4.74±0.07, 2.39±0.12, 3.99±0.01, 3.32±0.34 log CFU/g respectively, in PP samples, and 4.43±0.03, 4.31±0.14, 4.87±0.09, 4.41±0.06 log CFU/g, respectively, in BM samples.



		Storage day					
BM		0	3	7	14	30	60
TAM	60 MPa/10 °C	b	aB	cdC	dB	abA	cA
	75 MPa/25 °C	a	bC	bB	cC	eB	dB
	0.1 MPa/4 °C	c	bA	bA	aA*	+	+
ENT	60 MPa/10 °C	a	bB	cB*	cB*	cA*	cA*
	75 MPa/25 °C	a	bB	cB*	cB*	cA*	cA*
	0.1 MPa/4 °C	b	dA	cA	aA	+	+
LAB	60 MPa/10 °C	a	bB	cB	abB	cA	cA
	75 MPa/25 °C	a	bC	cC	dC	eB	fB*
	0.1 MPa/4 °C	c	cA	bA	aA	+	+
YM	60 MPa/10 °C	a	aB	bB	bB	cA	cA*
	75 MPa/25 °C	a	aC	aB	aB	aA*	aA*
	0.1 MPa/4 °C	d	cA	bA	aA	+	+

not applicable

Figure 5.1. Total aerobic mesophiles **(**TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on bovine minced meat (BM) expressed in Log N/N_0 (mean ± SE), up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-f) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively, and * represent samples with microbial counts below or above the considered limit.



PP		Storage day 0	3	7	14	30	60
TAM	60 MPa/10 °C	a	abB	bB	cC	cB	cA
	75 MPa/25 °C	a	bB	cB	dB	dA	eA
	0.1 MPa/4 °C	d	cA	bA	aA*	+	+
ENT	60 MPa/10 °C	a	bA	cC*	cB*	cA*	cA*
	75 MPa/25 °C	a	bA	cB	dB*	dA*	dA*
	0.1 MPa/4 °C	a	aA	aA	aA	+	+
LAB	60 MPa/10 °C	a	abB	abAB	bcB	cB	cA
	75 MPa/25 °C	a	aAB	bB	cB	bA	cA
	0.1 MPa/4 °C	b	bA	bA	aA	+	+
YM	60 MPa/10 °C	a	bB	bB	bB	bA	cA
	75 MPa/25 °C	a	bB	bB	cC	cB*	cB*
	0.1 MPa/4 °C	b	bA	abA	aA	+	+

not applicable

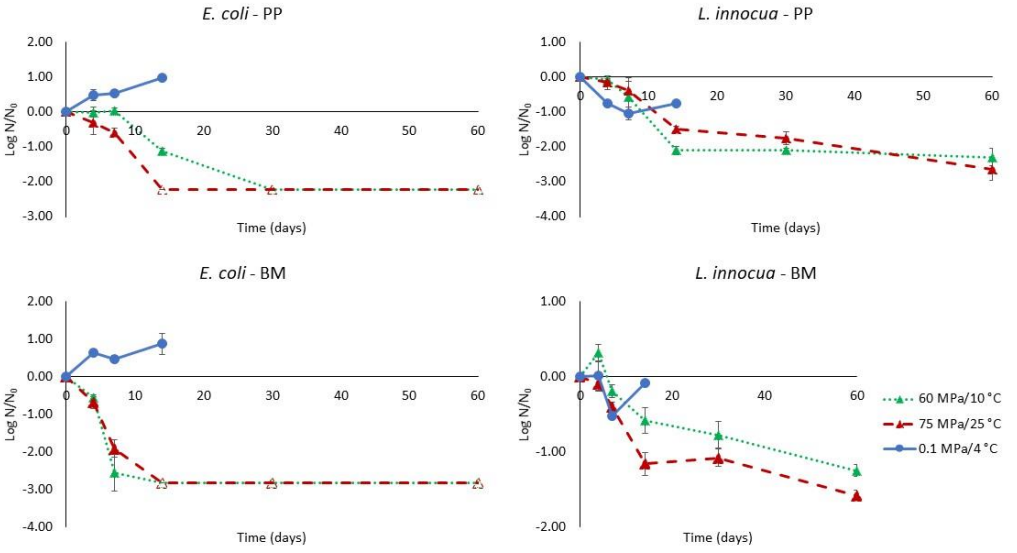
Figure 5.2. Total aerobic mesophiles **(**TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on pork meat in pieces (PP) expressed in Log N/N_0 (mean ± SE), up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-e) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively, and * represent samples with microbial counts below or above the considered limit.

Over 15 days of AP/4 ºC storage, both samples (BM and PP) presented an overall microbial load increase ($p < 0.05$) for TAM, ENT, LAB and YM, where values above 7.00 log CFU/g were obtained for TAM at the 15th day (Figure 5.1 and Figure 5.2). Notwithstanding, ENT, LAB and YM did not reach the considered limits in this study over that time when samples were stored under AP/4 ºC, being the increase observed below 1.00 log unit for all of them, except YM in BM samples (2.01±0.03 log CFU/g).

The comparison between these results and samples stored under pressure allows concluding an overall equal to better microbial preservation in the latter for both conditions, 60 MPa/10 ºC and 75 MPa/25 ºC. Hence, in PP samples microbial inactivation ($p < 0.05$) was observed for TAM, ENT, LAB, and YM, being detected reductions of >2.00, >1.30, >1.50, and >1.70 log units, respectively, at the 60th day of storage, while values below detection limit (1.00 log CFU/g) were observed in ENT at 60 MPa/10 ºC from the 7th storage

day, and in ENT and YM at 75 MPa/25 ºC from the 14th and 30th day of storage, respectively. Similar results were obtained in BM samples since microbial loads reductions were verified along storage for all microorganisms, except at 60 MPa/10 ºC for TAM, where just a slight decrease was detected (-0.32±0.09 log CFU/g at the 60th storage day). Apart of this result, reductions of >2.30, >3.30, >1.80 and >3.40 log units were observed for TAM, ENT, LAB and YM, respectively, over 60 days.

This work aimed also studying the HS effect on pathogenic microorganisms surrogates, and to do so, PP and BM samples were inoculated with *E. coli* and *L. innocua* being the initial microbial load for PP 3.21±0.12 and 3.89±0.04 log CFU/g, respectively, and for BM 3.82±0.07 and 3.69±0.09 log CFU/g. Over storage, 60 MPa/10 ºC and 75 MPa/25 ºC were capable to inactivate *E. coli* ($p < 0.05$), being found values below the detection limit (1.00 log CFU/g) in most of the cases after 15 days of storage, while RF was not capable to inhibit its growth. The HS effect in *L. innocua* (both conditions) allowed its inactivation ($p < 0.05$) however in a minor extent than in *E. coli* since microbial reductions of >2.20 and >1.20 log units were obtained for PP and BM, respectively (Figure 5.3).



			Storage day				
	PP						
E. coli	60 MPa/10 °C	a	aAB	aB	bB	cA*	cA*
	75 MPa/25 °C	a	abB	bC	cC*	cA*	cA*
	0.1 MPa/4 °C	c	bA	bA	aA	+	+
L. innocua	60 MPa/10 °C	a	aA	aA	bC	bB	bA
	75 MPa/25 °C	a	aA	aA	bB	bA	cA
	0.1 MPa/4 °C	a	bB	bA	bA	+	+
	BM	0	3	7	14	30	60
E. coli	60 MPa/10 °C	a	bB	cB	cB*	cA*	cA*
	75 MPa/25 °C	a	bB	cB	dB*	dA*	dA*
	0.1 MPa/4 °C	d	bA	cA	aA	+	+
L. innocua	60 MPa/10 °C	ab	aA	bA	cB	cA	dA
	75 MPa/25 °C	a	aB	bB	cC	cA	dB
	0.1 MPa/4 °C	a	aAB	bB	aA	+	+

not applicable

Figure 5.3. *Escherichia coli* and *Listeria innocua* counts on pork meat in pieces (PP) and bovine minced meat (BM) expressed in Log N/N_0 (mean ± SE), up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively, and * represent samples with microbial counts below or above the considered limit.

Differences between gram-positive bacteria (*L. innocua*) and gram-negative bacteria (*E. coli*) resistance to pressure were already pointed for instance in the work of Wuytack, Diels and Michiels [98] where gram-positive bacteria seemed to be more resistant to high hydrostatic pressure (200-400 MPa at 25 ºC during 15 min) than gram-negative bacteria, although it depends on the specific microorganism.

Notwithstanding, as the present work intended to compare HS as an alternative (75 MPa/25 ºC) and a complement (60 MPa/10 ºC) to RF, it was possible to perceive a similar behaviour between HS conditions at the end of storage (except 60 MPa/10 ºC for TAM in BM sample as already mentioned), and when differences were observed, for instance YM in PP and LAB in BM samples at the 30th day, 60 MPa/10 ºC presented lower microbial reductions than 75 MPa/25 ºC. Nonetheless, minor differences were also obtained at the

same day of storage (30th) in PP samples for TAM and LAB, and in this case, 60 MPa/10 ºC allowed a lower microbial load reduction than 75 MPa/25 ºC.

As was pointed in previous studies, for instance in Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] and Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] works, in this study it was possible to conclude that 75 MPa/25 ºC could be used as an alternative to RF for raw meat preservation over 60 days of storage since at these conditions not only an inhibition effect was observed but also microorganisms inactivation was achieved. Therefore, the results obtained were in accordance to Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] where 50 MPa allowed a microbial effect similar to RF and 100/150 MPa led to a microbial inactivation over 12h, as well as 10 days at 50 MPa at variable RT pointed to a possible microbial shelf-life extension. Moreover, raw minced pork meat was also preserved up to 24h at 100 MPa and variable RT by microbial inactivation [11].

Hence, this work allowed concluding that HS at low temperatures could be used as a complement to RF, since lower microbial counts were obtained over storage when compared to samples stored under RF, leading to similar results of 75 MPa/25 ºC condition at a lower pressure level.

Physicochemical analyses. The raw meat preservation conditions, as well its initial microbial load have an impact on pH variations along storage since some of them could be explained by microorganism metabolic activity and their metabolites [90, 91]. In this work the pH analysis allowed to observe similar behaviours over storage between the two tested samples (Table 5.1). The initial pH for PP and BM was 5.51±0.02 and 5.62±0.03, respectively. Throughout storage, pH presented an increase tendency for 60 MPa/10 ºC, reaching values of 5.70±0.01 and 5.93±0.04 at the 60th day for PP and BM, respectively, being the same tendency detected in 75 MPa/25 ºC storage condition (5.85±0.03 and 5.98±0.05, respectively, at 60th day).

Table 5.1. Results (values presented as mean ± SE) of pH, moisture content (%), drip loss (%), and thiobarbituric acid reactive substances (TBARS, µg MDA/g of meat) obtained for pork meat in pieces (PP) and bovine minced meat (BM), when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		pH		**Moisture Content (%)**		**Drip loss (%)**		**TBARS (µg MDA/g of meat)**	
Storage Condition	**Storage Day**	PP	BM	PP	BM	PP	BM	PP	BM
60 MPa - 10 ºC	**0**	5.51±0.02 b	5.62±0.03 c	69.87±0.51 a	68.12±0.76 a	#	#	0.488±0.097 c	0.695±0.105 c
	3	5.52±0.04 bB	5.64±0.05 bcB	68.00±1.88 aA	66.62±0.82 aA	8.20±2.25 aA	1.19±0.18 aA	0.516±0.199 cA	0.956±0.061 bcA
	7	5.45±0.06 bB	5.66±0.03 bcA	66.87±0.10 aB	68.46±1.08 aA	8.82±0.80 aA	0.94±0.12 aA	0.681±0.096 bcA	0.892±0.194 bcA
	14	5.54±0.02 bB	5.69±0.03 bcA	68.86±0.74 aA	68.63±1.05 aA	12.53±1.18 aA	0.59±0.05 aB	1.157±0.382 bB	1.094±0.213 bA
	30	5.52±0.03 bB	5.73±0.05 bA	67.30±1.70 aAB	68.06±0.55 aA	11.06±2.77 aA	0.89±0.50 aB	1.795±0.217 aA	1.154±0.095 bB
	60	5.70±0.01 aB	5.93±0.04 aA	66.97±0.98 aA	68.11±0.36 aA	8.97±1.17 aA	0.75±0.35 aB	1.917±0.198 aA	1.675±0.141 aA
75 MPa - 25 ºC	**0**	5.51±0.02 e	5.62±0.03 c	69.87±0.51 ab	68.12±0.76 ab	#	#	0.488±0.097 b	0.695±0.105 b
	3	5.60±0.01 cdA	5.81±0.02 bA	70.71±0.66 aA	68.56±0.75 abA	6.03±0.51 aA	1.21±0.02 bA	0.841±0.154 bA	0.832±0.324 bA
	7	5.58±0.01 dA	5.65±0.04 cA	68.02±0.44 bAB	69.93±0.36 aA	6.25±1.22 aA	1.31±0.45 bA	0.922±0.239 bA	0.927±0.083 bA
	14	5.66±0.01 bcA	5.79±0.07 bA	67.96±0.91 bA	67.98±0.85 bA	10.46±0.16 aA	1.83±0.25 abA	1.942±0.158 aA	1.019±0.162 bA
	30	5.71±0.04 bA	5.77±0.01 bA	65.67±0.71 cB	65.98±0.81 cB	10.37±3.63 aA	2.87±0.59 aA	2.169±0.117 aA	1.598±0.129 aA
	60	5.85±0.03 aA	5.98±0.05 aA	64.93±0.85 cA	65.67±0.34 cB	9.39±0.09 aA	2.76±0.85 aA	2.215±0.237 aA	1.621±0.194 aA
0.1 MPa - 4 ºC	**0**	5.51±0.02 a	5.62±0.03 a	69.87±0.51 a	68.12±0.76 a	#	#	0.488±0.097 b	0.695±0.105 b
	3	5.46±0.02 abB	5.60±0.01 aB	68.36±0.58 aA	67.09±2.73 aA	6.99±3.40 aA	2.44±0.87 aA	0.536±0.147 bA	0.796±0.212 abA
	7	5.42±0.04 abcB	5.44±0.02 bB	69.28±1.01 aA	68.26±2.99 aA	8.12±2.36 aA	0.72±0.30 bA	0.578±0.115 abA	0.963±0.172 abA
	14	5.37±0.05 bcC	5.39±0.01 bcB	69.15±0.37 aA	69.89±0.73 aA	6.15±1.14 aB	0.77±0.07 bB	0.694±0.196 abB	1.197±0.087 aA
	30	5.33±0.03 cC	5.38±0.02 cB	69.21±0.86 aA	68.69±0.06 aA	7.35±0.93 aA	1.06±0.04 bB	0.895±0.084 aB	0.975±0.169 abB

not applicable

The same was not observed in refrigerated samples since a decrease of the initial values was detected for both samples, reaching values of 5.33±0.03 and 5.38±0.02 at the 30th day. The comparison between the two tested HS conditions permitted to conclude an overall higher pH for samples stored at 75 MPa/25 ºC mainly in PP samples, being the AP/4 ºC the one that presented lower pH values at the 30th day when compared to both HS conditions.

Concerning moisture content (Table 5.1), the initial values (%) of 69.87±0.51 and 68.12±0.76 for PP and BM, respectively, remained unchanged ($p > 0.05$) over storage at 60 MPa/10 ºC and AP/4 ºC, while for 75 MPa/25 ºC moisture content has decrease ($p < 0.05$) along time (64.93±0.85 and 65.67±0.34 for PP and BM, respectively, at the 60th day of storage). At the 30th day AP/4 ºC presented higher moisture content ($p < 0.05$) than 75 MPa/25 ºC for both samples, being the differences between the two HS conditions only perceptible ($p < 0.05$) in BM samples (lower values for 75 MPa/25 ºC).

On the other hand, drip loss (%) only presented differences ($p < 0.05$) for BM samples at 75 MPa/25 ºC and AP/4 ºC during storage since the value obtained at the 3rd day increased and decreased, respectively, being also found higher values ($p < 0.05$) for 75 MPa/25 ºC at the end of storage when compared to AP/4 ºC and 60 MPa/10 ºC (Table 5.1). Meanwhile, PP did not reveal significant differences ($p > 0.05$) over storage neither between storage conditions at each storage day (except in the 14th day at AP/4 ºC).

The moisture content and drip loss results revealed to be related due to the higher drip occurred during storage at 75 MPa/25 ºC that led to lower moisture contents at the end of storage at that condition (Table 5.1). Furthermore, these two analyses presented higher differences for BM samples than in PP, being this in accordance to Fjelkner-Modig and Tornberg [99] that reported for pork meat a higher water-retaining capacity than for bovine, however being the variations between species larger for pork than for bovine samples, and keeping in mind that in the present work was used minced bovine meat and not in pieces as in pork samples. Notwithstanding, the variations observed over storage could be due to lipid and protein oxidation [94], as well the presence of oxygen inside the packages that could trigger lipids and proteins oxidation since WHC and drip loss are correlated, for instance, to oxidation processes [88]. The decrease of moisture content observe for HS should be taken in account in further studies to search for its causes.

The colour analyses performed (Table 5.2) allowed concluding that colour differences represented by ΔE at the end of each storage ranged between 3.21±0.93 and

4.83±2.22 for PP and 2.09±0.76 and 4.37±1.71 for BM. Generally, no significant differences ($p > 0.05$) were observed in ΔE parameter along time at each storage condition, neither between conditions at each storage day, with few exceptions, as for BM and the 30th day that presented a higher value ($p < 0.05$) at 75 MPa/25 ºC than at 60 MPa/10 ºC and AP/4 ºC.

Table 5.2. Results of *L**, *a**, *b** and ΔE colour parameters (values presented as mean ± SE) for pork meat in pieces (PP), and bovine minced meat (BM) when stored up to 60 at different storage conditions: 60 MPa-10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

		L*		***a****		***b****		**ΔE**	
Storage Condition	**Storage Day**	PP	BM	PP	BM	PP	BM	PP	BM
60 MPa - 10 ºC	**0**	59.41±1.41 a	55.19±0.83 a	2.45±0.91 a	15.42±1.11 a	15.57±0.80 bc	19.87±0.24 b	#	#
	3	61.44±1.99 aA	56.15±2.28 aA	3.57±0.69 aA	12.58±0.41 bA	15.24±0.30 bcA	19.14±1.56 bA	4.28±1.23 aA	4.01±1.05 aA
	7	61.76±1.37 aAB	57.41±0.06 aA	3.28±1.09 aA	14.32±0.83 abA	16.81±0.53 abAB	20.64±1.09 abA	2.80±0.04 abA	2.66±1.00 aB
	14	60.00±1.34 aB	54.85±2.30 aA	3.36±0.97 aA	14.89±1.31 abA	14.99±0.31 cB	19.16±0.49 bA	2.06±0.21 bB	4.07±0.01 aAB
	30	59.04±0.25 aAB	54.76±2.46 aA	1.94±0.16 aB	13.71±1.23 abA	13.95±0.85 cB	19.42±0.48 bA	2.52±0.12 abA	2.53±0.33 aB
	60	60.68±0.61 aA	55.68±1.39 aA	2.47±0.49 aB	15.58±0.09 aA	17.89±0.74 aA	22.72±0.60 aA	3.21±0.93 abA	3.77±0.52 aA
75 MPa - 25 ºC	**0**	59.41±1.41 bc	55.19±0.83 ab	2.45±0.91 bc	15.42±1.11 a	15.57±0.80 ab	19.87±0.24 ab	#	#
	3	63.70±0.35 abA	55.26±2.60 abA	1.07±0.89 cB	11.69±0.50 bcA	13.75±0.49 bB	17.62±0.14 cdA	6.68±1.10 aA	4.72±0.98 aA
	7	62.83±3.10 abA	57.27±1.26 aA	3.63±1.32 bcA	10.31±0.53 cC	18.05±2.14 aA	17.22±0.01 dB	5.14±3.06 aA	6.15±0.43 aA
	14	64.52±0.99 aA	53.75±0.06 abA	2.95±0.97 bcA	12.71±0.91 abcAB	17.36±0.39 aA	18.41±0.57 bcdAB	6.18±1.49 aA	3.51±0.35 aB
	30	60.39±0.89 abcA	52.52±0.09 bA	4.39±0.38 abA	13.71±2.46 abA	17.13±1.10 aA	18.88±1.19 abcA	3.22±1.55 aA	5.07±1.00 aA
	60	58.14±1.43 cB	54.22±2.67 abA	6.26±0.94 aA	11.50±0.33 bcB	18.15±0.61 aA	20.34±0.29 aB	4.83±2.22 aA	4.37±1.71 aA
0.1 MPa - 4 ºC	**0**	59.41±1.41 ab	55.19±0.83 b	2.45±0.91 ab	15.42±1.11 a	15.57±0.80 a	19.87±0.24 a	#	#
	3	57.05±1.82 bB	55.73±0.22 bA	1.23±0.56 bB	11.97±0.46 bcA	11.86±0.66 cC	18.30±0.08 bcA	5.47±1.21 aA	3.87±1.61 aA
	7	56.65±2.12 bB	57.09±0.21 aA	2.76±0.22 aA	12.38±0.49 bB	14.19±0.78 abB	19.11±1.03 abAB	3.43±1.14 aA	3.82±1.84 aAB
	14	62.27±1.91 aAB	55.27±0.04 bA	2.11±0.13 abA	10.60±0.11 cB	14.34±0.24 abB	17.45±0.03 cB	3.43±0.04 aB	5.47±0.99 aA
	30	58.43±0.68 abB	55.04±0.31 bA	1.96±0.16 abB	14.20±0.01 aA	13.50±0.36 bcB	19.06±0.30 abA	3.38±0.63 aA	2.09±0.76 aB

not applicable

Although no clear trends were found on ΔE in both HS conditions, the comparison of both at each storage day revealed that when significant differences were observed lower values ($p < 0.05$) were found at 60 MPa/10 ºC than at 75 MPa/25 ºC, being these similar to AP/4 ºC.

When *L**, *a** and *b** parameters were closely analysed several fluctuations were detected along storage but at the end of storage period in each storage condition, generally, there were not found significant differences ($p > 0.05$) from the first day. This could be due to the lower pressure level used in HS experiments since a whitening effect (increase of *L** and decrease of *a**) is commonly observed when raw meat is high pressure treated [100]. The HS impact in colour parameter could be due to the long time period that samples are under pressure, although being much lower than 150 MPa, pressure level at which colour changes similar to those in cooked meat can occur [2, 92] probably due to globin denaturation and/or to heme displacement or release, and oxidation of ferrous myoglobin to ferric metmyoglobin [93].

One of the most important meat degradation mechanisms is oxidation and it can result in sensorial degradation (e.g., color, aroma and flavour changes) and in nutritional value losses [101]. Generally, it is considered that meat oxidation can be initiated endogenously via metallic ions (mainly heminic iron), and via exogenous reactive oxygen species, while other parameters, such as animal species, muscle type, diet, post-slaughter processes, among others, can influence this reaction [101].

Regarding secondary lipid oxidation (Table 5.1), studied by TBARS, allowed observing that HS at both conditions led to an oxidation increase ($p < 0.05$) during storage time, since values increased from 0.488±0.097 and 0.695±0.105 to 1.917±0.198 and 1.675±0.141 µg MDA/g of meat for PP and BM, respectively at 60 MPa/10 ºC over 60 days. This increase was also observed at 75 MPa/25 ºC condition, reaching values of 2.215±0.237 and 1.621±0.194 µg MDA/g for PP and BM, respectively, at end of storage. Notwithstanding, a tendency increase was also observed at AP/4 ºC however in a minor extent, since generally lower ($p < 0.05$) TBARS values (0.895±0.084 and 0.975±0.169 µg MDA/g at the 30th day) were detected at this storage conditions when compared to both HS.

Concerning high pressure effect on lipid oxidation, several hypotheses have been suggested, including the release of iron from heme and conformational changes in hemoproteins resulting in greater exposure of unsaturated fatty acids to catalytic heme

groups [101]. Moreover, Cheah and Ledward [102] reported that water-soluble components of meat, such as low molecular weight iron compounds and myoglobin, hemoglobin and ferritin are important initiators of lipid oxidation after high pressure treatment. Although this lipid oxidation promoted by pressure is usually recognized for pressures >300-400 MPa, as Cheah and Ledward [103] observed pork minced meat pressure treated up to 800 MPa during 20 min at RT, HS uses lower pressure levels (in the present work up to 75 MPa) however over longer time periods (up to 60 days in this case), that could led to an impact on this parameter.

5.4.Conclusion

The present work allowed concluding that HS could be used as an alternative to RF (75 MPa/25 ºC) or a complement to it (60 MPa/10 ºC) for raw meat preservation, since was capable to guarantee not only a microbial load inhibition but also microorganisms inactivation. Moreover, the lower pressure level tested in this work allowed to obtain a similar microbial load behaviour when compared to the higher pressure level over 60 days, leading both to lower microbial counts at the 30th day of storage than AP/4 ºC.

Although the overall physicochemical analyses performed did not reveal a clear tendency for better results on 60 MPa/10 ºC condition compared to HS at 25 ºC, by the results obtained the colour parameter could be better maintained, and lower drip loss values could be attained at 60 MPa/10 ºC. Moreover, it must be noted that lipid oxidation (TBARS) presented an increase tendency over time in all samples, being observed higher values at the end of storage in both HS conditions when compared to AP/4 ºC.

Further studies are of interest to be carried out in order to optimize and assess the HS impact at cold temperatures to completely understand the usefulness of HS at these temperatures since in this case this food preservation methodology is not so much environmentally friendlier than RF, being needed several advantages for the food product when these conditions are used.

Chapter 6 – Quality evolution of raw meat under hyperbaric storage – Fatty acids, volatile organic compounds and lipid oxidation profiles

This chapter is based on the paper written during the PhD thesis and under revision as "Mauro D. Santos, Susana Casal, Ivonne Delgadillo, Jorge A. Saraiva (2019). Quality Evolution of Raw Meat under Hyperbaric Storage – Fatty Acids, Volatile Organic Compounds and Lipid Oxidation Profiles, in *Journal of Food Science* (submitted)".

6.1. Overview

Lately, several studies regarding a new food preservation methodology known as HS have been published, claiming the shelf-life extension of several food products when compared with conventional RF process, at the same time that a lower carbon footprint and a lower energy consumption can be attained [9, 22]. Up to now, several products were already tested by HS, such as, fruit juices [10, 15, 47], sliced cooked ham [39], raw meat [11, 13], raw fish [12, 32], ready-to-eat meals [40, 49], among others.

This new food preservation methodology relies on food storage under pressure (usually between 50 and 220 MPa) and has been tested at variable RT (≈18-25 ºC) as an alternative to RF or at cold temperatures (>8-10 ºC) as a complement to it [11, 22], with some advantages pointed on the latter for the global food product quality [31, 32]. All the works, although depending on the food product and pressure level applied, have concluded for the HS feasibility on the microbial control over storage, being possible to observe not only microbial growth slowdown/inhibition, but also microorganisms inactivation, including spores, over days to weeks [13, 14, 22, 46]. Moreover, several physicochemical parameters, such as pH, total soluble solids, browning degree, colour, enzymes activity, lipid oxidation, or fatty acid profile, are generally maintained or changed in the same or lower proportion than in RF samples [11, 15, 22, 37].

Concerning raw meat storage under pressure, Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] and Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] verified microbial control of samples using pressure levels between 50 and 150 MPa, observing simultaneously overall preservation of most

physicochemical parameters. No considerable differences in the fatty acids profile and lipid oxidation along storage were reported, except for SFA and *trans*-MUFA that presented some differences after 6 days of storage at HS, although without a consistent pattern relating HS and fatty acid composition [13].

Notwithstanding, these results on lipid oxidation and fatty acids profiles are related to short storage periods, as 24h under pressure (Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11]. Thus, there is a lack of information regarding lipid oxidation over longer storage periods as HS seems to allow, being this kind of knowledge of upmost importance to evaluate HS feasibility for the preservation of raw meat.

In fact, fatty acids are associated to several aspects in meat quality, influencing the fat tissue firmness (hardness), shelf-life (lipid and pigment oxidation), flavour, tenderness and juiciness [104]. For instance, firmness is affected by the different melting points of fatty acids (as unsaturation increases the melting point declines), and the relation between fatty acids and shelf-life relies on the propensity of unsaturated fatty acids (especially those with more than two double bonds) to oxidise, leading to the development of rancidity along time [104].

Likewise, oxidation is one of the important parameters related to meat and meat products degradation, since the newly formed compounds can react with proteins, vitamins, or other components, leading to loss of nutritional value and organoleptic characteristics modification [101]. Several analyses, such as, peroxides value (PV), TBARS, fluorescent compounds, have been used to study lipid oxidation behaviour (primary, secondary and tertiary, respectively) on food products [12]. The relationship between lipid oxidation and volatile organic compounds (VOCs) development during storage is reported in the literature, with VOCs profile being used as a meat spoilage indicator [105-107]. Several compounds, such as aldehydes, ketones, alcohols, hydrocarbons, have been identified as spoilage indicators due to their presence/increase over storage in different types of meat [107-114].

Since, as far the authors are aware, there are no studies regarding VOCs profile of raw meat stored by HS, as well there is a lack of knowledge regarding raw meat fatty acid and lipid oxidation profiles when preserved by this methodology over longer periods, the aim of this work was to study the HS impact on fatty acids, VOCs and lipid oxidation profiles of pork meat in pieces (PP) and bovine minced meat (BM), both at 75 MPa/25 ºC and 60

MPa/10 ºC up to 60 days, being these results compared to samples stored at RF (4 ºC) up to 30 days (control samples).

6.2. Materials and Methods

Sample preparation and storage experiments. Raw PP, ca. 2x1x1 cm and BM, *Longissimus thoracis et lumborum*, were purchased from a local butcher shop, divided into small portions (≈10-12 g) under aseptic conditions and packaged into low permeability PA/PE bags (90, Plásticos Macar – Indústria de Plásticos Lda., Palmeira, Portugal), by heat-sealing, with air being manually removed as much as possible before. Pieces of meat and mincing are among the most common commercialized forms of these meat types, being this the reason for its use in this work. Storage experiments were carried out in triplicate (for each storage day/meat sample/storage condition).

Two high-pressure equipment were used in this work, one for each storage condition over 60 days. A SFP FPG13900 (Stansted Fluid Power Ltd., Essex, UK), with a pressure vessel of 37 mm inner diameter and 52 cm height (0.4 L volume), was used for the 60 Mpa/10 ºC storage condition, and a FPG7100 (Stansted Fluid Power Ltd., Essex, UK), with a pressure vessel of 100 mm diameter and 250 mm height a (2 L volume), for the 75 Mpa/25 ºC storage condition, both using a mixture of propylene glycol and water (40:60 v/v) as pressurizing fluid. These storage conditions were selected from previous studies, since it was found to be possible to obtain good results for fresh raw meat preservation, taking into account microbial and physicochemical parameters [115]. For assessment of HS feasibility, control samples were stored at 4 ºC and AP (RF) for the same time period, in the dark and surrounded by the same fluid to mimic the same storage conditions.

Fatty acids profile. For the fatty acids profile determination the O'Fallon, Busboom, Nelson and Gaskins [116] methodology was adopted. Briefly, 1 g of each sample was placed into a screw-cap Pyrex culture tube to which 1.0 mL of the C11:0 triglyceride internal standard (0.5 mg of TriC11:0/mL of methanol), 0.53 mL of 10 N potassium hydroxide in water, and 4.0 mL of methanol were added. Tubes were incubated in a shaking water bath at 55 ºC for 1.5 hours, with vigorous handshaking for 5 sec every 20 min. Subsequently, the tubes were cooled in a cold tap water bath and it was added 0.44 mL of 24 N sulfuric acid (in water), being then vortex-mixed until potassium sulfate precipitation. After more 1.5

hours at 55°C with handshaking for 5 sec every 20 min, the tubes were cooled, and 2.0 mL of hexane was added at each tube being vortex-mixed for 5 min and centrifuged for 5 min. The hexane layer was collected to vials containing anhydrous sodium sulphate, transferred into GC vials and stored at -20 ºC until GC analyses.

The supernatant was injected (1 µL; 250 °C) at a slit ratio of 1:50 in a Chrompack CP 9001 gas chromatograph (Middelburg, The Netherlands) with flame ionization detection (FID) at 260 °C. Separation was achieved on a FAME CP-Select CB column (Agilent, 0.25 µm, 100 m × 0.25 mm, USA) heated from 179 (3 min hold) to 240 °C (2 min hold) at a 3 °C/min rate. FID response factors for each individual fatty acid were calculated using a certified standard mixture of fatty acids methyl esters (TraceCert – Supelco 37 component FAME mix, USA). Fatty acids were expressed in relative percentage of total calibrated response areas.

Volatile organic compounds profile. VOCs profile determination was performed by HS-SPME using a fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm) from Supelco (Bellefonte, USA). Samples were weighted (2.0 g) and placed in vials, where 3.0 mL of ultrapure water, 1.0 mL of cyclohexanone (5.0 µg/mL, internal standard), 1.44 g of sodium hydroxide, and a magnetic stirring rod were added being the vials immediately sealed with a polypropylene cap with silicon septum. The volatiles were released at 60 ºC during 20 min with continuous agitation. After that, the fiber was exposed during 20 min at the same temperature with constant agitation, for volatiles adsorption.

An Agilent GC-7890 gas chromatographer equipped with a mass spectrometer Agilent 5977B, and a DB-5 MS Capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent, USA) were used for volatiles determination.

The fiber was inserted into the injection port of the GC system for thermal desorption in the injector port held at 250 ºC in the splitless mode, over 5 min, with helium at a linear velocity of 30 cm/sec and a total flow of 24.4 mL/min as mobile phase.

The oven temperatures were the following: 40 °C (3 min); 3 °C/min until 235 °C (total 60 min). The transfer line was maintained at 280ºC and the ionization source at 230 °C with ionization energy of 70 eV, and with an ionization current of 0.1 kV. All mass spectra were acquired by electron ionization in the m/z 40–650 range set in full scan mode. Tentative

identification was based on the match value with a mass spectra database (NIST 11) and individual standards when available. The volatile profiles were evaluated on the basis of the relative ratios of each m/z sum area to the total area of the chromatogram.

Lipid oxidation profile

Primary lipid oxidation – Peroxide Value (PV). The lipids were extracted by the Folch method with slight modifications [117]. Five g of sample were homogenized with 50 mL of a chloroform:methanol mixture (2:1) for 2 min at 4 °C (MICCRA D-9 Homogenizer, MICCRA GmbH, Deutschland). At the end, Whatman N°1 filter paper was used for filtration being then added 5 mL of distilled water followed by homogenization. The tubes were centrifuged at 3000 *g* at 4 °C for 15 min (Laboratory Centrifuge 3 K30, Sigma, Germany), being the bottom layer collected for a round-bottom flask and the solvent evaporated at 40 °C.

The IDF Standard method by Gheisari, Møeller, Adamsen and Skibsted [118] was used for PV determination with few modifications. The extracted lipids (0.1 g) were added to 8.9 mL of chloroform/methanol (7:3, v/v) and vortex-mixed. Then, 50 μL of ammonium thiocyanate solution was added and mixed, and after, 50 μL of iron (II) solution was also added and mixed (2–4 sec). After 5 min of incubation at RT and in the absence of light, the absorbance of the sample was measured at 500 nm (Microplate Spectrophotometer Multiskan GO, Thermo Scientific) against a blank containing all chemicals except the sample. The PV was expressed as mg Fe/kg lipids.

Standard ferric solutions (5–50 μg Fe/mL) were prepared for the determination of the calibration curve used in calculations. For that, 0.5 g of iron powder was dissolved in 50 mL of hydrochloric acid (10 N) and 1–2 mL of hydrogen peroxide (30%). The solution was boiled (5 min) to remove the excess of hydrogen peroxide, and after cooling it, distilled water was added to a final volume of 500 mL

Secondary lipid oxidation – Thiobarbituric acid reactive substances (TBARS). Briefly, trichloroacetic acid (25 mL, 7.5%) was added to each sample (5 g) followed by homogenization over 1 min (MICCRA D-9 Homogenizer, MICCRA GmbH, Deutschland). The resulted suspension was filtered and centrifuged at 3600 *g* at 4 °C for 20 min (Laboratory Centrifuge 3 K30, Sigma, Germany). The extracted samples (5 mL) were added to 5 mL of

2-thiobarbituric acid (0.02 M in acetic acid 99%) and the mixture immersed in a boiling water bath for 40 min being, at the end, cooled down by cold water. The absorbance was measured at 538 nm (Microplate Spectrophotometer Multiskan GO, Thermo Scientific, USA) and a standard curve was prepared using 1,1,3,3-tetramethoxypropane as standard at concentrations ranging from 0 to 4 μM. TBARS results were expressed as μg MDA/g sample.

Tertiary lipid oxidation – Fluorescent compounds. Fluorescent compounds analysis was carried in accordance to Fidalgo, Lemos, Delgadillo and Saraiva [12] and Aubourg [119] works with few modifications. Briefly, the upper layer obtained after centrifugation using the Folch procedure (lipid content extraction in section 2.4.1) was used for fluorescent compounds determination using a Hitachi F2000 fluorescence spectrophotometer (Tokyo, Japan). The relative fluorescence was calculated as follows: Relative fluorescence = F/Fst, where F is the fluorescence measured at each excitation/emission maximum, and Fst is the fluorescence intensity of a quinine sulphate solution (1 mg/ml in 0.05 M H2SO4) at the corresponding wavelength. The Fluorescence Ratio (FR) was calcuted by Eq. 6.1.

$$Fluorescence\ Ratio = \frac{Relative\ Fluorescence\left(\frac{393\ nm}{463\ nm}\right)}{Relative\ Fluorescence\left(\frac{327\ nm}{415\ nm}\right)} \quad \text{Eq. 6.1}$$

Statistical analysis. All analyses were performed in triplicate and samples stored in triplicate for each storage condition/storage day of analysis. Statistical data analysis was performed using the one-way analysis of variance ANOVA, followed by a Tukey's HSD Test, at a 5% level of significance to identify differences between storage conditions at each day and throughout storage time at each condition.

6.3. Results and Discussion

Fatty acid profile. Meat fatty acid composition varies in accordance with the muscle fiber type, with "red" muscles having higher proportions of phospholipids than "white" muscles resulting in higher percentage of PUFAs [104].

Despite the natural differences between pork and bovine meat fatty acids profiles (Table 6.1 and Table 6.2), it was possible to observe a higher percentage of MUFAs

(43.8±1.8 and 39.8±1.2, respectively), followed by SFAs content (36.5±0.2 and 34.2±0.7, respectively) and PUFAs (19.1±1.6 and 24.5±0.5, respectively). Moreover, the fatty acids identified in higher amounts for PP and BM, as % of total fatty acids, were oleic (C18:1) (40.1±1.5 and 37.2±0.9), palmitic (C16:0) (22.8±0.1 and 20.11±0.26), stearic (C18:0) (11.6±0.1 and 10.4±0.5), and linoleic (C18:2) (13.9±1.4 and 20.6±0.2), respectively. These profiles are in accordance to previous studies and published reports where meat is reported to have higher amounts of MUFAs and SFAs, ranging PUFAs content between 11-29% of the total fatty acids [11, 120, 121].

Over storage, significant differences ($p < 0.05$) were observed in SFAs, MUFAs and PUFAs contents, including different behaviours between both samples. Moreover, total fatty acid content faced a decrease ($p < 0.05$) tendency in all storage conditions, except for 60 MPa/10 ºC in both samples ($p > 0.05$), the best HS condition in what concerns the preservation of fatty acids.

Regarding SFAs proportion in PP samples, a decrease ($p < 0.05$) was observed for all storage conditions, with similar profiles between HS and RF at the 30th day of storage similar ($p > 0.05$), as well between both HS conditions tested at the 60th day. However, for BM samples, a SFAs increased ($p < 0.05$) was observed over storage. This fact could be related to PUFAs decrease ($p < 0.05$) over time in BM samples while PP presented a maintenance of PUFAs content (or even an increase over time). The occurrence of a counterbalance behaviour between SFAs and PUFAs was already described in literature by Guyon, Meynier and de Lamballerie [101].

Table 6.1. Fatty acid profile in relative percentage (% ± SE) and total fatty acid (relative value ± SE) obtained for pork meat in pieces (PP) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

Storage Condition	60 MPa/10 °C					75 MPa/25 °C					RF (0.1 MPa/4 °C)			
Storage Day	0	7	14	30	60	0	7	14	30	60	0	7	14	30
Fatty acid														
C14:0	1.33±0.01 a	1.17±0.01 bB	1.35±0.01 aA	1.37±0.08 aA	1.26±0.09 abA	1.33±0.01 b	1.30±0.03 bcA	1.42±0.03 aA	1.24±0.02 cdB	1.21±0.04 dA	1.33±0.01 a	1.31±0.04 aA	1.16±0.08 bB	1.27±0.03 abAB
C16:0	22.80±0.14 a	21.11±0.03 dC	22.38±0.02 bAB	21.93±0.01 cA	22.14±0.24 bcA	22.80±0.14 a	22.30±0.01 bB	22.72±0.17 aA	22.14±0.14 bA	21.95±0.14 bA	22.80±0.14 a	22.51±0.06 aA	21.88±0.35 bB	22.22±0.27 abA
C17:0	0.28±0.01 ab	0.28±0.01 abA	0.31±0.02 aA	0.28±0.01 abA	0.27±0.02 bB	0.28±0.01 ab	0.26±0.01 bA	0.26±0.02 bA	0.28±0.01 abA	0.31±0.01 aA	0.28±0.01 ab	0.26±0.01 bA	0.30±0.02 aA	0.28±0.01 abA
C18:0	11.65±0.04 a	10.05±0.16 dB	11.15±0.03 bA	10.56±0.29 cA	10.40±0.06 cdA	11.65±0.04 a	11.12±0.23 bcA	11.15±0.12 bA	10.81±0.06 cA	10.34±0.07 dA	11.65±0.04 a	11.14±0.24 bA	10.94±0.05 bB	10.79±0.09 bA
C20:0	0.15±0.01 a	0.09±0.03 bB	0.13±0.02 abAB	0.15±0.01 aA	0.10±0.03 abA	0.15±0.01 a	0.14±0.01 abA	0.15±0.01 aA	0.12±0.01 bcB	0.11±0.01 cA	0.15±0.01 a	0.12±0.01 abAB	0.10±0.02 bB	0.12±0.01 abB
Total SFA	36.47±0.19 a	32.96±0.15 dC	35.58±0.06 bA	34.55±0.18 cA	34.41±0.41 cA	36.47±0.19 a	35.38±0.19 cB	35.97±0.25 bA	34.84±0.18 dA	34.27±0.24 eA	36.47±0.19 a	35.62±0.14 bA	34.63±0.38 cA	34.94±0.40 cA
C16:1	3.85±0.27 b	4.10±0.09 abB	3.92±0.19 bB	4.41±0.03 aA	4.25±0.13 abA	3.85±0.27 b	4.34±0.05 aA	4.35±0.05 aA	4.18±0.06 abAB	4.32±0.17 aA	3.85±0.27 a	4.19±0.05 aAB	3.85±0.13 aB	3.98±0.18 aB
C18:1	40.12±1.53 ab	39.07±0.14 bC	39.63±0.79 abB	41.78±0.06 aA	40.82±0.73 abA	40.12±1.53 ab	41.48±0.20 abA	42.51±0.52 aA	40.02±0.37 abB	38.93±1.45 bA	40.12±1.53 ab	40.85±0.15 aB	38.42±0.51 bB	38.74±0.10 abC
Total MUFA	43.84±1.79 bc	42.95±0.21 cC	43.45±0.93 cB	46.05±0.10 aA	45.03±0.87 abA	43.84±1.79 c	45.66±0.14 abA	46.78±0.57 aA	44.08±0.40 bcB	43.62±1.50 cB	43.84±1.79 ab	44.87±0.07 aB	41.92±0.65 cC	42.47±0.10 bcC
C18:3n3	0.94±0.11 a	0.72±0.06 aA	1.08±0.10 aA	0.88±0.10 aA	0.79±0.20 aA	0.94±0.11 a	0.69±0.03 bA	0.88±0.09 aA	0.77±0.04 abA	0.68±0.02 bA	0.94±0.11 a	0.74±0.04 aA	0.74±0.11 aA	0.80±0.12 aA
C20:5n3	0.14±0.02 a	0.23±0.01 aA	0.14±0.01 aB	0.14±0.02 aB	0.21±0.07 aA	0.14±0.02 cd	0.16±0.01 bcB	0.10±0.01 dC	0.19±0.02 bAB	0.25±0.02 aA	0.14±0.02 b	0.18±0.01 abB	0.22±0.02 aA	0.21±0.02 aA
C22:5n3	0.33±0.01 b	0.55±0.03 aA	0.33±0.05 bB	0.33±0.03 abA	0.44±0.11 bB	0.33±0.01 bc	0.38±0.05 bB	0.24±0.01 cB	0.42±0.04 bA	0.56±0.04 aA	0.33±0.01 b	0.41±0.01 abB	0.50±0.06 aA	0.46±0.05 aA
C22:6n3	0.12±0.01 b	0.20±0.01 aA	0.13±0.04 abAB	0.12±0.01 bB	0.17±0.04 abA	0.12±0.01 bc	0.13±0.02 bB	0.09±0.01 cB	0.18±0.01 aA	0.21±0.01 aA	0.12±0.01 b	0.19±0.02 aA	0.19±0.01 aA	0.17±0.01 aA
C18:2n6	13.93±1.42 a	15.65±0.29 aA	15.08±0.89 aA	13.56±0.35 aB	13.80±0.32 aB	13.93±1.42 ab	12.75±0.19 bB	12.33±0.93 bB	14.10±0.30 abAB	15.03±0.61 aA	13.93±1.42 ab	12.93±0.27 bB	15.21±0.42 aA	14.88±0.33 abA
C18:3n6	0.12±0.01 b	0.18±0.01 aA	0.12±0.01 bB	0.13±0.01 abA	0.15±0.04 bB	0.12±0.01 c	0.14±0.01 bcB	0.09±0.01 dC	0.16±0.01 abA	0.18±0.01 aA	0.12±0.01 b	0.14±0.01 abB	0.17±0.01 aA	0.17±0.02 aA
C20:2n6	0.45±0.05 a	0.38±0.02 aA	0.46±0.03 aA	0.41±0.03 aA	0.37±0.08 aA	0.45±0.05 a	0.35±0.01 bA	0.39±0.03 abA	0.38±0.02 abA	0.35±0.01 bA	0.45±0.05 a	0.36±0.01 bA	0.40±0.03 abA	0.39±0.03 abA
C20:3n6	0.35±0.02 b	0.55±0.02 aA	0.35±0.01 bB	0.36±0.03 bB	0.45±0.10 abA	0.35±0.02 c	0.40±0.01 bcB	0.26±0.01 dB	0.46±0.03 bA	0.57±0.04 aA	0.35±0.02 b	0.43±0.01 abB	0.54±0.07 aA	0.51±0.05 aA
C20:4n6	2.36±0.01 b	4.32±0.03 aA	2.41±0.07 bB	2.49±0.31 bB	3.23±0.88 abA	2.36±0.01 ab	2.96±0.11 abB	2.10±0.31 bB	3.42±0.24 abA	3.72±1.13 aA	2.36±0.01 b	3.02±0.08 abB	3.89±0.52 aA	3.71±0.45 aA
C22:4n6	0.36±0.01 b	0.58±0.02 aA	0.33±0.01 bB	0.36±0.03 bB	0.45±0.10 bA	0.36±0.01 c	0.39±0.02 bcB	0.26±0.01 dB	0.43±0.02 bAB	0.55±0.03 aA	0.36±0.01 b	0.43±0.01 abB	0.51±0.06 aA	0.47±0.05 aA
Total PUFA	19.12±1.61 bc	23.42±0.36 aA	20.46±0.92 bB	18.81±0.08 cC	20.09±1.08 bcA	19.12±1.61 bc	18.40±0.33 cdC	16.76±0.76 dC	20.55±0.58 abB	21.51±1.75 aA	19.12±1.61 b	18.90±0.23 bB	22.54±0.91 aA	21.84±0.32 aA
n3/n6	0.09±0.01 a	0.08±0.01 bB	0.09±0.01 aA	0.09±0.01 aA	0.09±0.01 aA	0.09±0.01 a	0.08±0.01 bB	0.09±0.01 abAB	0.08±0.01 abA	0.09±0.01 abA	0.09±0.01 ab	0.09±0.01 aA	0.08±0.01 cB	0.08±0.01 bcA
Total fatty acids	1.00 a	0.38±0.10 aA	0.77±0.25 aAB	0.78±0.26 aA	0.74±0.37 aA	1.00 a	0.64±0.19 bA	1.28±0.20 aA	0.55±0.09 bA	0.31±0.09 bA	1.00 a	0.56±0.15 bA	0.44±0.16 bB	0.52±0.14 bA

Table 6.2. Fatty acid profile in relative percentage (% ± SE) and total fatty acid (relative value ± SE) obtained for bovine minced meat (BM) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-c) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

Storage Condition	60 MPa/10 °C					75 MPa/25 °C					RF (0.1 MPa/4 °C)			
Storage Day	0	7	14	30	60	0	7	14	30	60	0	7	14	30
Fatty acid														
C14:0	1.30±0.10 c	2.38±0.02 abA	1.89±0.12 bA	2.41±0.12 aA	2.04±0.37 abA	1.30±0.10 b	1.96±0.03 aB	2.01±0.01 aA	2.27±0.26 aA	2.26±0.05 aA	1.30±0.10 a	1.15±0.04 aC	1.26±0.10 aB	1.18±0.03 aB
C16:0	20.11±0.26 c	23.23±0.18 aA	21.40±0.60 bcAB	22.86±0.55 abA	21.34±1.27 bcA	20.11±0.26 b	21.52±0.27 abB	21.67±0.37 aA	22.37±1.03 aA	22.59±0.32 aA	20.11±0.26 ab	19.53±0.11 bcC	20.35±0.32 aB	18.99±0.25 cB
C17:0	0.92±0.16 a	1.23±0.09 aA	0.99±0.07 aAB	1.22±0.02 aB	1.12±0.21 aA	0.92±0.16 b	1.10±0.01 abA	1.12±0.03 abA	1.15±0.04 aB	1.00±0.01 ab	0.92±0.16 bA	0.77±0.07 bB	0.73±0.20 bB	1.62±0.19 aA
C18:0	10.37±0.51 b	17.03±0.48 aA	16.40±0.80 aA	17.30±0.28 aA	17.07±1.32 aA	10.37±0.51 c	16.93±0.25 aA	17.07±0.18 aA	17.02±0.41 aA	14.37±0.49 bB	10.37±0.51 b	9.68±0.11 bB	11.01±0.66 bB	13.67±0.80 aB
C20:0	1.20±0.02 a	0.37±0.05 bB	0.52±0.06 bB	0.36±0.04 bB	0.46±0.14 bA	1.20±0.02 a	0.42±0.01 bcB	0.41±0.03 bcC	0.38±0.01 cB	0.57±0.14 bA	1.20±0.02 a	1.25±0.15 aA	0.96±0.03 bA	0.93±0.02 bA
Total SFA	34.20±0.74 c	44.89±0.73 aA	41.74±1.53 bA	44.79±0.33 aA	42.60±3.12 abA	34.20±0.74 c	42.44±0.07 abB	42.81±0.67 aA	43.71±1.74 aA	41.38±0.04 bA	34.20±0.74 b	32.63±0.17 cC	34.61±0.89 abB	35.19±0.36 aB
C16:1	3.16±0.12 a	2.93±0.10 abB	2.56±0.01 cC	2.99±0.14 abA	2.70±0.18 bcA	3.16±0.12 a	2.66±0.06 cB	2.67±0.03 cB	2.72±0.06 bcA	2.89±0.04 bA	3.16±0.12 a	3.24±0.15 aA	3.26±0.01 aA	2.52±0.02 bB
C17:1	0.39±0.02 c	0.78±0.03 abA	0.83±0.04 aA	0.76±0.02 abA	0.74±0.02 bA	0.39±0.02 d	0.80±0.01 abA	0.81±0.01 aA	0.75±0.02 bcA	0.71±0.03 cA	0.39±0.02 b	0.34±0.01 bB	0.43±0.03 bB	0.80±0.06 aA
C18:1	37.22±0.88 a	36.09±0.33 abAB	33.76±1.33 bA	35.93±0.55 abA	34.10±1.56 bA	37.22±0.88 a	35.32±0.70 bB	34.91±0.27 bcA	34.68±0.59 bcA	33.39±0.72 cA	37.22±0.88 a	37.89±1.14 aA	35.65±2.85 aA	34.06±1.70 aA
Total MUFA	39.84±1.25 a	34.36±0.43 bB	32.78±0.87 bB	34.22±0.85 bA	32.72±0.56 bA	39.84±1.25 a	34.13±0.81 bB	33.60±0.10 bcB	33.48±0.78 bcA	32.73±1.07 cA	39.84±1.25 a	40.76±1.23 aA	39.66±0.18 aA	31.74±1.35 bB
C18:3n3	0.73±0.02 a	0.30±0.01 bB	0.27±0.01 bB	0.29±0.03 bAB	0.28±0.04 bA	0.73±0.02 a	0.28±0.01 bB	0.24±0.01 bC	0.22±0.02 bB	0.28±0.08 bA	0.73±0.02 b	0.97±0.09 aA	0.70±0.01 bA	0.36±0.04 cA
C20:5n3	0.47±0.05 ab	0.49±0.03 abA	0.61±0.11 aA	0.35±0.01 bB	0.33±0.05 bB	0.47±0.05 a	0.52±0.03 aA	0.52±0.01 aA	0.50±0.04 aB	0.55±0.07 aA	0.47±0.05 b	0.46±0.03 bA	0.56±0.02 bA	0.84±0.12 aA
C22:5n3	0.34±0.04 b	0.43±0.09 bAB	0.73±0.13 aA	0.39±0.02 bC	0.50±0.18 abA	0.34±0.04 b	0.56±0.02 aA	0.59±0.02 aAB	0.58±0.04 aB	0.66±0.08 aA	0.34±0.04 c	0.32±0.02 cB	0.44±0.02 bB	0.77±0.02 aA
C22:6n3	0.09±0.01 a	0.06±0.02 aB	0.10±0.02 aA	0.06±0.01 aB	0.06±0.03 aA	0.09±0.01 a	0.07±0.01 aAB	0.08±0.01 aA	0.06±0.01 aB	0.11±0.02 aA	0.09±0.01 b	0.10±0.01 abA	0.11±0.01 abA	0.15±0.03 aA
C18:2n6	20.62±0.19 a	10.41±0.30 bB	14.41±1.77 bB	11.59±1.12 bB	15.06±3.41 bA	20.62±0.19 a	12.89±0.48 cB	12.88±0.80 cB	12.41±0.75 cB	15.20±0.16 bA	20.62±0.19 a	21.29±1.44 aA	18.65±0.55 aA	18.97±1.80 aA
C18:3n6	0.16±0.01 a	0.19±0.03 aA	0.15±0.01 aA	0.17±0.01 aA	0.16±0.05 aA	0.16±0.01 ab	0.18±0.01 aA	0.15±0.01 bA	0.14±0.01 bAB	0.09±0.01 cA	0.16±0.01 a	0.14±0.04 aA	0.16±0.01 aA	0.12±0.02 aB
C20:2n6	0.36±0.04 b	0.43±0.10 ab	0.72±0.12 a	0.40±0.01 b	0.52±0.18 ab	0.36±0.04 b	0.62±0.04 a	0.64±0.04 a	0.64±0.04 a	0.71±0.12 a	0.36±0.04 b	0.48±0.23 b	0.46±0.03 b	1.04±0.14 a
C20:4n6	1.93±0.19 b	2.41±0.51 abAB	3.54±0.62 aA	1.95±0.04 bC	2.51±0.91 abA	1.93±0.19 b	3.15±0.19 aA	3.18±0.11 aAB	3.09±0.21 aB	3.65±0.69 aA	1.93±0.19 b	1.86±0.21 bB	2.59±0.12 bB	4.99±0.70 aA
PUFA	24.49±0.47 a	14.56±0.92 cC	20.27±2.90 bB	14.77±1.01 cC	18.92±4.82 bA	24.49±0.47 a	17.78±0.76 cB	17.81±1.02 cC	17.16±1.06 cB	20.73±0.70 bA	24.49±0.47 bc	25.43±1.22 bA	23.44±0.76 cA	29.57±1.80 aA
n3/n6	0.07±0.01 a	0.10±0.01 aA	0.10±0.01 aA	0.08±0.01 aA	0.07±0.01 aB	0.07±0.01 b	0.09±0.01 aB	0.09±0.01 aB	0.09±0.01 aA	0.09±0.01 aA	0.07±0.01 b	0.08±0.01 aC	0.08±0.01 aC	0.08±0.01 aA
Total fatty acids	1.00 a	0.60±0.24 abB	0.32±0.09 bB	0.63±0.09 abA	0.61±0.30 abA	1.00 a	0.36±0.13 bB	0.52±0.18 bAB	0.37±0.12 bAB	0.31±0.12 bB	1.00 ab	1.32±0.35 aA	0.72±0.18 bcA	0.23±0.09 cB

Moreover, in BM samples, both HS conditions presented higher ($p < 0.05$) and lower ($p < 0.05$) SFAs and PUFAs values, respectively when compared to RF, without significant differences ($p > 0.05$) detected between both HS conditions. The composition differences between bovine and pork meat, as well the mincing of bovine meat that could have potentiated lipid oxidation (since this processing can liberate membrane-bound phospholipids where an important amount of unsaturated fatty acids are present thus becoming easily oxidized), could explain in a certain extent the differences observed between PP and BM [122].

On the other hand, PP's MUFAs at the end of each storage condition were similar ($p > 0.05$) to the initial ones, although slight variations ($p < 0.05$) along storage were observed; at the end of storage, RF presented always the lower ($p < 0.05$) values of MUFAs while 60 MPa/10 ºC evidenced always the higher ($p < 0.05$) ones. Differences on MUFAs behaviour between PP and BM samples were also observed, with BM presenting higher decrease tendencies along time for all storage conditions, being these in accordance with overall PUFAs behaviour.

When the most predominant fatty acids were analysed individually over storage, some differences were observed. Palmitic and stearic, for instance, tended to decrease over time in PP, without considerable differences between storage conditions, while in BM they increased ($p < 0.05$) with always higher ($p < 0.05$) values in HS than in RF samples. A different behaviour was also observed for oleic, without significant differences ($p > 0.05$) over storage in PP, and a decrease tendency in BM both HS conditions (while RF was capable to maintain the initial value). On the other hand, linoleic acid decreased ($p < 0.05$) in BM for both HS conditions (where RF presented the higher values), while for PP the values were maintained over storage for all storage conditions tested.

Globally, no consistent variations regarding the impact of this methodology on the fatty acid profile of raw meat were observed at the end of storage in both HS studies. Fernandes, Moreira, Santos, Duarte, Santos, Inácio, Alves, Bessa, Delgadillo and Saraiva [11] reported similar results, pointing then the short storage period of 24 hours as a reason for these findings, while Freitas, Pereira, Santos, Alves, Bessa, Delgadillo and Saraiva [13] observed only differences in SFA (higher value when preserved at 150 MPa/21ºC over 6 days when compared to the initial sample) and *trans*-MUFA (lower value when preserved

at 150 MPa/21ºC over 6 days when compared to the initial sample), probably due to a lower enzymatic/microbial activity, not being possible to foresee a pattern from these differences.

Volatile organic compounds profile. Meat spoilage and oxidation can be evaluated by VOCs profile over storage, being this profile a mixture of several compounds of different origins, e.g. bacterial metabolism, biochemical reactions, among others [107]. In fact, the fatty acid composition has also an important role in volatiles generation, particularly PUFAs due to their increased sensitivity to oxidation [114]. Furthermore, some studies reported VOCs and non-volatile compounds (i.e., biogenic amines) primarily a result of enzymatic decarboxylation of the amino acids by exogenous enzymes of bacterial origin [123].

As far the authors are aware there are no studies regarding HS impact on VOCs profile of raw meat. The 17 and 20 VOCs identified for PP and BM samples (Table 6.3 and Table 6.4), respectively, were already described and discussed in other studies, for instance, studies concerning chicken meat [108, 109], cooked ham [110], duck meat [111], beef [107, 112, 113], and pork meat [114]. However, it must be noted that packaging type, storage temperature, and meat characteristics (pH, moisture content, microorganisms presence, among others) have important roles in VOCs development [124, 125].

In the present study same aldehydes were identified in both samples: 3-methylbutanal, pentanal, hexanal, heptanal, benzaldehyde, octanal, and nonanal. The one that presented the higher percentage over time was hexanal (30.4±1.1 and 30.8±6.6, for PP and BM, respectively, at time zero), leading to aldehydes total content of 36.0±0.3 and 38.4±5.9 for PP and BM, respectively. Significant changes were observed throughout storage. For instance, 3-methylbutanal was not detected in PP samples at 60 MPa/10 ºC up to 60 days, in BM it was only identified at the 30th day of storage, while at 75 MPa/25 ºC and RF presented an increase ($p < 0.05$) over time just after the 7th/14th day of storage for PP/BM at 75 MPa/25 ºC and at the 3rd day in refrigerated samples, being the values always higher ($p < 0.05$) in RF storage. On the other hand, the compound in higher amounts (hexanal), did not show significant differences ($p > 0.05$) at 60 MPa/10 ºC over time (except in BM at the 60th day of storage), while at 75 MPa/25 ºC and RF it presented similar to lower values over storage for both samples, probably associated to an higher spoilage level of the samples, since a sharp decrease was observed (values of ≈2 to ≈6 were detected at the 60th day of storage at 75 MPa/25 ºC). Globally, benzaldehyde, octanal and nonanal faced an increase over time in

all storage conditions tested, with 60 MPa/10 ºC presenting similar to lower percentage of these compounds over storage when compared to 75 MPa/25 ºC and RF. Casaburi, Piombino, Nychas, Villani and Ercolini [125] pointed that aldehydes could derive from triglycerides hydrolysis and fatty acid metabolism imparting fatty flavour. Hexanal, nonanal, heptanal, benzaldehyde, and 3-methylbutanal are commonly found in raw meat during storage being this fact in accordance to the results obtained in the present study [125].

Regarding alcohols in PP and BM samples, the former presented a lower total proportion (5.4±0.4) than the latter (12.0±0.4) prior to any storage period. At this stage, the specific alcohols identified for both samples were: 1-pentanol, 1-hexanol, 1-heptanol, 1-octen-3-ol, 2-ethyl-1-hexanol, 1-octanol (and 1-butoxy-2-propanol only detected in BM samples). From all of these compounds, 1-octen-3-ol (1.1±0.2), 2-ethyl-1-hexanol (1.0±0.1), and 1-pentanol (1.2±0.1) were the ones that presented higher percentages in PP samples, and for BM, 1-octen-3-ol (4.4±1.3) evidenced from the others.

Table 6.3. Volatile organic compounds (VOCs) profile in relative percentage (% ± SE) obtained for pork meat in pieces (PP) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-c) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

	60 MPa/10 °C						75 MPa/25 °C						RF (0.1 MPa/4 °C)				
VOCs	0	3	7	14	30	60	0	3	7	14	30	60	0	3	7	14	30
3-methylbutanal	#	#	#	#	#	#	#	#	0.03±0.01 bB	0.07±0.01 bB	0.16±0.02 bB	0.99±0.35 aA	#	0.27±0.13 bA	2.38±0.86 abA	1.61±0.53 abA	4.70±2.21 aA
Pentanal	0.93±0.39 a	0.88±0.24 aA	0.44±0.08 aC	0.77±0.24 aB	0.94±0.14 aB	1.05±0.24 aA	0.93±0.39 b	1.22±0.01 bA	1.81±0.06 aA	1.31±0.14 abA	1.79±0.26 aA	0.17±0.08 cB	0.93±0.39 a	0.91±0.03 aA	0.93±0.07 aB	1.24±0.03 aAB	0.84±0.23 aB
Hexanal	30.41±1.07 a	25.89±1.80 aA	20.73±0.96 aAB	26.50±5.57 aA	23.06±3.05 aA	21.49±8.55 aA	30.41±1.07 a	25.49±3.96 abA	28.53±1.96 aA	23.08±2.09 abA	18.24±2.83 bA	6.24±3.22 cA	30.41±1.07 a	22.57±2.75 abA	15.66±5.05 bB	20.51±1.18 bA	22.79±2.61 abA
Heptanal	1.51±0.30 ab	0.98±0.10 abA	0.79±0.11 bB	0.78±0.12 bB	1.28±0.06 abA	1.73±0.48 aA	1.51±0.30 ab	1.48±0.36 abA	1.67±0.27 aA	1.57±0.29 abA	1.46±0.32 abA	0.68±0.25 bA	1.51±0.30 a	0.95±0.11 abA	0.78±0.10 bB	1.27±0.27 abAB	1.12±0.28 abA
Benzaldehyde	0.38±0.04 c	0.39±0.01 cB	0.42±0.03 cB	0.54±0.15 bcB	0.89±0.01 aA	0.72±0.05 abB	0.38±0.04 c	0.67±0.06 cA	0.86±0.18 bcA	1.23±0.15 abA	1.02±0.18 abA	1.45±0.20 aA	0.38±0.04 b	0.33±0.03 bB	0.77±0.07 aA	0.92±0.11 aA	0.87±0.23 aA
Octanal	0.81±0.22 bc	0.77±0.26 bcB	0.69±0.12 cB	0.61±0.07 cB	1.40±0.16 bA	2.08±0.35 aA	0.81±0.22 a	1.52±0.34 aA	1.53±0.26 aA	1.51±0.29 aA	1.61±0.34 aA	0.90±0.34 aB	0.81±0.22 a	1.01±0.15 aAB	0.84±0.12 aB	1.13±0.16 aAB	1.03±0.37 aA
Nonanal	1.11±0.11 b	2.28±1.17 abA	2.03±0.44 bA	1.65±0.19 bB	2.63±0.07 abA	3.73±0.51 aA	1.11±0.11 c	2.98±0.34 aA	2.65±0.05 abA	2.50±0.29 abA	3.53±0.35 aA	1.61±0.64 bcB	1.11±0.11 b	2.92±0.52 aA	2.90±0.41 aA	2.39±0.08 aA	2.92±0.72 a
Total Aldehydes	35.99±0.33 a	31.08±0.05 aA	25.10±1.58 bB	34.39±2.95 aA	30.21±2.63 abA	25.72±2.87 bA	35.99±0.33 a	33.29±5.64 aA	35.94±2.37 aA	31.62±2.92 aA	27.49±3.05 aA	14.71±2.27 bB	35.99±0.33 a	28.96±1.83 bA	20.69±2.38 cB	29.05±1.09 bA	33.27±0.45 aA
1-pentanol	1.17±0.04 a	1.05±0.04 aA	0.38±0.03 cB	0.61±0.09 bcA	0.68±0.14 bA	0.70±0.14 bA	1.17±0.04 a	0.73±0.15 bAB	0.82±0.11 bA	0.61±0.05 bA	0.56±0.02 bA	0.61±0.08 bA	1.17±0.04 a	0.69±0.12 bcB	0.45±0.12 cB	0.53±0.01 cA	1.13±0.07 abA
1-hexanol	0.27±0.01 b	0.25±0.06 bAB	0.19±0.10 bB	0.25±0.07 bB	0.34±0.01 abB	0.50±0.07 aB	0.27±0.01 b	0.31±0.02 bA	0.34±0.01 bA	0.33±0.03 bB	0.39±0.13 bB	1.38±0.32 aA	0.27±0.01 c	0.18±0.02 cB	0.33±0.01 cAB	1.03±0.13 bA	1.55±0.12 aA
1-heptanol	0.33±0.01 bc	0.32±0.01 bcA	0.23±0.04 cB	0.26±0.02 bcA	0.35±0.03 abA	0.44±0.07 aA	0.33±0.01 a	0.37±0.11 aA	0.36±0.04 aA	0.37±0.17 aA	0.47±0.15 aA	0.40±0.11 aA	0.33±0.01 b	0.30±0.05 bA	0.33±0.02 bA	0.62±0.14 aA	0.58±0.03 aA
1-octen-3-ol	1.06±0.15 a	1.53±0.47 aAB	1.74±0.21 aB	2.04±0.45 aAB	2.67±0.04 aA	2.75±0.74 aA	1.06±0.15 b	3.48±0.32 bA	5.10±0.73 aA	3.20±0.58 abA	2.59±0.26 bA	1.42±0.67 bA	1.06±0.15 a	1.31±0.05 aB	1.34±0.64 aB	1.73±0.16 aB	1.90±0.28 aB
2-ethyl-1-hexanol	0.99±0.11 d	2.27±0.15 aA	1.94±0.18 abB	1.74±0.30 bcB	1.58±0.05 bcB	1.34±0.07 cdB	0.99±0.11 d	1.88±0.28 cdA	2.59±0.13 bcA	3.40±0.72 bA	3.57±0.03 bA	5.08±0.69 aA	0.99±0.11 b	2.45±0.65 aA	1.86±0.22 abB	1.39±0.20 abB	1.28±0.44 bB
1-octanol	0.51±0.10 ab	0.61±0.18 abA	0.59±0.01 abA	0.43±0.06 bB	0.68±0.01 abA	0.77±0.05 aA	0.51±0.10 a	0.77±0.10 aA	0.67±0.15 aA	0.57±0.08 aAB	0.57±0.13 aA	0.54±0.07 aB	0.51±0.10 a	0.58±0.16 aA	0.63±0.01 aA	0.62±0.03 aA	0.67±0.20 aA
Total Alcohols	5.44±0.38 a	5.96±0.96 aA	5.07±0.42 aB	5.12±1.26 aB	6.30±0.26 aB	6.67±1.07 aA	5.44±0.38 c	6.52±1.28 bcA	9.72±0.89 aA	8.71±0.13 abA	8.38±0.34 abA	9.84±1.76 aA	5.44±0.38 ab	5.52±0.81 abA	4.59±0.20 bB	5.92±0.01 abB	6.79±1.09 aAB
2,2,4,6,6-pentamethylheptane	9.02±0.27 ab	9.83±1.75 aA	6.24±0.37 abcA	5.55±0.66 bcA	4.26±1.46 cA	4.23±2.67 cA	9.02±0.27 a	9.10±0.35 aA	5.00±1.10 bA	4.42±2.09 bA	4.28±1.26 bA	7.14±1.10 abA	9.02±0.27 a	1.19±0.20 cB	0.94±0.24 cB	6.81±0.89 bA	0.72±0.05 cB
Decane	0.19±0.06 a	0.19±0.05 aB	0.11±0.02 aB	0.23±0.06 aA	0.15±0.07 aA	0.20±0.02 aA	0.19±0.06 ab	0.20±0.02 abB	0.13±0.02 bB	0.22±0.02 abA	0.27±0.07 aA	0.22±0.01 abA	0.19±0.06 b	0.46±0.07 abA	0.64±0.09 aA	0.21±0.10 bA	0.34±0.13 bA
2,2,4,4-tetramethyloctane	0.46±0.23 b	1.48±0.27 aA	1.01±0.04 abA	0.94±0.13 abA	0.79±0.21 bB	0.55±0.32 bB	0.46±0.23 c	1.51±0.10 aA	0.66±0.22 bcA	0.64±0.33 bcA	0.74±0.23 bcB	1.31±0.22 abA	0.46±0.23 c	0.83±0.16 bcB	1.98±0.50 bcA	1.09±0.44 bA	1.89±0.20 aA
Total Hydrocarbons	9.68±0.45 ab	11.50±1.97 aA	7.36±0.40 bcA	7.13±0.65 bcA	5.19±1.60 cAB	9.51±1.56 bcA	9.68±0.45 ab	10.80±0.43 aA	5.94±1.24 cA	5.41±2.40 cA	6.31±0.40 bcA	8.68±1.31 abcA	9.68±0.45 a	2.48±0.29 bB	2.35±0.02 bB	8.19±1.16 aA	5.65±2.58 bB
2,5-octanedione	2.48±0.22 bc	3.43±0.19 bA	2.72±0.18 bcB	4.85±0.25 aA	2.27±0.63 cA	2.92±0.66 bcA	2.48±0.22 abc	3.78±1.03 abA	4.26±0.73 aA	1.88±0.66 bcdB	1.25±0.30 cdA	0.40±0.15 dB	2.48±0.22 a	1.98±0.50 abA	1.09±0.44 bC	1.48±0.13 abB	1.74±0.36 abA

not detected

Table 6.4. Volatile organic compounds (VOCs) profile in relative percentage (% ± SE) obtained for bovine minced meat (BM) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-c) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

	60 MPa/10 °C						75 MPa/25 °C						RF (0.1 MPa/4 °C)				
VOCs	0	3	7	14	30	60	0	3	7	14	30	60	0	3	7	14	30
3-methylbutanal	#	#	#	#	1.14±0.50 aAB	0.11±0.08 bA	#	#	#	0.06±0.01 aB	0.13±0.01 aB	0.50±0.12 aA	#	0.26±0.01 bA	0.24±0.05 bA	2.06±0.06 aA	1.71±0.36 aA
Pentanal	1.74±0.34 a	0.96±0.06 abcA	1.13±0.29 abA	0.69±0.39 bcA	0.55±0.32 bcB	0.16±0.10 cA	1.74±0.34 a	0.84±0.25 abA	1.56±0.31 aA	1.04±0.33 abA	0.52±0.21 bB	#	1.74±0.34 a	1.00±0.19 aA	1.31±0.32 aA	0.99±0.24 aA	1.70±0.01 aA
Hexanal	30.83±6.61 a	32.30±4.44 aA	33.36±7.63 aA	25.43±8.33 aA	38.45±2.18 aA	6.49±3.74 bA	30.83±6.61 ab	36.06±2.20 aA	34.15±4.97 abA	28.96±3.07 abA	24.63±0.63 bB	2.52±1.80 cA	30.83±6.61 a	32.58±8.19 aA	38.42±3.67 aA	24.79±2.69 aA	28.30±3.64 aB
Heptanal	2.03±0.55 a	1.27±0.06 abA	1.60±0.26 aA	1.55±0.60 abA	1.69±0.14 aA	0.43±0.24 bA	2.03±0.55 a	1.51±0.10 aA	1.66±0.25 aA	2.11±0.17 aA	1.37±0.16 aA	0.33±0.21 bA	2.03±0.55 a	1.32±0.43 aA	2.15±0.20 aA	1.83±0.37 aA	1.96±0.42 aA
Benzaldehyde	0.43±0.01 ab	0.28±0.02 abB	0.38±0.09 abB	0.43±0.16 abA	0.52±0.04 aB	0.18±0.09 bB	0.43±0.01 b	0.47±0.06 bA	0.65±0.05 abA	0.78±0.47 abA	1.31±0.20 abA	1.54±0.60 aA	0.43±0.01 c	0.50±0.03 cA	0.64±0.06 bcA	1.04±0.22 abA	1.33±0.31 aA
Octanal	1.32±0.31 ab	1.24±0.11 abA	0.86±0.54 abA	1.86±0.66 aA	1.46±0.08 abA	0.56±0.01 bA	1.32±0.31 b	1.29±0.09 bA	1.42±0.20 bA	2.39±0.24 aA	1.07±0.51 bA	0.53±0.07 bA	1.32±0.31 ab	0.81±0.05 bB	1.92±0.36 aA	1.40±0.11 abA	1.45±0.41 abA
Nonanal	2.26±0.12 a	2.19±0.29 aA	1.51±0.14 abB	1.52±0.48 abC	2.32±0.41 aA	1.20±0.05 bA	2.26±0.12 b	2.35±0.03 bA	2.62±0.28 bAB	3.65±0.14 aA	2.32±0.58 bA	0.81±0.03 cB	2.26±0.12 a	2.13±0.45 aA	3.33±0.69 aA	2.68±0.23 aB	2.57±0.49 aA
Total Aldehydes	38.37±5.89 ab	38.68±5.63 abA	44.24±4.31 abAB	31.58±9.41 bA	47.41±0.39 aA	7.42±4.33 cA	38.37±5.89 ab	43.09±2.10 aA	38.33±2.10 abB	39.31±3.96 abA	31.64±1.60 bC	7.56±1.42 cA	38.37±5.89 ab	40.89±8.72 abA	49.79±4.23 aA	34.79±1.57 bA	41.54±3.05 abB
1-pentanol	1.61±0.16 b	2.11±0.62 abA	2.39±0.36 abA	2.04±0.26 abA	2.82±0.12 aA	0.28±0.21 cA	1.61±0.16 ab	2.76±0.27 aA	2.51±0.55 abA	2.06±0.13 abA	1.65±0.37 bB	0.19±0.15 cA	1.61±0.16 a	1.73±0.49 aA	2.00±0.07 aA	1.08±0.01 aB	1.45±0.23 aB
1-hexanol	0.55±0.03 ab	0.83±0.24 aA	0.75±0.03 aB	0.45±0.11 abB	0.78±0.12 aB	0.14±0.09 bA	0.55±0.03 a	0.53±0.10 aA	0.49±0.11 aB	0.45±0.25 aB	0.37±0.07 aB	0.44±0.34 aA	0.55±0.03 c	0.27±0.20 cA	1.50±0.07 bA	1.44±0.22 bA	2.81±0.25 aA
1-butoxy 2-propanol	0.99±0.06 ab	0.67±0.22 bAB	1.35±0.22 aA	1.33±0.03 aA	0.79±0.32 abA	#	0.99±0.06 a	0.47±0.10 bB	0.12±0.10 cC	#	#	#	0.99±0.06 ab	1.62±0.54 aA	0.79±0.01 bB	0.80±0.03 bB	0.90±0.06 abA
1-heptanol	0.66±0.10 a	0.53±0.18 aA	0.52±0.21 aAB	0.66±0.19 aA	0.32±0.29 aA	0.27±0.01 aA	0.66±0.10 a	0.32±0.21 aA	0.45±0.09 aB	0.55±0.19 aA	0.43±0.24 aA	#	0.66±0.10 bc	0.60±0.05 cA	0.87±0.01 aA	0.80±0.12 abcA	0.81±0.01 abA
1-octen-3-ol	4.36±1.27 b	6.17±1.09 abA	4.98±1.10 abA	7.88±0.69 aA	6.67±0.40 abA	1.28±0.69 cA	4.36±1.27 ab	4.89±0.37 abAB	4.55±0.94 abA	7.07±1.05 aB	2.56±1.10 bcB	0.40±0.26 cA	4.36±1.27 a	3.07±1.04 aB	4.86±0.20 aA	3.48±0.08 aC	4.17±0.79 aAB
2-Ethyl-1-hexanol	1.40±0.25 ab	2.15±0.85 aA	0.87±0.39 bB	1.54±0.29 abB	1.54±0.24 abB	0.30±0.16 bB	1.40±0.25 c	2.79±0.34 cA	1.80±0.27 cA	3.22±0.56 bcA	4.70±0.70 bA	8.78±1.03 aA	1.40±0.25 b	3.31±0.85 aA	0.87±0.64 bAB	1.82±0.09 bB	1.15±0.25 bB
2-octen-1-ol	0.66±0.08 ab	0.81±0.23 aA	0.55±0.09 abB	0.84±0.14 aA	0.80±0.06 aB	0.28±0.02 bA	0.66±0.08 ab	0.58±0.04 abcA	0.53±0.08 bcB	0.88±0.10 aA	0.33±0.15 cC	#	0.66±0.08 b	0.63±0.11 bA	1.01±0.04 aA	0.96±0.04 aA	1.14±0.09 aA
1-octanol	0.98±0.02 a	0.85±0.11 aA	0.81±0.04 aB	0.83±0.25 aA	0.76±0.27 aAB	0.23±0.13 bA	0.98±0.02 a	0.68±0.06 bA	0.72±0.10 bB	0.91±0.02 aA	0.32±0.04 cB	#	0.98±0.02 a	1.20±0.16 aA	1.06±0.09 aA	1.10±0.15 aA	1.08±0.07 aA
Total Alcohols	11.97±0.43 b	13.83±0.76 abA	13.45±0.01 abA	15.73±1.02 aA	14.58±1.04 aA	2.50±1.43 cB	11.97±0.43 bc	13.82±0.51 abA	10.97±2.09 bcdA	15.05±1.34 aA	10.67±0.27 cdB	8.76±0.86 dA	11.97±0.43 b	12.23±0.45 bB	13.41±0.17 aA	11.48±0.06 bB	13.53±0.94 aA
2,2,4,6,6-pentamethylheptane	2.91±0.82 ab	7.23±2.25 abA	9.93±3.65 abA	10.52±3.68 aA	2.91±1.66 abB	2.09±1.10 bB	2.91±0.82 b	8.87±3.25 abA	9.51±4.00 abA	9.70±2.25 abA	11.45±1.65 abA	13.43±2.89 aA	2.91±0.82 a	4.09±1.20 aA	3.51±1.02 aA	3.29±0.25 aA	1.86±0.51 aB
Decane	0.26±0.11 a	0.21±0.02 aA	0.11±0.03 aB	0.33±0.11 aA	0.11±0.02 aB	#	0.26±0.11 ab	0.25±0.10 abA	0.20±0.04 bB	0.32±0.01 abA	0.35±0.11 abA	0.48±0.05 aA	0.26±0.11 a	0.48±0.13 aA	0.40±0.09 aA	0.58±0.19 aA	0.41±0.01 aA
2,2,4,4-tetramethyloctane	0.49±0.16 b	1.20±0.37 abA	1.43±0.47 abA	2.31±0.57 aA	0.66±0.19 bB	0.37±0.20 bB	0.49±0.16 b	1.32±0.46 abA	1.48±0.73 abA	1.65±0.55 abAB	1.99±0.30 abA	2.20±0.53 aA	0.49±0.16 a	0.69±0.26 aA	0.64±0.19 aA	0.61±0.05 aB	0.34±0.09 aB
Dodecane	0.35±0.07 a	0.37±0.07 aB	0.27±0.03 aB	0.36±0.14 aB	0.30±0.18 aA	#	0.35±0.07 a	0.40±0.16 aAB	0.27±0.10 aB	0.31±0.09 aB	0.39±0.08 aA	0.61±0.22 aA	0.35±0.07 b	0.75±0.10 abA	0.66±0.16 abA	0.96±0.04 aA	0.76±0.19 abA
Total Hydrocarbons	4.74±0.33 bc	8.98±1.85 bA	9.14±2.21 bAB	16.31±1.89 aA	4.17±2.02 cB	2.17±1.52 cB	4.74±0.33 b	11.29±4.80 abA	14.25±3.29 aA	12.29±2.75 abA	12.67±0.36 abA	16.72±3.59 aA	4.74±0.33 ab	6.43±1.90 aA	4.63±1.22 abB	5.45±0.08 abB	2.86±0.43 bB
3-hydroxy butanone	0.74±0.23 bc	0.81±0.42 bA	1.76±0.38 aA	1.73±0.19 aA	0.98±0.03 bA	0.05±0.05 cA	0.74±0.23 ab	1.26±0.13 aA	0.63±0.01 abcB	0.47±0.11 bcB	0.74±0.44 abA	0.07±0.05 cA	0.74±0.23 a	0.64±0.05 aA	#	#	#

The total content of alcohols for PP pointed that 60 MPa/10 ºC and RF conditions were more stable over storage, without significant differences ($p > 0.05$) throughout storage, while the 75 MPa/25 ºC condition exhibited an increasing tendency ($p < 0.05$). On the other hand, in BM samples, a rise was observed over time ($p < 0.05$), for HS conditions but at the 60th day a sharp decrease was observed, probably due to the high level of deterioration. Furthermore, when storage conditions were compared at each storage day, higher values could be found in the 75 MPa/25 ºC condition.

When these results are compared to others found in literature, it was possible to conclude that the presence of several compounds is related to meat spoilage, as for instance (and associated to): butanol, 3-methyl-1-butanol (whiskey-like odor), 1-octanol, 1-octen-3-ol (mushrooms), 2-octen-1-ol, 2-ethyl-1-hexanol (resin, flower and green), 2,3-butanediol, 1-hexanol (chemical wine, fatty, fruity and weak metallic), and heptanol (fragrant, woody, oily, green, fatty, winey, sap and herb) [125]. The presence of these compounds is associated with many metabolic pathways, as proteolytic activity and amino acid metabolism, methyl ketone reduction, and reduction of aldehydes coming from lipid oxidation [125]. Additionally, 1-octen-3-ol is a common oxidative product (and identified in PP and BM samples) with linoleic and linolenic acids proposed as precursors [126].

Concerning hydrocarbons identified in PP and BM samples, the initial total amount was higher for PP (9.7±0.4) than for BM (4.7±0.3), where 2,2,4,6,6-pentamethylheptane (9.0±0.3 and 2.9±0.8, for PP and BM, respectively) and 2,2,4,4-tetramethyloctane (0.5±0.2 for both) contributed in a higher extent for the total hydrocarbons content. Over storage, hydrocarbons presented an increasing tendency in the first days, tending to lower their percentage at the end of HS (except for BM at 75 MPa/25 ºC where an increase was observed). Several of these compounds were also identified in raw meat when stored at cold temperatures. For instance, Insausti, Beriain, Gorraiz and Purroy [113] performed trials regarding beef storage in MAP up to 15 days and detected an increase of decane and 2,2,4,6,6-pentamethylheptane over storage, having also identified 2,2,4,4-tetramethyloctane.

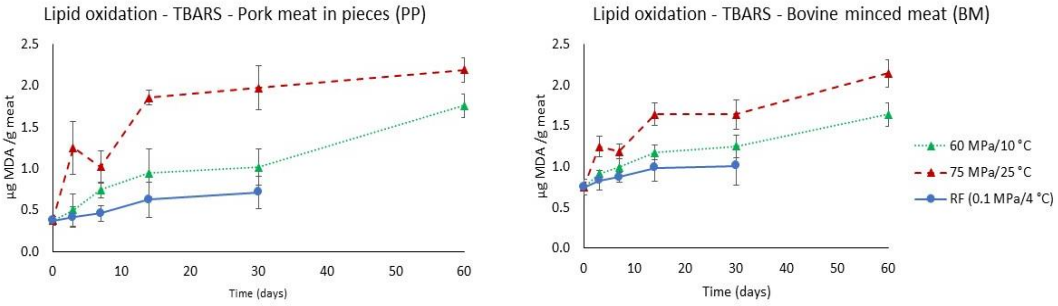
Likewise, two ketones were detected, 2,5-octadione and 3-hydroxybutanone (associated to buttery creamy dairy milky fatty sweet) in PP and BM samples, respectively. Both compounds in HS revealed an increasing tendency, decreasing at the end. These compounds were already identified as meat spoilage tracers, being 3-hydroxybutanone the ketone most commonly found. The pathways to their formation could derive from lipolytic

activity, alkane degradation by bacteria, or bacterial dehydrogenation of secondary alcohols [125].

Lipid oxidation profile. Autoxidation, a free-radical chain reaction, is one of the most important degradation mechanisms in meat products, and it can be described in 3 phases: initiation, propagation, and completion [101, 122]. Briefly, initiation and propagation are recognized for the development of radicals that transform into non-radical compounds such as conjugated dienes and hydroperoxides, the primary products of lipid oxidation [101]. These compounds are recognised for their instability, leading to secondary oxidative products, such as carbonyl compounds, aldehydes, ketones and alcohols, from where aldehydes are crucial due to their fast reaction with proteins [101]. These reactions of secondary products with proteins, phospholipids, nucleic acids may produce chromophores leading to fluorescence spectra [127].

The PVs observed in PP and BM samples before storage experiments were at low and similar amounts, with 0.10±0.04 and 0.11±0.06, respectively, with variations along storage (Table 6.5). Briefly, 60 MPa/10 ºC and RF exhibited similar tendencies for both meat samples, with an initial increase followed by a decrease. It was observed that 60 MPa/10 ºC allowed to better control lipid oxidation, since lower PVs ($p < 0.05$) were determined in both samples when compared to RF. On the other hand, no primary oxidation products were observed in PP samples at 75 MPa/25 ºC, indicative of a faster lipid oxidation. Notwithstanding, BM at this HS condition presented PVs values similar to 60 MPa/10 ºC, but sooner, at the 14th day instead of the 30th day. The overall values obtained for PV allowed concluding that both HS conditions led to a lower ($p < 0.05$) PV content than RF.

In Figure 6.1 is possible to observe TBARS values evolution over 60 days of storage for both HS conditions, as well for RF up to 30 days. The initial value for PP and BM were 0.37±0.05 and 0.75±0.09 µg MDA/g of meat, respectively. This difference could be due to several factors, as meat composition, grinding/chopping of bovine meat that accelerate lipid oxidation (since it liberates more membrane-bound phospholipids aldehydes easily oxidizable), among others [122].



		Storage day					
		0	3	7	14	30	60
PP	60 MPa/10 °C	d	cdA	bcdAB	bcAB	bB	aB
	75 MPa/25 °C	c	bA	bA	aA	aA	aA
	RF (0.1 MPa/4 °C)	a	aA	aB	aB	aB	#
BM	60 MPa/10 °C	d	cdB	bcdAB	bcB	bAB	aB
	75 MPa/25 °C	d	cA	cA	bA	bA	aA
	RF (0.1 MPa/4 °C)	a	aB	aB	aB	aB	#

Figure 6.1. Results obtained in thiobarbituric acid reactive substances (TBARS) analyses (µg MDA/g of meat ± SE) for pork meat in pieces (PP) and bovine minced meat (BM) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-B) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

An increase tendency was observed over 60 days of storage for both HS conditions ($p < 0.05$) and meat samples, reaching values of 1.76±0.14 and 2.19±0.15 µg MDA/g of meat for PP at 60 MPa/10 ºC and 75 MPa/25 ºC, respectively, and 1.64±0.13 and 2.14±0.20 µg MDA/g of meat in BM samples at 60 MPa/10 ºC and 75 MPa/25 ºC, respectively. Moreover, RF samples presented always similar ($p > 0.05$) values to 60 MPa/10 ºC and lower ($p < 0.05$) than 75 MPa/25 ºC. For both HS conditions it was possible to conclude that 60 MPa/10 ºC allowed to better preserve both raw meat samples than 75 MPa/25 ºC. These results are in accordance to previous HS studies [12], with Atlantic salmon preserved under pressure at 25 ºC, where higher TBARS values were obtained for all HS conditions tested (50, 60 and 75 MPa) when compared to RF. Furthermore, Cheah and Ledward [102] reported that pressure processing for pasteurization can promote meat oxidation since water-soluble components of meat, such as low molecular weight iron compounds and myoglobin, hemoglobin and ferritin are important initiators of lipid oxidation after high pressure treatments. However it must be noted that this occurs at higher pressure levels (>300 MPa) than the ones used in HS and over short periods of time (sec to min) [101].

In this study, a frequently used methodology to study fluorescent compounds development by lipid oxidation in fish samples [128-130] was adapted, being the first work where fluorescent compounds development were studied in raw meat samples stored under pressure over long storage periods. The results obtained presented similar behaviours for all storage conditions for both meats (Table 6.5). The initial value (1.33±0.08 and 1.14±0.01) decreased ($p < 0.05$) after 7 days of storage in all samples, although a lower decrease was observed for BM. The overall results allowed observing a similar to lower FR for HS conditions when compared to RF, mainly up to 14 days of storage since at the end no differences were observed between storage conditions.

The results obtained are not in accordance to literature concerning several food products. For instance, when fish samples when stored at cold temperatures at AP tended to increase the ratio of fluorescent compounds with storage time [128-130]. Furthermore, Fidalgo, Lemos, Delgadillo and Saraiva [12] reported initial values between 0.22 and 0.28 for Atlantic salmon, without differences throughout storage time in samples preserved under pressure and RF up to 10 days. Veberg, Vogt and Wold [127] investigated the formation of fluorescent compounds resulting from reactions between protein and carbonyl compounds at 4 ºC in biological systems (including minced turkey, pork, and cod meat). Different fluorescence spectra were obtained for different combinations of aldehydes and amino acids, and the overall intensity increased logarithmically for 14 days.

Table 6.5. Peroxides value (mg Fe/kg lipids ± SE) and Fluorescent compounds (Fluorescent ratio ± SE) obtained for pork meat in pieces (PP) and bovine minced meat (BM) when stored up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-d) and different upper case letters (A-C) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

	Peroxides value						Fluorescent compounds					
	PP			BM			PP			BM		
Storage Condition	60 MPa/10 °C	75 MPa/25 °C	RF (0.1 MPa/4 °C)	60 MPa/10 °C	75 MPa/25 °C	RF (0.1 MPa/4 °C)	60 MPa/10 °C	75 MPa/25 °C	RF (0.1 MPa/4 °C)	60 MPa/10 °C	75 MPa/25 °C	RF (0.1 MPa/4 °C)
Days												
0	0.10±0.04 d	0.10±0.04 a	0.10±0.04 d	0.11±0.06 c	0.11±0.06 c	0.11±0.06 d	1.33±0.08 a	1.33±0.08 a	1.33±0.08 a	1.14±0.01 a	1.14±0.01 a	1.14±0.01 a
7	0.12±0.03 dB	0.09±0.03 aB	2.53±0.29 bA	0.25±0.11 cB	0.38±0.17 bB	8.41±2.38 cA	0.45±0.04 cB	0.50±0.03 bB	0.89±0.04 bA	0.94±0.05 bA	0.80±0.01 bB	1.01±0.03 bA
14	18.83±5.57 aB	0.15±0.02 aC	62.12±15.36 aA	11.53±2.35 bC	26.84±9.41 aB	71.59±14.29 aA	0.80±0.11 bA	0.50±0.02 bB	0.64±0.04 cAB	0.87±0.06 bAB	0.71±0.09 bcB	0.89±0.05 cA
30	2.74±0.46 bA	0.12±0.05 aC	1.24±0.21 cB	22.98±6.78 aA	0.33±0.10 bB	26.84±10.52 bA	0.47±0.08 cA	0.51±0.03 bA	0.51±0.02 dA	0.53±0.08 cA	0.57±0.01 dA	0.55±0.04 dA
60	0.28±0.08 cA	0.17±0.02 aA	#	0.19±0.08 cA	0.14±0.05 cA	#	0.49±0.01 cA	0.51±0.01 bA	#	0.55±0.07 cA	0.61±0.06 cdA	#

not applicable

As well, the authors observed that the degree of aldehydes saturation led to an increase of fluorescence intensity, and aldehydes with double bonds in 2,4 position gave generally higher intensity than aldehydes with double bond in 2,6 position [127]. Furthermore, although MDA, one of the aldehydes studied by Veberg, Vogt and Wold [127], presented a strong fluorescence when added to amino acids, the same did not occur when it was added to minced meat.

In the VOCs analyses of this work, the aldehydes identified (unsaturated) tended to decrease their content mostly at the end of the storage (at the 30th or 60th day of storage), not being found a relation between the results of these analyses. It must be noted that fluorescence spectra of meat samples is a result of different contributions as oxidation products, adipose tissue, connective tissue, porphyrins, and other possible fluorophores, as well various pigments in meat can reabsorb parts of the fluorescence [131, 132].

6.4.Conclusion

The results obtained in this study allowed to better perceive the HS impact on raw meat samples over 60 days of storage when used at a room like temperature (75 MPa/25 ºC) and at cold temperatures (60 MPa/10 ºC). Up to now, there were no studies regarding the effect of this new preservation methodology on fatty acid, VOCs and lipid oxidation profiles. It was possible to conclude that 60 MPa/10 ºC condition should be considered for raw meat preservation, since the results obtained in all analyses demonstrated a similar to better preservation of the samples when compared to conventional RF. Moreover, 75 MPa/25 ºC does not allow proper preservation of PP and BM samples. In the future, the use of HS at cold temperatures should be considered for longer storage periods to better preserve food products and their characteristics. Although HS at RT already proved its capability for microbial growth slowdown and inactivation and several physicochemical parameters maintenance, in this work it was observed that lower temperatures and lower pressure levels could lead to better preservation results.

Chapter 7 – Evaluation of enzymes activity in raw meat and texture characteristics of cooked meat both preserved by hyperbaric storage

This chapter is based on the paper written during the PhD thesis and under revision as "Mauro D. Santos, Ana Salgueiro Carta, José A. Lopes-da-Silva, Ivonne Delgadillo, Jorge A. Saraiva (2019). Hyperbaric storage effect on enzymes activity and texture characteristics of raw meat, in *Food and Bioproducts Processing* (submitted)".

7.1. Overview

Hyperbaric storage has been considered a good alternative to refrigerated preservation processes, when used at variable uncontrolled RT, or as a complement to it when applied at lower temperatures, possibly leading to shelf-life extensions, lower carbon footprints, and lower energy costs, as demonstrated by the growing number of published studies on this subject [9, 22]. This way, HS has been tested in recent years for the preservation of several food products, such as fruit juices [10, 15, 41], ready-to-eat meals [40], raw fish [12], and meat [11, 13]. Notwithstanding, many of these studies have only focused on important microbial (e.g., TAM, ENT, YM, *E. coli*, *L. innocua*, *B. subtilis*, *A. acidoterrestris*), and certain physicochemical parameters (e.g., pH, lipid oxidation, color, cloudiness, browning degree). Thus, there is a need of broad and deeper studies in what concerns HS effect on food products to assess its real feasibility as a new food preservation methodology and explain possible effects on foods, like for instance, the effect on food quality-related enzymes activity.

Regarding meat products, texture is one of the most important quality attributes for consumers, specifically its tenderness, being the post-mortem period crucial since the degradation of muscle properties contributes to the softening of flesh [68]. Calpains and cathepsins are recognized as two proteolytic systems, acting synergistically, capable of hydrolyzing myofibrillar proteins during post-mortem storage [67-69]. The acid proteases cathepsins, that are located in the lysosomes, can be liberated to the cytoplasm and intracellular spaces after lysosomal disruption/cell dead due to pH decrease, leading to muscle ageing [68]. From all cathepsins, cathepsin B, L, H and D have a prominent involvement in muscle ageing, and in the case of mammalian meat B, D and L are the most important due to meat pH (≈6.5 decreasing to 5.7-5.4 after 24h of storage after death) and

cathepsins optimal pH [68, 80, 81]. Moreover, calpains activity is known to be calcium dependent, and calpains are so subclassified into μ-calpains and m-calpains, depending on their sensitivity to calcium ions [68]. Calpains have a large and a small subunit, and although the active site is present in the large subunit, the presence of the smallest one is fundamental for activity.

Considering that HS relies on food storage under pressure, it must be noted that some studies regarding HPP, envisaging pasteurization of raw meat already concluded that enzymes could be affected by pressure, activated or inactivated depending on the pressure level, temperature and enzyme [133]. However, although HPP is being increasingly applied widely at food industries, uses pressure levels between 300 and 600 MPa during sec to few min, while HS is applied at much lower pressure (50-150 MPa), however over days/weeks [2, 11], thus being difficult to infer conclusions from HPP to HS.

Since that texture plays an important role on consumer acceptance of meat products, some methods are currently used as an indicator of meat sensory parameters, such as Warner–Braztler (WB) shear test, or texture profile analysis (TPA), being the latter commonly used in the food sector, since it can assess several texture parameters by a double compression cycle and mimics the conditions to which food is subjected throughout the mastication process [134, 135].

Considering the importance of the enzymatic activity profile in/and texture properties of meat for consumers, and the absence of published studies about HS on these, this work aimed to study the HS impact on enzymes activity (cathepsin B, D, L and calpains), on texture properties of cooked meat previous preserved by HS, as well on meat microstructure by scanning electron microscopy (SEM). For that, two different HS conditions were tested, 60 MPa/10 ºC and 75 MPa/25 ºC up to 60 days and compared to conventional RF.

7.2. Materials and Methods

Sample preparation and storage experiments. Raw PP, in two different sizes (ca. 2x1x1 and ca. 8x4x3 cm) and BM, *Longissimus thoracis et lumborum*, were purchased in a local butcher shop. Portions of 10-12 g for BM and smaller portions of 2x1x1 cm size for PP were prepared under aseptic conditions and packaged into low permeability PA/PE bags (90, Plásticos Macar – Indústria de Plásticos Lda., Palmeira, Portugal). Air was manually removed before the heat-sealing process to minimize the amount of air in the packages.

Previous HS preservation studies of raw pork and bovine meats, allowed concluding that 60 MPa/10 ºC and 75 MPa/25 ºC were the best conditions for meat preservation, capable not only to inhibit microbial growth, but also to additionally cause inactivation of microorganisms, while maintaining the overall physicochemical characteristics analyzed (pH, moisture content, drip loss, color, and lipid oxidation) [136]. The effects of these two HS conditions were so studied in the present work for up to 60 days.

For that, two high-pressure equipment were used, a SFP FPG13900 (Stansted Fluid Power Ltd., Essex, UK) with a pressure vessel of 37 mm inner diameter and 52 cm height (0.4 L volume), and a FPG7100 (Stansted Fluid Power Ltd., Essex, UK), with a pressure vessel of 100 mm diameter and 250 mm height a (2 L volume), for the 60 MPa/10 ºC and 75 MPa/25 ºC conditions, respectively. In both cases a mixture of propylene glycol and water (40:60 vol/vol) was used as pressurizing fluid. The results obtained were compared to control samples stored at RF (0.1 MPa/4 ºC), in the dark and surrounded by the same fluid to mimic the same storage conditions. Storage experiments were carried out in triplicate for each storage day/meat sample/storage condition and the analyses carried out in triplicate.

Enzymes activity

Preparation of the enzymatic extract. The extracts used for the enzymatic activity analyses were obtained applying the methodology reported by Lakshmanan, Patterson and Piggott [137] and Homma, Ikeuchi and Suzuki [138] with some modifications. For each storage day/meat sample/storage condition, 10 g of meat (BM and 2x1x1 cm PP samples) were homogenized with 40 mL of ice-cold distilled water for 2 min, kept during 30 min on ice with occasional stirring, and centrifuged at 14.600 *g* and 4 ºC for 20 min. The supernatant was filtered through a Whatman Nº1 filter and used as the enzymatic extract, being stored at - 20 °C prior to enzymatic activity quantification.

Cathepsin B. Cathepsin B activity assessment was performed following the method of Lakshmanan, Patterson and Piggott [137] with some modifications. Briefly, 0.1 mL of the enzyme extract and 0.1 mL of the substrate solution containing 0.063mM of Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride in 100 mM Bis-Tris buffer with 20 mM ethylenediaminetetraacetic acid (EDTA) and 4 mM dithiothreitol (DTT) at pH 6.5 were incubated up to 5 min. One mL of 3% sodium dodecyl sulfate (SDS, w/v) in 50 mM Bis-

Tris (pH 7.0) was then added to the mixture to stop the reaction. The released 7-amino-4-methylcoumarin (AMC) was measured at an excitation/emission wavelength of 360/460 nm (Hitachi F2000 fluorescence spectrophotometer, Japan) and the activity expressed as fluorescence units (FU)/min/g of meat, being the quantifications carried out in triplicate.

Cathepsin L. Cathepsin L activity quantification was carried out using the method of Homma, Ikeuchi and Suzuki [138] with some adjustments. In this case carbobenzoxy-L-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA) was used as substrate. To 100 µL of the enzyme extract were added 450 µL of distilled water, 50 µL potassium phosphate (0.8 M, at a pH of 5.5), 32 µL EDTA (32mM) and 20 µL DTT (120 mM). After incubation at 37 °C for 5 min, 100 µL of the substrate solution was added to the reaction mixture, with the reaction being stopped after 10 min by the addition of 2 mL of 100 mM monochloroacetic acid in 100 mM acetate buffer (pH 4.3). After, the fluorescence was measured at 370/460 nm (Hitachi F2000 fluorescence spectrophotometer, Japan) and the activity expressed as FU/min/g of meat, being the quantifications carried out in triplicate.

Cathepsin D. Cathepsin D was evaluated following the method used by Anson [139] and Teixeira, Fidalgo, Mendes, Costa, Cordeiro, Marques, Saraiva and Nunes [140] with some modifications. An aliquot of the enzyme extract previously obtained (0.5 mL) was incubated with 1.5 mL of denatured hemoglobin from bovine blood (20 mg/mL) in 0.2 M citrate buffer, pH 3.7. After 3 hours of incubation at 37 ºC the reaction was stopped adding 1.5 mL of trichloroacetic acid (0.1 g/mL). After being stirred for 1 min the precipitate was removed by centrifugation (14000 *g* for 15 min), and the soluble peptides measured at 280 nm (Microplate Spectrophotometer Multiskan GO, Thermo Scientific, USA). Cathepsin D activity was expressed as absorbance units (AU)/h/g of muscle being the analyses performed in triplicate.

Calpains. Some modifications to the method used by Lakshmanan, Patterson and Piggott [137] and Teixeira, Fidalgo, Mendes, Costa, Cordeiro, Marques, Saraiva and Nunes [140] were carried out to evaluate calpains activity in the samples. Briefly, 0.1 mL of enzyme extract and 0.1 mL of the substrate solution consisting of 0.125 mM L-methionine-7-amido-4-methylcoumarin triflouroacetic salt in Bis-Tris (100 mM), $CaCl_2$ (5 mM) at pH 6.5 and 4

ºC, were mixed. After 10 min at 4 ºC, the reaction was stopped adding 3 mL of monochloroacetic acid (30 mM), acetic acid (21 mM) and sodium acetate (9 mM) at pH 4.3 and 4 ºC. Fluorescence was measured at 360/460 nm (Hitachi F2000 fluorescence spectrophotometer, Japan) and the activity was expressed as FU/min/g being the analyses carried out in triplicate.

Texture profile analysis. Textural profile analysis (TPA) of grilled PP samples (ca. 8x4x3 cm) was performed for each preservation condition along storage time. For that, the methodology used by Caine, Aalhus, Best, Dugan and Jeremiah [141] was applied with some modifications. The samples were grilled in a stainless-steel pan placed in a hotplate at constant temperature, being cooked on one side to an internal temperature of 45 ºC and then turned and cooked to an internal final temperature of 75 ºC. Temperature was measured using a thermocouple (10 cm spear point, T-type) inserted horizontally at the midpoint of the samples. After cooking, samples were packed in polyamide-polyethylene bags (PA/PE-90, Plásticos Macar – Indústria de Plásticos Lda., Portugal) and cooled down in an ice bath. A TA-HDi texture analyser (Stable Micro Systems, United Kingdom) equipped with a cylindrical stainless probe of 2 mm diameter, was used to perform all the textural analyses. A two-cycle puncture test was performed perpendicularly to the muscle fibres orientation, to 50% deformation (relative to sample height) at a speed of 0.5 mm/sec, with a 2 sec pause between cycles.

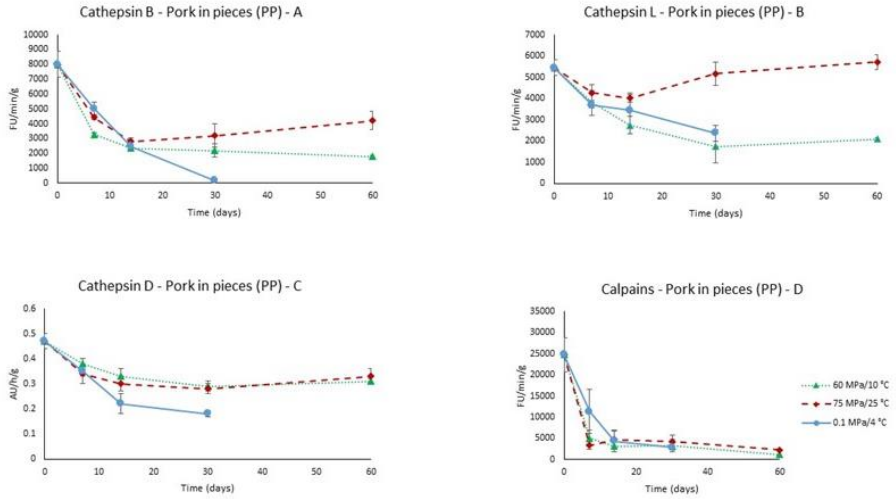
Each sample was analysed at four different points and TPA parameters were calculated from force-by-time data. Hardness (peak force in the first cycle), cohesiveness (ratio between the area of the second compression and the first compression), springiness (ratio of the time elapsed during the force input between the second and the first compression), resilience (ratio of the negative force input to positive force input in the first compression), adhesiveness (negative area between cycles), and chewiness (hardness multiplied by cohesiveness multiplied by springiness) were determined [141, 142].

Scanning electron microscopy. Longitudinal sections of freeze-dried pork meat in pieces (lyophilized at - 70 °C and 0.010 mbar for 72 hours; Telstar Benchtop Freeze Dryer LyoQuest, Spain) were observed by scanning electron microscopy (SEM) (Tabletop Microscope TM4000Plus, Hitachi, Japan), with an accelerating voltage of 15kV.

Statistical analysis. Triplicate of samples were analysed for each storage condition/storage day of analysis. Statistical data analysis was performed using one-way analysis of variance ANOVA, followed by a Tukey's HSD Test, at a 5% level of significance to identify differences between storage conditions and throughout storage time at each condition.

7.3. Results and Discussion

Enzymes activity. Changes in enzymes activities with storage time were clearly different for each type of meat, for the three different preservation conditions analysed, as shown in Figure 7.1 and Figure 7.2.



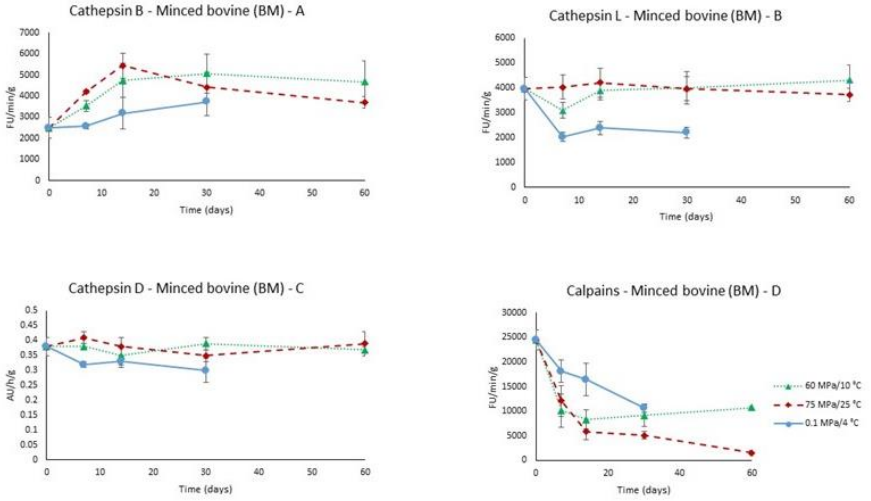
PP		0	7	14	30	60
CathepsinB	60 MPa/10 °C	a	bB	bcA	bcA	cB
	75 MPa/25 °C	a	bA	bA	bA	bA
	0.1 MPa/4 °C	a	bA	cA	cA	#
CathepsinL	60 MPa/10 °C	a	bA	bcA	cB	cB
	75 MPa/25 °C	a	bcA	cA	abA	aA
	0.1 MPa/4 °C	a	bA	bcA	cB	#
Cathepsin D	60 MPa/10 °C	a	bA	bcA	cA	cA
	75 MPa/25 °C	a	bA	bAB	bA	bA
	0.1 MPa/4 °C	a	bA	cB	cB	#
Calpains	60 MPa/10 °C	a	bA	bA	bA	bB
	75 MPa/25 °C	a	bA	bA	bA	bA
	0.1 MPa/4 °C	a	bA	bA	bA	#

the samples showed a microbial advanced state of degradation and so were not analysed

Figure 7.1. Evolution of cathepsin B, L, D and calpains activity on pork meat in pieces (PP) up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Results are expressed as mean ± SE in fluorescent units (FU)/min/g of meat for cathepsin B, L and calpains, and absorbance units (AU)/h/g of meat for cathepsin D. Different lower case letters (a-c) and different upper case letters (A-B) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

Overall, for PP samples all enzymatic activities decreased along time. In more detail, a decrease ($p < 0.05$) of cathepsin B and calpains (Figure 7.1A and Figure 7.1D) activities was observed for all preservation conditions just after 7 days of storage (for instance, for cathepsin B a decrease of ≈38, ≈45 and ≈59% was observed for RF, 75 MPa/25 ºC and 60 MPa/10 ºC, respectively). A less pronounced decrease ($p < 0.05$) was observed for cathepsins L and D (Figure 7.1B and Figure 7.1C; for example, in cathepsin L after 7 days, a decrease of ≈33, ≈32 and ≈22% was detected for RF, 75 MPa/25 ºC and 60 MPa/10 ºC, respectively). In general, after storage for two weeks, HS lead to lower enzymes activity, compared to the initial value, except for cathepsin L at 75 MPa/25 ºC (Figure 7.1B), which showed a similar value ($p > 0.05$) at the 60th day of storage (5712±370) when compared to the initial one (5450±372). When storage conditions were compared at each storage day, main differences were observed in cathepsins B and L. Moreover, although the analyses of refrigerated

samples were only performed up to 30 days of storage, due to samples microbial spoilage (data not shown), it was possible to detect similar behaviours at the beginning of storage when compared to samples stored under pressure, being obtained generally the lowest values at the 30th day of storage for refrigerated samples.



BM		0	7	14	30	60
CathepsinB	60 MPa/10 °C	b	abB	aAB	aA	aA
	75 MPa/25 °C	b	abA	aA	abA	bcA
	0.1 MPa/4 °C	a	aC	aB	aA	#
CathepsinL	60 MPa/10 °C	a	aB	aA	aA	aA
	75 MPa/25 °C	a	aA	aA	aA	aA
	0.1 MPa/4 °C	a	bC	bB	bB	#
Cathepsin D	60 MPa/10 °C	a	aA	aA	aA	aA
	75 MPa/25 °C	a	aA	aA	aAB	aA
	0.1 MPa/4 °C	a	abB	abA	bB	#
Calpains	60 MPa/10 °C	a	bB	bB	bA	bA
	75 MPa/25 °C	a	bAB	cB	cB	cB
	0.1 MPa/4 °C	a	bA	bcA	cA	#

the samples showed a microbial advanced state of degradation and so were not analysed

Figure 7.2. Evolution of cathepsin B, L, D and calpains activity on minced bovine meat (BM) up to 60 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Results expressed as mean ± SE in fluorescent units (FU)/min/g of meat for cathepsin B, L and calpains, and absorbance units (AU)/h/g of meat for cathepsin D. Different lower case letters (a-c) and different upper case letters (A-B) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition and between storage conditions at each storage time, respectively.

On the other hand, changes on activity of cathepsins B, L, and D were smaller for the BM samples (Figure 7.2). Contrarily to what was observed for the PM samples, cathepsin B activity increased under the storage conditions analysed, namely during the initial two-weeks of storage (Figure 7.2; increasing ≈28%, ≈118% and ≈90% in RF, 75 MPa/25 ºC and 60 MPa/10 ºC samples, respectively). Generally, cathepsin L and D showed a maintenance of activity values during storage under pressure conditions, while RF caused a decrease tendency just after 7 days (≈45% and ≈21%, respectively), but then also with no-significant

effects up to the end of storage. For the three cathepsins, both HS conditions revealed similar results over storage, being the activity values generally higher than for samples stored under RF conditions, mainly for cathepsin L (Figure 7.2B). As observed for PP samples, for BM, calpains also presented a considerable decrease of activity over storage (between a minimum of ≈57% for refrigerated samples and a maximum of ≈94% for 75 MPa/25 ºC), although with a more pronounced difference between storage conditions, with samples stored under RF conditions showing higher calpains' activity than that observed under HS conditions, for all storage times analysed (Figure 7.2D).

Although the absence of studies regarding the HS effect on meat enzymes it is known that a pressure treatment at higher pressure (100-200 MPa) over short periods of time increased cathepsin activities in mammals meat, due to lysosome membrane damage induced by pressure [133]. Furthermore, Homma, Ikeuchi and Suzuki [138] observed that cathepsins B, D and L of beef samples increased with pressure level (processing period of 5 min) up to 400 MPa, and even when a crude extract of enzymes were pressure treated cathepsins B, D and L revealed equal to higher activities from 100 MPa up to 400 MPa, decreasing for higher pressure levels.

For calpains, some studies verified that the activity depends also on several factors, as calpastatin concentration or myofibrils fragmentation [133]. For instance, in rabbit muscle calpains activity remained practically unchanged up to 100 MPa (5 min and 2 ºC), while for higher pressures, a reduction of activity was verified [143].

Notwithstanding, a published work performed by Fidalgo, Delgadillo and Saraiva [34] regarding the HS effect (50-75MPa at 10, 25 and 37 ºC) on raw fish enzymes up to 50 days concluded that generally cathepsins B and D activities could be affected by storage temperature (with higher reductions at 37 ºC), and on the other hand that pressure storage seemed to have no effect on these enzymes activities, being the activity decrease similar to control samples (at AP). The authors also observed an increase of these enzymes activity for longer storage periods (up to 50 days) attributed to lysosomes disruption. The same authors reported the lowest activities for calpains and being mainly influenced by storage time regardless of the pressure level applied [34].

The pressure levels studied in this work (60 and 75 MPa) were lower than those used for the available results in the literature (most of all >200 MPa by HPP), and additionally the latter were also obtained for shorter processing times (few min), thus hindering results

comparison. In this work, HS (60 and 75 MPa) took place for much longer times (up to 60 days) and the first sampling point was at the 7th day of storage. This way, the results are not comparable straightforwardly with results already published regarding HPP of raw meat. Furthermore, the level of activity measured for each enzyme, at each sampling point, indicates the level of activity at that storage time (being possibly affected by denaturation and action of proteases and microbial activity), but no straightforward conclusions can be taken, for the level of activity during HS, since it depends also on the degree on denaturation and action of proteases and microbial growth, and in addition is influenced by the temperature and pressure of storage.

Furthermore, the differences observed between PP and BM could be related not only to the animal species but also to the two meats state, since mince processing applied to bovine sample cause cells disruption, thus enhancing contact between cells components [122].

Texture profile analysis. Textural profile analysis of grilled PM samples allowed determining initial values of 30 ± 3 N, 0.58 ± 0.03, -2.82 ± 0.03 N.s, 0.75±0.03, 13.1 ± 0.8 N, and 0.20 ± 0.02 for hardness, cohesiveness, adhesiveness, springiness, chewiness, and resilience, respectively (Table 7.1).

Table 7.1. Results obtained in textural profile analysis (TPA) for cooked pork meat in pieces (PP), when stored up to 14 days at different storage conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC. Different lower case letters (a-c) and different upper case letters (A-B) indicate significant differences ($p < 0.05$) between storage times at each storage condition and between storage conditions at each storage time, respectively.

Storage Condition	Storage Day	Hardness (N)	Cohesiveness	Adhesiveness (N.s)	Springiness	Chewiness (N)	Resilience
60 MPa - 10 ºC	**0**	29.99±2.95 a	0.58±0.03 b	-2.82±0.03 b	0.75±0.03 b	13.09±0.77 a	0.20±0.02 b
	3	29.31±3.93 aA	0.56±0.02 bB	-2.99±1.13 bB	0.80±0.03 bA	13.29±1.91 aA	0.21±0.03 bB
	14	26.80±2.63 aA	0.63±0.04 aA	-1.42±0.69 aA	0.81±0.02 aA	13.18±1.49 aA	0.27±0.03 aB
75 MPa - 25 ºC	**0**	29.99±2.95 a	0.58±0.03 b	-2.82±0.03 b	0.75±0.03 b	13.09±0.77 a	0.20±0.02 c
	3	26.83±2.71 abAB	0.62±0.03 bA	-1.22±0.59 aA	0.80±0.01 aA	13.29±1.81 aA	0.26±0.03 bA
	14	24.26±2.18 bA	0.67±0.04 aA	-1.04±0.48 aA	0.83±0.04 aA	13.18±1.24 aA	0.31±0.03 aA
0.1 MPa - 4 ºC	**0**	29.99±2.95 a	0.58±0.03 a	-2.82±0.03 b	0.75±0.03 a	13.09±0.77 a	0.20±0.02 a
	3	23.85±3.46 bB	0.62±0.04 aA	-1.57±0.60 aA	0.78±0.04 aA	11.48±1.86 aA	0.20±0.03 aB

In general, there was a tendency for hardness to decrease with storage time. However, no statistically significant differences were observed under HS 60 MPa/10 ºC ($p > 0.05$). A significant decrease in hardness ($p < 0.05$) was obtained under RF conditions, decreasing to 24 ± 4 N just after 3 days of storage. The strength of the internal structure of meat, assessed by cohesiveness, showed an increase ($p < 0.05$) tendency at the 14th day of storage for all storage conditions, just being observed a slight benefit of 60 MPa/10 ºC at the 3rd day since the value was better maintained (0.56±0.02) when compared to the initial one (0.58±0.03).

Considering the adhesiveness changes, HS storage under 60 MPa/10 ºC also revealed to be the better condition regarding the maintenance of meat texture characteristics, at least during the first 3 days of storage, since no significant differences ($p > 0.05$) were obtained for adhesiveness when compared to day 0 (although at the 14th day, both HS conditions led to similar values, $p > 0.05$).

Springiness and chewiness parameters did not reveal significant differences ($p > 0.05$) over time at each storage condition and between storage conditions at each storage time, except a slight increase of springiness observed at the 3rd day in all storage conditions. Regarding resilience 60 MPa/10 ºC demonstrated to be again the best HS condition since it allowed a smaller increase of the values when compared to 75 MPa/25 ºC.

Globally, it is possible to observe that 60 MPa/10 ºC could maintain better the texture characteristics of PP grilled samples compared to 75 MPa/25 ºC and RF.

Scanning electron microscopy. Figure 7.3 presents SEM images of PP samples before storage, after 3 days of storage and at the 30th day of storage. As it is possible to see, when control sample (day 0) was compared to all storage conditions at the 3rd day, higher differences were observed in 75 MPa/25 ºC since fibres gained a smother appearance probably due to proteins denaturation, seeming less individualized. On the other hand, it was possible to verify that fibres are tighter and closer together in 60 MPa/10 ºC storage condition when compared to 0.1 MPa/4 ºC and control sample. When both HS conditions were compared at the 30th day of storage, 75 MPa/25 ºC continued to demonstrate considerable differences when compared to 60 MPa/10 ºC, being the latter more similar to fibres appearance at the beginning of the study. In what concerns pressure effect on meat structure, a higher pressure level, although over a short period of time was tested by Elgasim and Kennick [144], where high pressure treated (103.5 MPa at 37 ºC and 2 min) beef meat presented

a distorted endomysial and sarcolemmal sheath, interfibrillar spaces, intermyofibrillar spaces and globular material on the surface of the fibres.

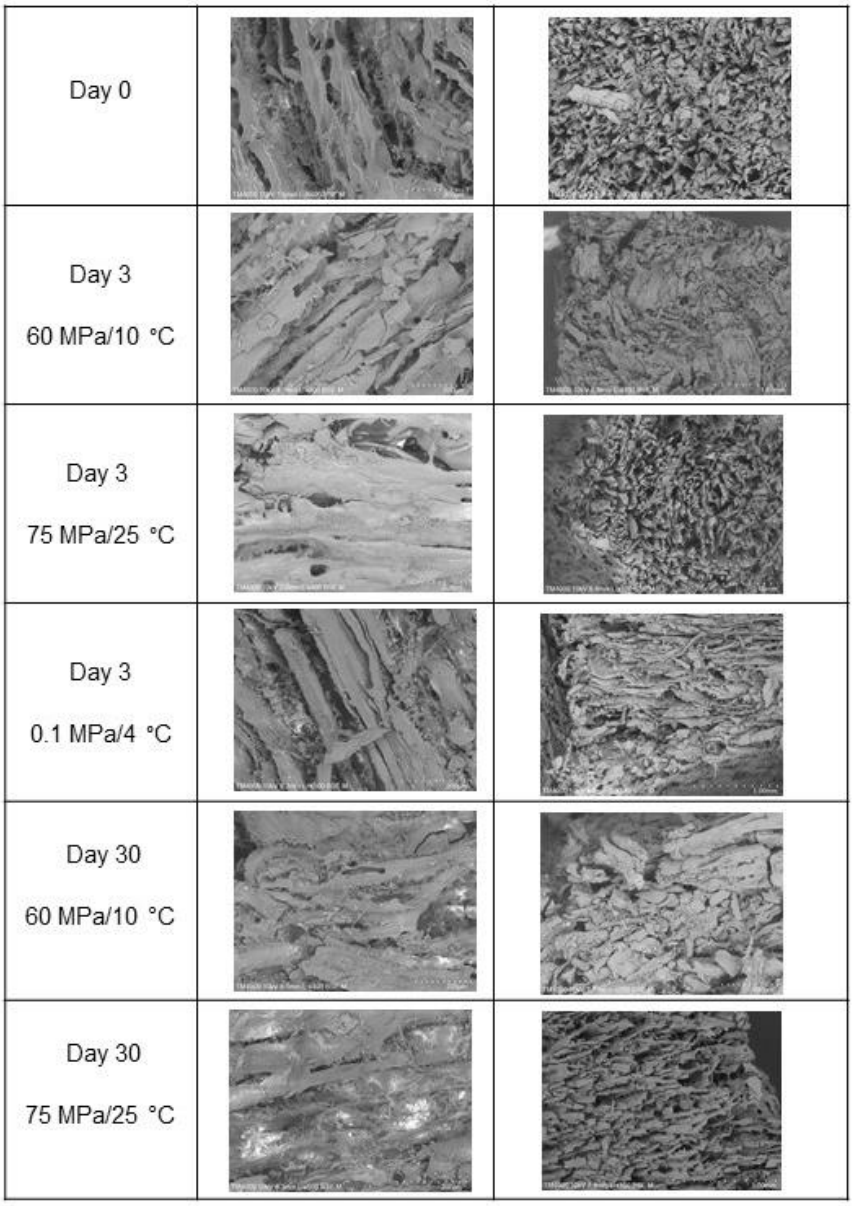


Figure 7.3. Scanning electron microscopy (SEM) images of pork meat in pieces (PP) prior to any storage time, and after 3 and 30 days of storage at different conditions: 60 MPa/10 ºC, 75 MPa/25 ºC, and 0.1 MPa/4 ºC.

7.4.Conclusion

This study aimed evaluating HS impact at room-like (75 MPa/25 ºC) and refrigerated temperatures (60 MPa/10 ºC) on enzymes activity, meat structure and texture properties of cooked meat preserved by HS. It was possible to observe different behaviours between both samples on enzymes activities, probably not only due to the fact of being from different species, but also by the fact that bovine sample was previously minced. Generally, PP revealed a decrease tendency of cathepsin B, D, L and calpains activities, presenting both HS conditions similar values over storage except for cathepsin L. On the other hand, BM samples revealed the maintenance of enzymes activities over storage except for calpains for which a decrease was observed.

When samples were cooked, it was verified that 60 MPa/10 ºC allowed a better maintenance of the initial texture properties. In PP samples, SEM images revealed that 75 MPa/25 ºC led to extensive changes on meat structure when compared to 60/10 ºC and RF that kept better the initial fibres characteristics.

Thus, 60 MPa/10 ºC demonstrated to be the better HS condition proving that HS at refrigerated temperatures should be considered as an alternative/complement to the traditional RF.

Chapter 8 – Concluding remarks and future work

The feasibility of the HS methodology has been increasingly tested for several food products, such as for fruit juices, ready-to-eat meals, dairy products, and meat/fish products. Among these studies important conclusions have been made, for instance, the capability, not only to inhibit microbial growth but also to inactivate microorganisms, and the possibility to keep the overall physicochemical parameters nearly unchanged when compared to the initial sample, or that sample when preserved at RF condition.

Moreover, it should be highlighted that HS could become a possible alternative/complement to RF, turning food preservation into a greener process since when used at variable RT no energy is required over storage after being reached the desired pressure level. Notwithstanding, if low temperature is needed coupled with HS, even so, less energy will be spent since usually these temperatures are higher than ≈5 ºC (the normal RF temperature), while a longer shelf-life period could be obtained.

Though, there was a lack of studies regarding its capability for raw meat at long term as well the impact on several important parameters such as, fatty acids profile, VOCs, texture after cooking, enzymes, among others.

Thus, this PhD thesis aimed studying the HS feasibility as a new food preservation methodology for raw fresh bovine and pork meat, minced and in pieces, observing its impact on several microbial, physicochemical and textural properties, correlating how it could affect the final quality of the product. The work plan for this thesis consisted in the HS optimization for these food products, as well it was intended a comparison of this new methodology to the traditional RF process. For that, three pressure levels were used at variable uncontrolled RT for HS as an alternative to RF: 50, 75 and 100 MPa; and in order to study HS as a complement to RF, 50, 60 and 70 MPa at 10 ºC (Annex I) were also tested. During HS optimization, it was assessed a possible microbial shelf-life period to be used in the next phases of the work. At the end of this thesis several conclusions can be made:

► Raw fresh bovine and pork meat, both minced and in pieces, were better preserved microbiologically (TAM, ENT, LAB, YM) at 75/100 MPa at variable RT and 50/60/70 MPa at 10 ºC than at RF conditions. In this study it was possible to detect not only the microbial growth inhibition at these conditions, but also microorganisms inactivation up to 60 days.

The same was not observed for 50 MPa at variable RT and for RF since samples reached microbial loads in the first 3 to 7 days of storage that turned it unsafe for consumption.

In what concerned the physicochemical impact of HS on samples, it was possible to observe an overall equal to better pH maintenance and similar colour (ΔE) differences between HS and RF. The increase of pressure level on HS at variable RT led to a tendency of lower and higher moisture and drip loss values, respectively, over storage. Furthermore, HS at variable RT revealed a decrease of sarcoplasmic protein values in samples stored under pressure, being observed similar trends in some samples stored at RF (mainly on bovine meat).

► At this point of work, the results allowed defining as optimum conditions, 75 MPa at variable RT (in further studies it was used a set temperature of 25 ºC) as an alternative to RF, and 60 MPa at 10 ºC as a complement to RF.

► The microbial analyses performed in the samples stored at the optimized conditions, 75 MPa at 25 ºC and 60 MPa at 10 ºC up to 60 days, revealed the same trend previously observed. Furthermore, it was concluded that the initial inoculated loads of *E. coli* and *L. innocua* were also better controlled by HS, through its inactivation over time, while RF was not capable to inhibit its growth.

It must be noted that in the initial studies, when lipid oxidation was assessed only by TBARS analysis, HS at variable RT revealed higher secondary products of lipid oxidation when compared to RF. This fact was further analysed by a complete set of lipid oxidation analyses (peroxides value, TBARS and fluorescent compounds). In the latter set of analyses, 60 MPa/10 ºC resulted in a better lipid oxidation control (primary, secondary and tertiary) than in HS at RT throughout storage, with TBARS values similar to RF storage.

► The fatty acid profile analyses allowed to conclude that its general total content decreased throughout time for all samples and storage conditions, except for 60 MPa at 10 ºC. Even so, when SFAs, MUFAs and PUFAs were globally analysed, no consistent variations were observed between both HS conditions. Moreover VOCs profiles allowed to identify several compounds related to meat spoilage and lipid oxidation, with hexanal, 3-methylbutanal, 1-octen-3-ol, 2-ethyl-1-hexanol, 2,2,4,6,6-pentamethylheptane, and 2,2,4,4-

tetramethyloctane showing high variations during storage, with a global similar to better preservation performance at 60 MPa and 10 ºC when compared to RF.

► Regarding enzymes analyses performed in the samples stored at optimized conditions, revealed, generally, for PP samples, that both HS conditions caused a decrease in enzymes activities over storage alike RF, except for cathepsin L and 75MPa at 25 ºC, for which higher values were observed. On the other hand, for BM samples, no major differences were observed overall for the two HS conditions, and the values were higher compared to RF, except for calpains for which the opposite was observed. Activities along storage increased for cathepsin B, while cathepsins D and L showed no variations (except for the latter in RF).

► After cooking raw meat preserved under pressure, 60 MPa at 10 ºC revealed to be the preservation condition that allowed a similar to better maintenance of meat textural characteristics during storage when compared to 75 MPa at 25 ºC and RF. Furthermore, analysis by SEM showed a more pronounced effect of 75 MPa at 25 ºC on meat microstructure, with muscular fibres showing a smother appearance and a less individualized organization.

As it is possible to observe, 60 MPa at 10 ºC demonstrated to be the best HS preservation condition leading to shelf-life periods up to 60 days, instead of 3 to 7 days obtained at RF. Although HS at RT could be efficaciously used for shorter shelf-life periods, the application of HS at low temperatures allowed to better preserve several characteristics of meat as the fatty acids profile, VOCs profile, texture, and lipid oxidation control. Hyperbaric storage at low temperatures led to better results This could be a turnover on the food preservation field since a great advantage could be obtained by food companies and consumers. However future work should be performed to complete the results obtained during this PhD thesis, such as:

► Generally, this study should be continued to increase the number of replicates using for that meat samples from several suppliers taking into consideration the conditions at which the animal was slaughtered;

► The HS optimization for poultry meat (among other types of meat) should be also carried out;

► Evaluation of the HS impact on foodborne microorganisms/spores, e.g., *Staphylococcus aureus, L. monocytogenes, among others*;

► Analyses of the HS effect on proteins by protein muscle profile determination using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) should be performed;

► Evaluation of the possible HS impact on the nutritional properties of cooked meat preserved under pressure;

► A sensorial analysis should be carried out thus allowing the validation of this new food preservation methodology by consumers.

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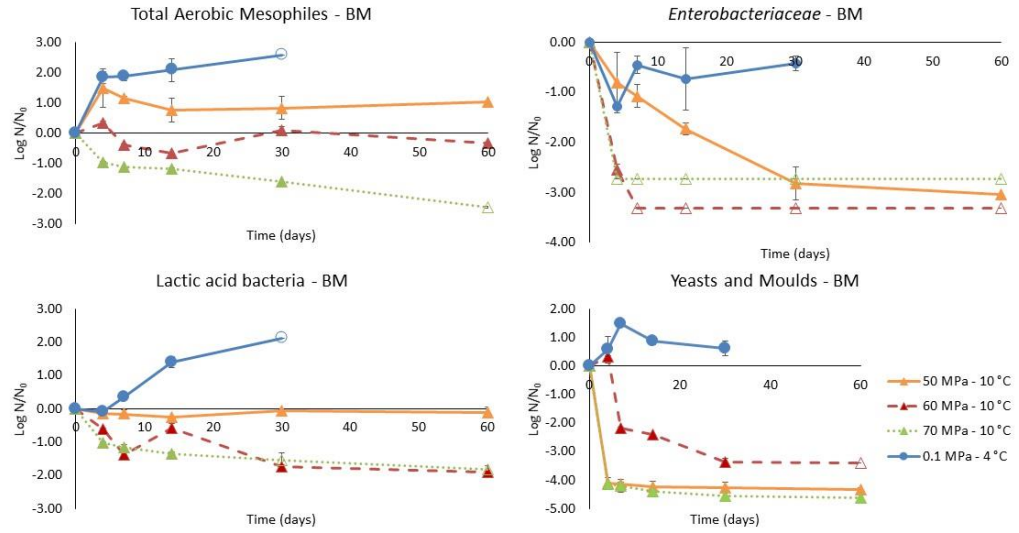
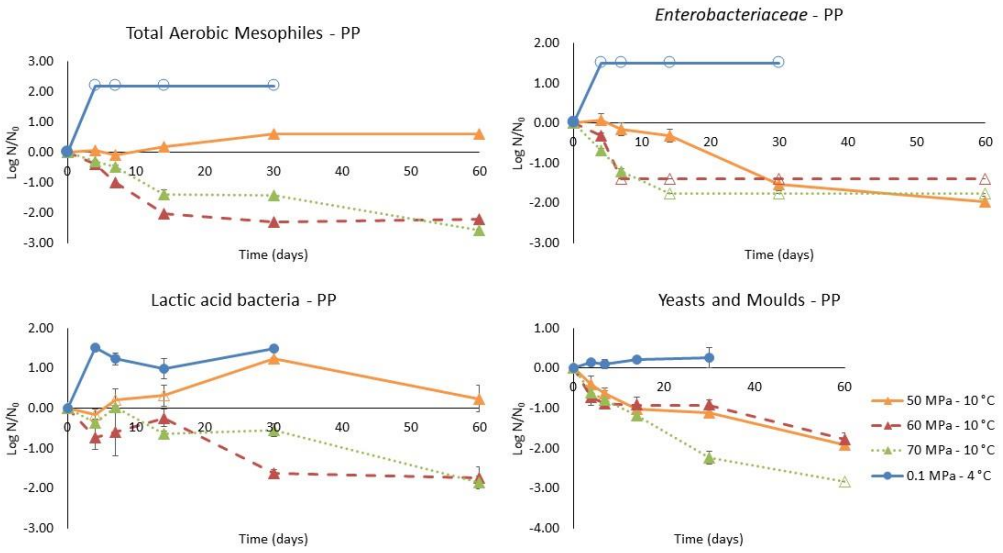
Annex I

The current annex (I) intended to present the results obtained during HS optimization coupled with low temperatures. To do so, 50, 60 and 70 MPa were tested at 10 ºC up to 60 days, being the results compared to RF.



		Storage day					
BM		0	3	7	14	30	60
TAM	50 MPa/10 °C	b	aA	aB	abB	aB	aA
	60 MPa/10 °C	b	aB	cC	dC	bC	cB
	70 MPa/10 °C	a	bC	cD	dC	eD	fC*
	0.1 MPa/4 °C	c	bA	bA	bA	aA*	#
ENT	50 MPa/10 °C	a	bA	bB	cB	dB	dB
	60 MPa/10 °C	a	bC	cD*	cC*	cC*	cC*
	70 MPa/10 °C	a	bD*	bC*	bC*	bB*	bA*
	0.1 MPa/4 °C	a	cB	abA	bcA	abA	#
LAB	50 MPa/10 °C	a	abA	abB	bB	abB	abA
	60 MPa/10 °C	a	bB	cD	bC	dC	eB
	70 MPa/10 °C	a	bC	bcC	cdD	dC*	eB*
	0.1 MPa/4 °C	d	dA	cA	bA	aA*	#
YM	50 MPa/10 °C	a	bB	bC	bC	bC	bB
	60 MPa/10 °C	b	aA	cB	dB	eB	eA*
	70 MPa/10 °C	a	bB	bC	bcC	cC	cC
	0.1 MPa/4 °C	c	bcA	aA	abA	bcA	

Figure 0.1. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on minced bovine meat (BM) expressed in Log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 60 MPa, 70 MPa at 10 ºC, atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) Different lower case letters (a-f) and different upper case letters (A-D) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.



PP		Storage day 0	3	7	14	30	60
TAM	50 MPa/10 °C	cd	cB	dB	bB	aB	aA
	60 MPa/10 °C	a	bC	cD	dD	fD	eB
	70 MPa/10 °C	a	bC	cC	dC	dC	eC
	0.1 MPa/4 °C	e	dA*	cA*	bA*	aA*	#
ENT	50 MPa/10 °C	a	aB	bB	cB	dC	eC
	60 MPa/10 °C	a	aB	bD*	bC*	bB*	bA*
	70 MPa/10 °C	a	bB	cC	dD*	dD*	dB*
	0.1 MPa/4 °C	b	aA*	aA*	aA*	aA*	#
LAB	50 MPa/10 °C	b	bB	bB	bB	aA	bA
	60 MPa/10 °C	a	aC	aB	aBC	bC	BB
	70 MPa/10 °C	a	bBC	aB	cC	bcB	dB
	0.1 MPa/4 °C	c	aA	abA	bA	aA	#
YM	50 MPa/10 °C	a	bB	bB	cB	cB	dA
	60 MPa/10 °C	a	bB	bB	bB	bB	cA
	70 MPa/10 °C	a	bB	bB	cB	dC	eB*
	0.1 MPa/4 °C	a	aA	aA	aA	aA	#

Figure 0.2. Total aerobic mesophiles (TAM), *Enterobacteriaceae* (ENT), Lactic acid bacteria (LAB), and yeasts and moulds (YM) counts on pork meat in pieces (PP) expressed in Log CFU/g (mean ± SE), up to 60 days at different storage conditions: 50 MPa, 60 MPa, 70 MPa at 10 ºC, atmospheric pressure/4 ºC (0.1 MPa - 4 ºC) Different lower case letters (a-e) and different upper case letters (A-D) indicate significant differences ($p < 0.05$) between storage times at each tested storage condition (different columns) and between storage conditions at each storage time (different lines), respectively, and * represent samples with microbial counts below or above the established limit.

As it is possible to observe in Figure 0.1 and Figure 0.2, all HS conditions allowed a better microbial control over storage than RF since the growth of the studied microorganism was inhibited in most of the cases, leading also to microorganism inactivation when 60 and 70 MPa were applied. An exception must be noted for 50 MPa at 10 ºC since TAM in BM samples and LAB in PP samples presented an increase of the microbial load. Notwithstanding, even the latter result was microbiologically better than RF due to a fast microbial growth observed on samples at RF. Thus, HS optimization at low temperature was fixed at 60 MPa at 10 ºC due to its potential feasibility on raw meat storage.

At the end, it was possible to conclude that at a microbial control level, 60 MPa at 10 10 ºC was capable to preserve meat samples up to 60 days, being for some microorganism the results similar between that condition and 70 MPa/10 ºC.