Departamento de Quimica

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# ARMAZENAMENTO SOB PRESSÃO DE REQUEIJÃO SEM REFRIGERAÇÃO

# STORAGE UNDER PRESSURE OF *REQUEIJÃO* WITHOUT REFRIGERATION

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, no Ramo de Biotecnologia Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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**Palavras-chave** Armazenamento hiperbárico, armazenamento sob pressão, alta pressão, queijo de soro, Requeijão, crescimento microbiano, tempo de prateleira

**Resumo**

O armazenamento sob pressões moderadas de Requeijão, o queijo de soro português, um alimento lácteo altamente perecível, foi avaliado como uma possível alternativa energeticamente menos dispendiosa do que a refrigeração. A nível laboratorial, o queijo de soro foi armazenado durante 4 e 8 horas, a diferentes pressões (0.1, 100 e 150 MPa) e temperaturas (25, 30 e 37 ºC), sendo os resultados comparados com a refrigeração (4 ºC). Foi ainda feito um estudo num equipamento industrial, com Requeijão de dimensões semelhantes aos comercializados, durante períodos maiores de armazenamento, 12 e 24 horas, a 100 MPa, à temperatura ambiente (≈21 ºC) com o respectivo controlo sob refrigeração. Realizou-se ainda um estudo pós armazenamento hiperbárico, no qual as amostras sob refrigeração e à temperatura ambiente a 0.1 e 100 MPa, após as 24 horas de armazenamento, foram armazenadas durante três dias sob refrigeração à pressão atmosférica.

A análise microbiana demonstrou que o armazenamento durante 4 horas a 100 MPa manteve a carga microbiana semelhante à refrigeração e à carga inicial, ≈3 $Log_{10}$ CFU/g, em todas as temperaturas estudadas. Ao aumentar a pressão para 150 MPa e o período de armazenamento para 8 horas, observou-se inactivação microbiana para todos os microrganismos, sendo que as bactérias ácido lácticas e *Enterobacteriaceae* foram reduzidas para valores abaixo dos limites de detecção (<1 unidade logarítimica). O armazenamento hiperbárico manteve o pH, actividade da água e os valores de oxidação lipídica, semelhantes aos da refrigeração, mantendo também a cor. O estudo com Requeijão de tamanho semelhante aos comerciais, obteve resultados semelhantes, com melhoria a nível de inibição e inactivação microbiana comparativamente à refrigeração, sem alterações a nível físico-químico e de cor.

Considerando os resultados obtidos, o armazenamento sob pressão de alimentos altamente perecíveis, parece ser uma possível alternativa relativamente à refrigeração, com benefícios económicos para as indústrias e ecológicos para o ambiente.

**Keywords** Hyperbaric storage, storage under pressure, high pressure, whey cheese, *Requeijão*, high pressure, microbial growth, shelf-life

**Abstract**

**S**torage under mild pressure of *Requeijão*, the traditional Portuguese whey cheese, a highly perishable dairy food, was evaluated as a possible energy costless alternative to refrigeration. At a laboratorial scale, the whey cheese was stored during 4 and 8 hours, at different pressure levels (0.1, 100, and 150 MPa) and temperatures (25, 30, and 37 ºC), and the results were compared with refrigeration (4 ºC). A experiment in an industrial equipment was conducted, using *Requeijão* with similar sizes to those commercially available, for longer storage periods, 12 and 24 hours, under 100 MPa at room temperature (≈21 ºC), with the respective control under refrigeration. A post-hyperbaric storage study was performed, in which samples previously stored under refrigeration and at room temperatures under 0.1 and 100 MPa, for 24 hours, were then stored under refrigeration at atmosphere pressure, for three days.

Microbial analyses showed that storage for 4 hours at 100 MPa was able to maintain microbial counts similar to refrigeration and initial counts, ≈3 $Log_{10}$ CFU/g, at all tested temperatures. By increasing the pressure to 150 MPa and the storage time to 8 hours, a microbial inactivation effect was observed for all microorganisms, being the lactic acid bacteria and Enterobacteriaceae reduced to counts below the detection limit. Hyperbaric storage in general maintained pH, water activity and lipid oxidation values, at levels similar to refrigeration, retaining also the colour of *Requeijão*. The experiment in an industrial equipment obtained similar results to those mentioned above, with a clear microbial growth inhibition and inactivation for all microorganism studied, at 12 and 24 hours of storage, comparatively to refrigeration.

The results obtained in this study, shows that the storage under pressure of highly perishable foods, could be a possible energy costless alternative to refrigeration, with economical benefits for industries and ecological advantages.

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## LIST OF ABBREVIATIONS

**α-La** - α-lactalbumin

**ACE** - Angiotensin-I-converting enzyme

**β-Lg** - β-lactoglobulin

**CMP** - Caseino-macropeptide

**ENT** - *Enterobacteriaceae*

**FFA** - Free fatty acids

**HPP** - High pressure processing

**HP** – Hyperbaric storage

**Igs** - Immunoglobulins

**LAB** – Lactic acid bacteria

**LF** - Lactoferrin

**LP** - lactoperoxidase

**MAP** - Modified atmosphere packaging

**MDA** - Malondialdehyde

**PHS** – Post-hyperbaric storage

**SA** - Serum albumin

**TAM** – Total aerobic meshopliles

**TBARS** - Thiobarbituric acid reactive substances

**TKN** - Total Kjeldahl Nitrogen

**YM** – Yeasts and Moulds

**WPC** - Whey proteins concentrates

***ΔE**** - Total colour change

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## Contextualisation and Thesis Structure

This work is divided in six Chapters. Chapter I comprises a comprehensive literature review and state of the art of the cheese wheys, *Requeijão*, high pressure processing and hyperbaric storage. Chapter II describes the objectives of this work. Then, in Chapter III, the material and methods used in this study are described. In Chapter IV the results obtained for microbial, physicochemical and colour analysis are described and discussed. This Chapter is divided into two parts, one with the results obtained at the laboratorial scale experiment and the other with the results obtained in the industrial equipment experiment. Chapter V features the global conclusion and future work possibilities are presented in the last Chapter, Chapter VII.

# CHAPTER I – LITERATURE REVIEW

## 1. Cheese whey

Cheese whey is the main by-product of cheese manufacturing industries. It is the aqueous portion of milk obtained following acid-, heat- or rennet-driven coagulation. Cheese whey along ice-cream, butter and cheese production effluents are the most important sources of organic contamination in the dairy industry. Cheese manufacturing effluents can be divided in three main types: cheese whey-CW (resulting from cheese production), second cheese whey-SCW (resulting from cottage cheese production) and cheese whey wastewater-CWW (washing water that contains different fractions of cheese whey and/or second cheese whey) (Prazeres et al., 2012). Cheese effluents can represent a significant environmental impact in the dairy industry because of their physicochemical characteristics, namely, minerals (0.46-10%), total suspended solids (0.1-22 kg $m^{-3}$), pH (3.3-9.0), phosphorus (0.006-0.5 kg $m^{-3}$), Total Kjeldahl Nitrogen (TKN) (0.01-1.7 kg $m^{-3}$), organic load (0.6-102 kg $m^{-3}$), etc. The high value of organic matter is caused by lactose (0.18-60 kg $m^{-3}$), protein (1.4-33.5 kg $m^{-3}$) and fat (0.08-10.58 kg $m^{-3}$) contents (Ergüder et al., 2001). Organic matter present in cheese whey is around 99% biodegradable, according to conventional treatments that are based on biological processes. Cheese whey can cause negative impact on the environment due to an excess of oxygen consumption, impermeabilization, eutrophication, toxicity, among others.

Annual worldwide production of whey is in the order of 85 million tons and has risen sharply during the last decades along with the production of cheese (Zall, 1984).

### 1.1. Whey characterization

Proteins in milk can be divided into two major families, caseins (insoluble) and whey proteins (soluble). Whey represents approximately 85-95 % of total milk volume and the most important nutrients are lactose, proteins, mineral salts and residual fat, organic acids (lactic and citric acids) and B group vitamins (Pintado et al., 2001).

Whey composition depend on factors, such as milk source, type of whey (acid or sweet), time of the year, feed, stage of lactation and processing quality (de Wit, 1998).

Table 1 shows the different chemical composition of whey and respective whey protein concentrates (WPC), from ovine, caprine and bovine whey.

**Table 1 -** Chemical composition of WPC and their original value for ewe, caprine and bovine whey (g/100 g). Adapted from Pintado et al. (1999).

| | WPC | | | Whey | | |
|---|---|---|---|---|---|---|
| **Compound** | **Ovine** | **Caprine** | **Bovine** | **Ovine** | **Caprine** | **Bovine** |
| **Moisture** | 11.82 | 11.46 | 12.27 | 88.77 | 89.20 | 87.76 |
| **Lactose** | 8.19 | 17.03 | 26.13 | 76.76 | 67.80 | 71.16 |
| **Protein** | 73.24 | 63.44 | 56.05 | 16.30 | 12.06 | 10.59 |
| **Ash** | 3.96 | 4.30 | 7.46 | - | - | - |
| **Calcium** | 0.29 | 0.29 | 0.47 | 0.35 | 0.55 | 0.51 |
| **Sodium** | 0.35 | 0.32 | 0.40 | 1.81 | 0.54 | 0.56 |
| **Potassium** | 0.56 | 0.55 | 0.82 | 1.23 | 3.29 | 2.54 |

### 1.1.1. Whey proteins concentrates (WPC)

Whey proteins represent 20 % of total milk protein, whereas caseins are the most abundant proteins. Whey proteins are globular molecules with substantial contents of α-helix motifs, with acidic/basic and hydrophobic/hydrophilic amino acids balanced throughout their sequences. These proteins possess important nutritional and biological properties, particularly regarding to health promotion and prevention of diseases. Whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), serum albumin (SA), lactoferrin (LF), and lactoperoxidase (LP) (Table 2). Caseino-macropeptide (CMP) is released from κ-casein in the first step of enzymatic cheese making and found at lower concentrations (Pintado et al., 2001). Other components like lactose, oligosaccharides, minerals and vitamins are also important in whey composition.

**Table 2 -** Fractional concentration of each major whey protein of WPC from ovine, caprine and bovine whey (g/100 g). Adapted from Pintado et al. (1999).

| | WPC | | |
|---|---|---|---|
| **Protein** | **Ovine** | **Caprine** | **Bovine** |
| **IgG** | 7.32 | 9.80 | 6.16 |
| **BSA** | 7.97 | 7.82 | 6.12 |
| **β -Lactoglobulin** | 52.54 | 48.17 | 47.33 |
| **α -Lactalbumin** | 24.90 | 27.14 | 36.91 |

### 1.1.2. β -Lactoglobulin

β-Lactoglobulin (β-Lg) was first discovered at 1934, and is quantitatively the dominant whey protein found in cow, sheep, goat and other ruminants milk, approximately 50 % (w/w). It is a small globular protein that contains 162 amino acids in a single peptide chain with a molecular weight of 18.3 kDa and is synthesized in the mammary gland of ruminants and other species. β-Lg high nutritional and functional value made it an ingredient of choice in the formulation of modern foods and beverages (Chatterton, 2006). This protein high stability to proteolytic action of digestive enzymes and its tertiary structure homology with the plasma retinol-binding protein, suggests that it may play a role as resistant carrier of retinol (provitamin A) (Pérez and Calvo, 1995). β-Lg often binds to small hydrophobic ligands, such as fatty acids, vitamin D, cholesterol, palmitic acid, calcium, triacylglycerols, alkanes, aromatic compounds, acting probably as a transporter of this compounds. It also plays an important role in the regulation of mammary gland phosphorus metabolism and in transference of passive immunity (Hernández-Ledesma et al., 2011).

### 1.1.3. α-Lactalbumin

α-Lactalbumin (α-La) is the second most important protein in whey, 20 % (w/w) of total whey protein inventory, and is fully synthesized in the mammary gland. It is a small and globular protein, with 14 kDa that consists of a single polypeptide chain with eight cysteine residues, and is physiologically important because it acts as a coenzyme for lactose biosynthesis (Hernández-Ledesma et al., 2011).

### 1.1.4. Serum albumin

Serum albumin is a 582 amino acids protein with 66.271 kDa, not synthesized in the mammary gland that appears in milk after passive passage from blood stream. In vitro, serum albumin shows antioxidant activity, protecting lipids against phenolic-induced oxidation (Tong et al., 2000). Because of its size and structure serum albumin can bind to free fatty acids and other lipids, participating in lipids synthesis.

### 1.1.5. Immunoglobulins

Immunoglobulins (Igs) are a complex group of globular proteins produced by B lymphocytes and make a significant contribution to whey content. Because placenta does not allow the passage of macro molecules, Igs postnatal transference via colostrum play a key role in neonate own immune system (Hernández-Ledesma et al., 2011). Igs can be either monomers or polymers of a four-chain molecule, consisting of two light polypeptide chains (with a molecular weight in the range 25,000 kDa) and two heavy chains (with molecular weight of 50,000–70,000 kDa) (Akita and Li-Chan, 1998).

### 1.1.6. Lactoferrin

Lactoferrin (LF), also known as lactotransferrin, is an iron-chelating monomeric glycoprotein, with 80,000 kDa, to which two carbohydrate groups are attached (de Wit, 1998). This globular multi-functional protein binds, transports and supplies the organism with iron. Iron binding properties of LF vary between different species, being present at higher levels in sheep and goat milk, than in cow milk. LF antimicrobial activity is attributed to iron-binding properties, which make it unavailable, inhibiting Fe-dependent bacteria. Bacteria with iron requirements are not the only ones affected, because LF can also bind to microbial membrane and thereof release, causing structural changes, which include loss of membrane potential and integrity. This way, LF has a bactericidal power against a wide range of microorganisms (Shin et al., 2000).

### 1.1.7. Lactoperoxidase

Lactoperoxidase (LP) is an important part of the natural host defence system in mammals, present in tears, saliva and milk. It is a member of mammalian peroxidises

family, represents approximately 1% (w/w) of the total protein pool in whey, providing protection against microorganisms and virus. LP also act in the expression regulation of genes involved in metabolism, immunity, apoptosis, and mitosis of epithelial intestinal cells (Wakabayashi et al., 2007).

### 1.1.8. Caseino-macropeptide

Caseino-macropeptide (CMP) is a precursor of κ-casein, composed by the 64 C-terminal amino acids derived from the action of chymosin during the milk clotting process in cheese manufacturing. Amino acid sequences of CMP from different species are characterized by high levels of acidic and hydroxyl amino acids and the absence of aromatic amino acids (Phe, Trp, and Tyr) and Arg (Park et al., 2007). This unique amino acid composition makes it suitable for patients that require special diets, like phenylketonuria patients, by phenylalanine absence in CMP and hepatic diseases patients diet due to the branched-chain amino acids (valine and isoleucine) and low methionine content in CMP (El-Salam et al., 1996). CMP has several biological properties and some are mediated by the carbohydrate fraction. CMP interacts with toxins, viruses and bacteria, preventing them from adhering to cells by carbohydrate receptors recognition (Dziuba and Minkiewicz, 1996). CMP has the ability to promote the growth of *Bifidobacterium* genus: *B. breve*, *B. bifidum*, and *B. infantis*, thus contributing to a healthy gut microflora, making it a potential prebiotic in functional foods or as a supplement to simulate the beneficial bacteriological effects of breast milk (Bruck et al., 2003).

### 1.1.9. Lactose and oligosaccharides

Lactose is the major ingredient of non-fat dry milk and whey solids with a content of 4.1 g/100 mL in goat milk, 4.9 g/100 mL in sheep milk and 5.3 g/100 mL in bovine milk (Park et al., 2007). Lactose is a valuable nutrient that helps intestinal absorption of magnesium and phosphorous, calcium and the utilization of vitamin C. Oligosaccharides in ovine milk are in the range of 20–30 mg/mL while in caprine milk are in the range of 250–300 mg/mL (Park et al., 2007). Milk oligosaccharides possess prebiotic and anti-infective properties and many contain sialic acid [general name for N-

acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc)] that promotes infants brain development among other positive effects (Park et al., 2007).

### 1.1.10. Minerals

Milk contains many minerals that play an important role in the physiology and metabolism of the human body, and some of them are still present in whey. The most abundant elements are calcium, phosphorus, potassium, sodium, and magnesium; zinc, iron, copper and manganese are trace-elements (Raynal-Ljutovac et al., 2008). Sheep milk is richer in calcium, phosphorus, and zinc when compared to cow milk (Table 3). Sodium, potassium, and chloride are almost entirely soluble and fully available in whey, whereas calcium, magnesium, and phosphorus are associated in different proportions to the colloidal suspension of casein micelles and, therefore, are partly retained in the curd during cheese-making (Park et al., 2007).

**Table 3 -** Mineral composition of goat, sheep and cow milk. Data from goat and cow milk are per L, and sheep milk per kg. Adapted from Raynal-Ljutovac et al. (2008).

| Mineral (mg) | Goat | Sheep | Cow |
|---|---|---|---|
| Calcium | 1260 | 1950–2000 | 1200 |
| Phosphorus | 970 | 240–1580 | 920 |
| Potassium | 1900 | 1360–1400 | 1500 |
| Sodium | 380 | 440–580 | 450 |
| Chloride | 1600 | 1100–1120 | 1100 |
| Magnesium | 130 | 180–210 | 110 |
| Ca/P | 1.3 | 1.3–1.6 | 1.3 |
| Zinc | 3.4 | 5.2–7.470 | 3.8 |
| Iron | 0.55 | 0.720–1.222 | 0.460 |
| Copper | 0.3 | 0.4–0.680 | 0.220 |
| Manganese | 0.08 | 0.053–0.090 | 0.06 |
| Iodine | 0.08 | 0.104 | 0.07 |
| Selenium | 0.02 | 0.031 | 0.031 |

### 1.1.11. Vitamins

During cheese-making, most of the fat-soluble vitamins are incorporated to the curds, while water-soluble vitamins go mostly to the whey (Hernández-Ledesma et al., 2011). Vitamins are physiological, biochemical and metabolically bioactive compounds present in milk, which composition (breeding systems) has changed throughout the years. Ovine milk normally is richer than bovine and caprine milk for most of the vitamins (Table 4) (Park et al., 2007).

**Table 4 -** Vitamin content of ovine, caprine and bovine raw whole milks (per 100g). Adapted from Raynal-Ljutovac et al. (2008).

| **Fat soluble vitamins (µg)** | **Onive** | **Caprine** | **Bovine** |
|---|---|---|---|
| A | | | |
| Retinol | 40 | 80 | 40 |
| Beta carotene | 0 | 0 | 20 |
| D | 0.06 | 0.18 | 0.08 |
| E | | | |
| Tocopherol | 40 | 110 | 110 |
| Water soluble vitamins | | | |
| B1 | | | |
| Thiamin | 50 | 80 | 40 |
| B2 | | | |
| Riboflavin | 140 | 350 | 170 |
| B3 | | | |
| Niacin (PP) | 200 | 420 | 90 |
| B5 | | | |
| Pantothenic acid | 310 | 410 | 340 |
| B6 | | | |
| Pyridoxin | 50 | 80 | 40 |
| B8 | | | |
| Biotin | 2.00 | nd | 2.00 |
| B9 | | | |
| Folic acid | 1.00 | 5.00 | 5.30 |
| B12 | | | |
| Cobalamin | 0.06 | 0.71 | 0.35 |
| Ascorbic acid | 1300 | 5000 | 1000 |

### 1.2. Cheese whey management

As mentioned before, cheese whey is the main contaminating waste generated in the production of cheese and there are several ways to reuse it. Liquid whey can be supplied into drinking waters for farm animals, as good source of high-quality proteins and lactose, calcium, phosphorus, sulphur and water-soluble vitamins or and as agricultural fertilizer. Condensed or powdered whey forms (condensed whey, demineralised whey powder, deproteinized whey, etc.) can be applied in animal feeding and smaller quantities could be used in human foods (ice creams, baked goods, sauces, etc.); WPC functional and technological characteristics makes it suitable for manufacture of transformed food products; fermentation of lactose present in whey to ethanol; biogas production; and production of several organic acids for food uses (Siso, 1996).

One of the simplest ways to recover proteins from whey is precipitation by heat processing from sweet and acid whey. Whey proteins recovery requires large capital investments and only a few major dairy companies can afford it. Whey cheeses, like ricotta and *Requeijão* are probably the oldest and best known ways for protein recover by heat precipitation from whey (Pintado et al., 2001).

## 2. Whey cheeses

About 3000 years ago, warm milk was stored in a bag made of fresh stomach skin of sheep and through chymosin natural action, curds and whey were produced. Later, using copper kettles, nomad shepherds started boiling whey and eventually obtained a nourishing solid food (Smithers, 2008). Whey cheeses were produced all over the world according to traditional protocols, by protein precipitation and these cheeses have distinct names, depending on country and region where they are produced (Table 5). Whey cheese manufacture seems to be a good and easy method for whey protein recovery, especially for small dairy industries in which is difficult to implement mass production techniques (Pintado et al., 1999).

**Table 5 -** Whey cheeses produced worldwide. Adapted from Pintado et al. (2001).

| Country | Name of Whey Cheese |
|---|---|
| **Norway** | Mysost, Primost, Gjestost, Grubransdalsost |
| **Switzerland** | Schottenziegr, Hudelziger, Mascarpone |
| **Portugal** | Requeijão |
| **Spain** | Requesón |
| **France** | Serac, Brousse, Broccio, Greuil |
| **Germany** | Zieger, Schottenzieger, Schabzieger |
| **Greece** | Manouri, Myzithra, Anthotyros |
| **Italy** | Ricotta (gentile, pecorina or romana) |
| **Malta** | Cacio-ricotta |
| **Cyprus** | Anari |
| **Romania** | Ziger, Urda |
| **Macedonia** | Urda |
| **Tunisia** | Klila |
| **Northern Africa** | Nicotta |
| **Lebanon** | Kariche |
| **Israel** | Urda |
| **Iraq** | Lour |
| **Argentina** | Ricotta |
| **Brazil** | Requeijão do Norte, Ricotta fresca |
| **USA** | Ricotone, Ricotta |

## 3. *Requeijão*

Whey cheeses are very popular and consumed as table cheeses in the Mediterranean basin, due to their nutritional value, low salt content and good sensory characteristics. *Requeijão,* the Portuguese whey cheese is small (≈150 g), white and with a semi-sweet flavour, which is produced from the remaining cheese whey of cheese making industries, like *Serra da Estrela* cheese, *Castelo Branco* cheese, etc. It is well accepted by Portuguese consumers being consumed during a period of 24-48 h after production, alone or mixed with salt, honey, sugar or compote, depending on individual taste and region customs (Pintado et al., 2001). This whey cheese is produced usually from sheep whey, but mixture with sheep or caprine milk are also used. It is then heated at 90 to 100 °C for about 30 min, fo protein precipitation, followed by a decanting process and a cooling period.

### 3.1. *Requeijão* composition

Initial whey type and origin dramatically influences *Requeijão* final characteristics. *Requeijão* has a high protein (8.5 %) and fat (29 %) content, being this values dependent if milk is added. pH is close to 6, lactose and lactic acid content is 3.5 % and 0.5 %, respectively (Pintado and Malcata, 2000a). *Requeijão* moisture content is relatively variable (≈50 %) depending on package permeability, draining period and storage conditions. Compared with other whey cheeses, *Requeijão* seems to have similar composition in moisture and lactose content as Italian Ricotta (Pizzillo et al., 2005) and pH and moisture content as Greek Anthotyros (Papaioannou et al., 2007).

*Requeijão* is rich in short chain saturated free fatty acids (FFA), $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$, and in long chain FFA, $C_{16:0}$ and $C_{18:0}$ as Table 6 shows (Pintado and Malcata, 2000a). This is similar to what is found in Italian Ricotta, as for saturated and unsaturated FFA, however $C_{16:0}$ is the predominant unsaturated fatty acid in Italian Ricotta. *Requeijão* content in long chain FFA is important (15-18 % each) and the predominant unsaturated FFA are $C_{18:1}$ and $C_{18:2}$ (13-14 % each) (Pintado and Malcata, 2000a).

**Table 6 –** *Requeijão* free fatty acid, total and individual composition at 24h after production. Produced with traditional procedures, stored at 4°C, without package. Adapted from Pintado and Malcata (2000a).

| | **Concentration** | |
|---|---|---|
| **Free fatty acids** | mg/g $_{cheese}$ | % total |
| **C4:0** | 0.024 | 4.9 |
| **C6:0** | 0.026 | 5.1 |
| **C8:0** | 0.033 | 6.6 |
| **C10:0** | 0.042 | 8.4 |
| **C12:0** | 0.043 | 8.7 |
| **C14:0** | 0.056 | 11.3 |
| **C16:0** | 0.065 | 13.0 |
| **C18:0** | 0.070 | 14.0 |
| **C18:1** | 0.070 | 14.1 |
| **C18:2** | 0.066 | 13.3 |
| **C18:3** | 0.000 | 0.0 |
| **Total** | 0.498 | 100.0 |

### 3.2. *Requeijão* manufacturing process

In Portugal, *Requeijão* can be produced from sheep whey, mixed with caprine or sheep milk up to 10 % (for yield and organoleptic purposes) followed by heating at 90 to 100 °C for approximately 30 to 50 minutes under smooth stirring conditions. Curds rise and then a decantation method is performed in order to separate them from the remaining liquid (*Rescaldão*). *Requeijão* might than be packed, or not, in plastic that allow draining and cooling for several minutes.

Pintado et al. (1996) studied the technological optimization of *Requeijão* manufacturing process. Using possible combinations of initial temperature (90 to 95 °C), heating times (30 to 60 min) and two types of milk addition (10 % (v/v) of caprine milk and 10 % (v/v) of ovine milk). The effect of these different combinations on *Requeijão* quality were analysed by fat, nitrogen and moisture content analysis, rheological and sensorial evaluation. Milk addition (*m*) result in a higher amount of proteinaceous coagulum produced and curds were more cohesive, due possibly to co-precipitation of caseins present in milk with whey proteins. Initial comparison of traditional whey cheese, used as control in this study with experimental whey cheeses, showed that nitrogen content was similar. Differing on fat and moisture content, that was higher and lower respectively for traditional whey cheese, which might be due to the use of higher temperatures in it manufacture. Fat content is positively affected by time and heating temperature, as well as for milk addition, which leads to an increase fat retention. Moisture content is only affected by time and heating temperature. In sensorial analysis, traditional whey cheese was the firmest of all whey cheeses, but overall, experimental whey cheeses were preferred over the traditional one.

Higher yield and higher nitrogen content were the criteria analysed for optimal *Requeijão* production conditions, but this criteria did not coincide between different conditions used (optimal conditions selected: $T$ = 90 °C, $t$ = 48 min and $m$ = 8.2 % caprine milk, or $T$ = 95 °C, $t$ = 37.5 min and $m$ = 4.4 % caprine milk). If this criteria are conjugated with organoleptic preference, Pintado et al. (1996) conclude that the first condition is most suitable.

### 3.3. *Requeijão* Microbiology

Microorganism's growth depends on factors such as availability of nutrients, water activity, pH, ionic strength, temperature and overhead atmosphere composition.

Despite *Requeijão* being produced at high temperatures, its high content in protein, lactose, moisture, low salt content and almost neutral pH promote the growth of microorganisms that are result of contamination during post-production manipulation. These microorganisms promote quantitative and qualitative changes in physicochemical properties, which drastically constrain final consumer acceptability. The fact that whey cheeses are easily contaminated and were normally transported and sold without a closed package, contributed to a rather limited shelf-life of 2 ± 3 days, even under refrigeration (Pintado et al., 1996).

Moulds, yeasts and *Enterobacteriaceae*, especially under certain temperatures contribute to microbial spoilage. Even under anaerobic conditions rapid spoilage occurs, usually in less than 7 days (Pintado et al., 1996). When acidified whey is used, heated at 80 °C for 10 min, a high microbial spoilage is observed as a result of contamination and sporulation after heating process.

In a study made by Pintado and Malcata (2000c), after *Requeijão* manufacture, it showed that viable bacteria are virtually absent, as expected after thermal processing. However, after 2 days of storage, *Requeijão* exhibited high viable counts of *Pseudomonas* (4.3 x $10^3$ CFU/g), *Bacillus* (1.1 x $10^4$ CFU/g), *Staphylococcus* (7.2 x $10^3$ CFU/g) and lactic acid bacteria (especially *lactococci*) (1.4 x $10^4$ CFU/g). Later during storage, yeasts and moulds (3.6 x $10^3$ and 1.1 x $10^6$ CFU/g) and *Enterobacteriaceae* (8.7 x $10^3$ and 7.8 x $10^5$ CFU/g) increased, at 6 and 10 days, respectively. After 10 days of storage spore-forming clostridia (4.9 x $10^2$ CFU/g) were detected (Pintado and Malcata, 2000c). *Listeria monocytogenes* could have grown in *Requeijão*, but this pathogen was not tested because normal pasteurization prevents contamination of this cheeses with *Listeria spp*. (Beckers et al., 1987). Good hygienic handling, addition of salt and use of chilled storage are crucial steps for microbial spoilage prevention.

### 3.4. Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) is a process that combines food, gas and packaging. Have been successfully applied from the 50's in Europe and since 1980 in the United States (Lopes et al., 2004). It is a modern method of food preservation, given the demand of preservative-free and minimally processed foods. There are essentially two principal methods to perform MAP: vacuum packing and gas packaging. Vacuum packing is the primordial form of MAP processes, used widely nowadays. It is used in foods like fresh and cured meat, cheese and coffee granules, being inadvisable for bakery products and meats for long preservation periods since oxygen removal promotes browning as well as unwanted residual liquid (McMillin, 2008). Gas packaging increase food shelf life, by replacing atmospheric air, with an optimum mixture of $O_2/CO_2/N_2$, inhibiting microbial growth present in food, enzyme degradation and inhibition of tissue respiration (Tano et al., 2007).

Microbial spoilage after *Requeijão* processing is the biggest problem concerning it shelf life. Under vacuum packaging at 4°C performed by Pintado and Malcata (2000b), yeasts and *Staphylococci* showed growth inhibition, because these microorganisms are aerobic. Spore-forming clostridia (anaerobic bacterium), under vacuum packaging in *Requeijão* was also inhibited, acidification occurred, derived from lactose metabolism by lactic acid bacteria. Vacuum packaging also inhibited lipolysis, which prevent rancid and soapy flavours, contributing to a good sensorial quality and may extend storage life to 20–30 days (Pintado and Malcata, 2000b).

In another study conducted by Pintado and Malcata (2000c), the effect of MAP on *Requeijão* was evaluated. Different temperatures (4, 12 and 18 °C), storage times (2, 6, 10 and 15 days) and gas used for packaging (100% $CO_2$, 100% $N_2$, and a 50% mixture of $CO_2$ and $N_2$) were tested. The package prevented dehydration and consequently lost is texture, which was confirmed from moisture content and texture analysis, that didn't change significantly the storage times. Pure $CO_2$ atmosphere at 4 °C extend the shelf life of the product by 15 day, showing no signals of lipolysis, microbial inhibition and minimal outer and inner pH alterations. However, it is important to note that without adequate temperature control for extended conservation periods, and adequate hygiene during post-processing manipulation, increased shelf life as claimed cannot be achieved.

## 4. High Pressure Processing (HPP)

High pressure is a technology that has long been applied in several non-food industries, like production of plastics, ceramics and metal-forming, etc. This technology is also applied in food processing, owning to it unique advantages over thermal preservation methods (Mertens and Deplace, 1993). Thermal preservation (pasteurization and sterilisation) is the most used treatment for food preservation. However, this technology can cause undesired changes in foods, such as losses is texture, nutritional value, flavour and colour that overall leads to a reduction of final product quality (Tewari et al., 1999). Nowadays, consumers demand foods not only safe but also with high quality, more fresh, and shelf-stable. HPP may present a real solution for this problem. HPP is a non-thermal processing technology that can inactivate most microorganisms and enzymes causing negligible impairment in foods sensory properties and nutritional quality, as retention of flavour, colour and nutritional value.

Le Chatelier's Principle states that when a system at equilibrium is disturbed, the system responds in order to minimise the disturbance. According to this, any chemical reaction, conformational change, or phase transition that is accompanied by a decrease in volume will be enhanced by pressure, while reactions involving an increase in volume will be inhibited (Cheftel and Culioli, 1997). This principal plays a crucial role in microbial inhibition, by increasing the pressure, biological systems will reduce their volume, influencing the microbial structure. For example, high pressure can cause disruption of the cell membrane, affecting the production of energy, osmosis, reaction and communication with the external environment. It can also affect cell growth, DNA stability, promote subunit dissociation of the ribosomes compromising the cell viability (Norton and Sun, 2008).

Isostatic principle is another basic principles governing HPP effect in foods. Pressure is applied in HPP instantaneously and uniformly transmitted throughout the food, regardless of size, shape and composition, when the pressure is released, food returns to its original shape (Martínez Rodríguez et al., 2012). HPP acts independently of sample size and geometry during processing, allows low temperature treatment, and the availability of a waste-free, makes it an environment-friendly technology (Huppertz et al., 2006).

Typical pressure operating machines consist in a high-pressure vessel and its closure, a pressure-generating system, a temperature-control device and a material

handling system. Nowadays, industrial machines may reach 600 MPa, approximately 6 000 times atmospheric pressure, using vessels with a volume up to 525 L (Figure 2). The procedures, normally imply: placing the product (liquid or solid), with or without packaging, in an adequate vessel and, following vessel closure and then pressure is applied either through a piston or a pump (Huppertz et al., 2006).

Milk, fruits and vegetables were the first foods processed with high pressure by Hite, using 670 MPa for 10 min achieved five to six microbial logarithmic reductions, extending shelf life up to 4 days (Hite, 1899, Hite et al., 1914). However only in 1990, the first HPP product, a fruit jam, was introduced in to the market (Huppertz et al., 2002). The high cost, lack of investment in emerging technologies and commercial equipment capable of operating with minimal disruptions and processing large amounts of food were the causes for the slow evolution of this technology in the food industry (Torres and Velazquez, 2005). At the present HPP is commercially applied in a range of products, like oysters, fruit juice, smoothies, ready meals, guacamole and meat (Huppertz et al., 2006).

Figure 1 – Example of one HPP industrial-scale equipment, Hiperbaric 55. Courtesy of Hiperbaric.

## 5. Hyperbaric Storage

Refrigeration main role is to preserve foodstuffs and therefore reduce losses during processing, storage, transport and sale of foodstuffs (Segovia-Bravo et al., 2012). Effective management of cold chains utilizes about 50 % of total energy in food industry. Cooling process inherent high costs, makes it unaffordable for less developed countries (Coulomb, 2008). It is estimated that developing countries would be able to preserve over 200 million tons of perishable foods, if they get the same level of refrigerated equipment as that in industrialized countries (Coulomb, 2008). Hyperbaric storage arose by chance about 40 years, when about 10 mounts after submarine Alvin

sunken, well preserved foods were found (Jannasch et al., 1971). Hyperbaric storage utilizes high pressure (HP) technology that allows energy saving, since energy would only be used to pressurize the vessel, and eventually to maintain the pressure. This way, hyperbaric storage have lower costs when compared with refrigeration. As mentioned before, HP technology is a promising technology since it does not use heat, and so does not affect foods organoleptic and nutritional value (Tewari et al., 1999). Hyperbaric storage could be implemented to ensure the preservation of a wide variety of foods and other biomaterial, at different temperatures under pressure, in order to reduce the losses during storage, transportation, selling until consumption. To test hyperbaric possible utility several authors have studied it, under different temperature condition (sub-zero, low and room temperatures), pressures and storage times.

### 5.1. Hyperbaric storage at low temperature

The first reference related to low temperature hyperbaric storage is the submersible Alvin on 1 September 1969 (Jannasch et al., 1971). Alvin remained at 1540 m (15 MPa) deep at 3-4 ºC for 10 months and bouillon, sandwiches and apples were recovered at good conditions. Jannasch et al. (1971) conducted several trials to understand this phenomena. They realize that if food recovered from Alvin was compared to fresh food, under 3 and 30 ºC for 5 and 22 days, an improvement related to microbial spoilage, putrefactive odor, decreased tyrosinase activity and turbidity, for bread, meat, apples and bouillon was observed, respectively. Authors hypostatized that low temperatures and high hydrostatic pressure could have an inhibitory effect in the biochemical activity of microbial cells. This author conducted an experiment using organic matter marked radioactively with $14_C$ to a depth of 5300 m, observed a decreased 8-700 times in substrate decomposition rate in depth when compared to laboratory at 3 ºC and atmospheric pressure.

### 5.2. Hyperbaric storage at sub-zero temperatures

It is possible to change the freezing point of water below 0 ºC, using pressure (Figure 3), being this way possible to storage foods at sub-zero temperatures without nutritional and textural changes, normally associated with freezing and thawing processes (Kalichevsky et al., 1995).

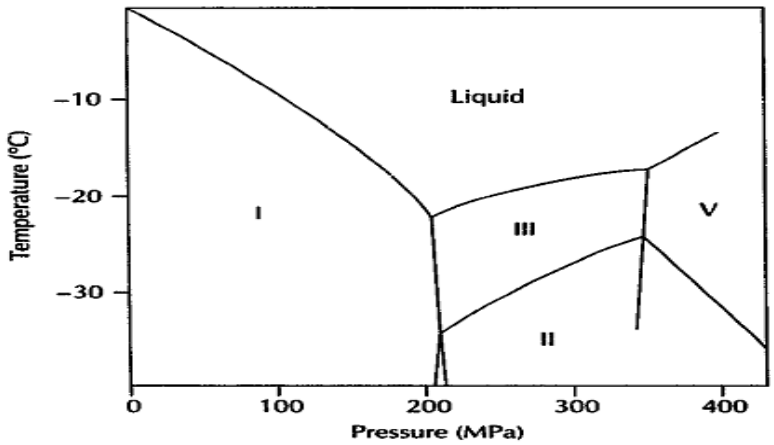


Figure 2 – Water equilibrium solid-liquid phase diagram. Adapted from Kalichevsky et al. (1995).

Charm et al. (1977) studied the effect of hyperbaric storage on the enzymes trypsin and peroxidase, from 10 to 60 days under different condition combinations, -3, 0, 4 and 23 °C and 0, 27.6, 34.5 and 41.3 MPa, respectively. The activity of these enzymes were tested, showing that under a constant temperature, enzyme activity decrease when pressure is raised. Peroxidase activity at 4 °C and 41.3 MPa decreases about 25-30% when compared with its activity at 0.1 MPa. But for trypsin, when pressure was raised at 23ºC, it resulted in an increased yield. The authors suggested that some enzymes under pressure are stimulated, resulting in an increase reaction rate. This study showed that it is possible to use hyperbaric storage to extend food shelf-life, under a certain combination of temperature and pressure in order to reduce enzyme activity. Charm et al. (1977) also studied the acceptability of perishable food (cod fish, pollock, beef and chicken) treated with -20, -3 and 1 °C at 0.1 and 24.12 MPa, by an expert panel. Expert panel classified pollock stored at 1 ºC and 0.1 MPa for a period of 12 days, as 12 days old whereas that stored under 24.12 MPa was scored as if it were only 6.7 days old. Cod fish stored at 1 ºC and 24.12 MPa after 21 days was classified as acceptable, whereas cod stored under atmospheric pressure at the same temperature was unacceptable. Cod fillets stored at -3 °C under pressure conditions remained unfrozen, being stable and classified if they were 7 days old.

Ooide et al. (1994) conducted experiments on chicken and carp muscles at -8 or -15 ºC and 170 MPa for 50 days. They were capable to preserve meat texture at low temperatures without extensive protein denaturation, however enzymes activity was conserved. Deuchi and Hayashi (1992) were able to store successfully ground beef at -20 °C and 200 MPa, with a reduction of the microbiological load (coliform,

*Enterobacteriaceae*, psychrotrophs, *Enterococci* and lactic acid bacteria). Some enzymes like catalase (3-amylase, cathepsin and lactate dehydrogenase) that are inactivated by freezing were not inactivated under experiment conditions and activity was reduced after non-frozen storage.

Hyperbaric storage at sub-zero temperatures could be applicable to prolong certain foods shelf-life, allowing reduction of nutritional and textural changes caused by freezing.

### 5.3. Hyperbaric storage at room temperature

Ko and Hsu (2001) compared normal tilapia fillets stored at 0.1 MPa, 25 ºC for 12 h with pressurized ones (203 MPa, 25 ºC for 12 h). HP treatment result in a total plate count of these fillets of 2.0 log CFU/g, lower when compared with tilapia fillets stored at room temperature, 4.7 log CFU/g. This effect was also observed for psychrophilic bacteria, however, reactivation of enzymes and microorganisms were observed after 12 h of pressurization.

Baba and Ikeda (2003) studied the effect of HP on mume fuit under 0.5 MPa for 5 days. Fruits were subjected to HP at 5, 4, 3, 1 and 0.5 MPa for 10 min, and then sequentially subjected to 0.5 MPa for 5 days. It is important to adequate the HP to the product being analyzed. Fruits and vegetables are more sensitive to pressure when compared to other foods. The authors were able to keep an acceptable color quality, decrease ethylene and $CO_2$ production for HP treatment. Queirós et al. (2014) study the feasibility of stored melon juice, under pressure (25, 50, 75 and 150 MPa) for 8h, at room and above temperature (25, 30 and 37 ºC). When compared to hyperbaric treatment, physicochemical parameters (pH, titratable acidity, total soluble solids, browning degree, and cloudiness) did not vary significantly for refrigeration storage. Pressures between 75 and 150 MPa were capable to achieve a greater microbial inhibition (total aerobic mesophiles, *Enterobacteriaceae*, yeasts and moulds), for all temperatures used (Figure 4). This may indicate that an inhibitory effect on microbial growth as well as inactivation is achieved with HPP treatment.

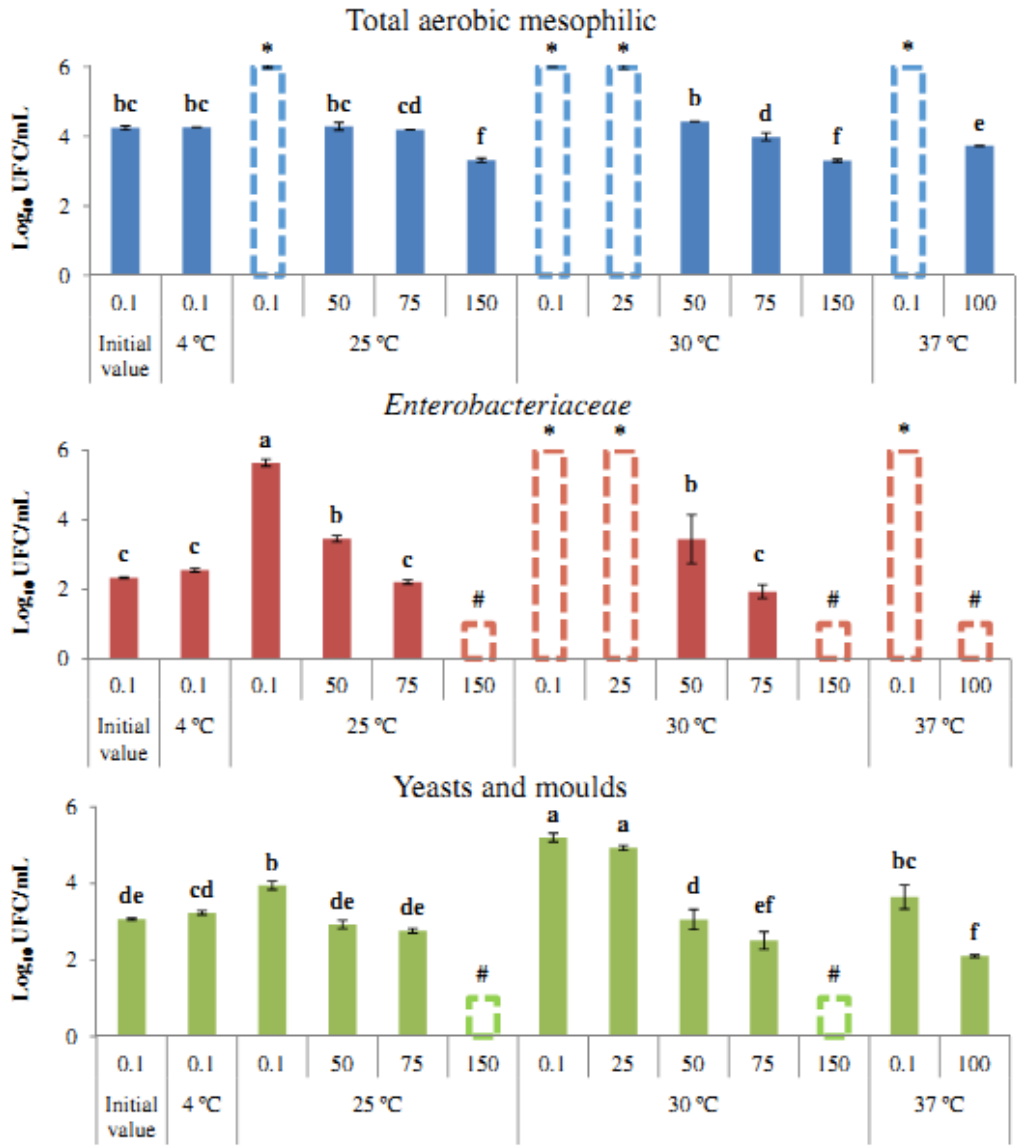


Figure 3 - Total mesophiles, *enterobacteriaceae* and yeasts and moulds counts (expressed in $Log_{10}$ CFU/mL) in melon juice initially and after 8 h of storage at different pressure (between 0.1 and 150 MPa) and temperature conditions, as present in the x-axis of the graphs. Bars with * and # are indicative of higher than 6 and lower than 1 $Log_{10}$ CFU/mL, respectively. Different letters between (a–g) indicate significant differences ($p < 0.05$). Addapted from Queirós et al. (2014).

Raw watermelon juice (*Citrullus lanatus*), a high perishable food with low acid pH and high water activity, was treated with 100 MPa at naturally variable room temperature conditions and above (18–21, 30 °C), throughout storage (up to 60 h) by Fidalgo et al. (2013). As similar to what was found by Queirós et al. (2014), 100 MPa for 8 hours was able to avoid microbial growth, however by increasing storage time it was possible to decrease the initial loads, without reaching higher pressures. Concerning physicochemical parameters, pressure attenuated the increase in titratable acidity, decreased browning degree and caused higher colour changes, specially a higher lightness. The authors also studied the post-hyperbaric storage at 0.1 MPa and 5 °C. After 7 days, the microbial load stayed low, showing an extended sheld-life when compared to refrigerated samples at 5 °C at 0.1 MPa without previously hyperbaric storage. After 14 days, microbial loads were above 6 log units for total mesophiles. This indicates that despite hyperbaric storage, inhibits and reduces the microbial load, the microorganisms left were able to grow at atmospheric pressure after a period of 14 days at 5ºC.

Hyperbaric storage under variable room and above temperature seems to be a good and very promising food preservation methodology. This would allow ensuring the safety of foods and other biomaterials, along the distribution chains, where variations on temperature normally occur, while reducing the costs and losses from refrigeration.

# CHAPTER II – Objectives

This study aims to evaluate the effect of storage under pressure, on a highly perishable diary food, using *Requeijão* as a case study, at and above room temperature, on:

- Microbial load: total aerobic mesophiles, *Enterobacteriaceae*, lactic acid bacteria, yeasts and moulds;
- Physicochemical parameters: pH-value, water activity and lipid oxidation;
- Colour properties

The results obtained will be compared with initial values before storage, and respective controls under refrigeration at atmospheric pressure, to see if hyperbaric storage of *Requeijão*, could be an efficient alternative to refrigeration.

# CHAPTER III - Material and Methods

## 1. Laboratorial experiments

Requeijão samples were stored under pressure using a hydrostatic pressure equipment (High-pressure system U33, Institute of High Pressure Physics, Warsaw, Poland) with a pressure vessel of 35 mm inner diameter and 100 mm height, surrounded by an external jacket connected to a thermostatic bath (Huber Compatible Control CC1, New Jersey, USA) for temperature control. A mixture of propyleneglycol and water (40:60) was used as a pressurizing fluid.

Laboratorial storage experiments were performed at different temperature conditions (25, 30 and 37 ºC), under 100 and 150 MPa during 4 and 8 hours (**Table 7** shows the preservation conditions studied). For each case, control samples at 0.1 MPa were immersed in the same fluid used for pressurization for the same time period and temperature, in a thermostatic bath (SELECTA FRIGITERM 6000382, Barcelona, Spain), of the samples stored under pressure in a dark environment, to mimic exactly the conditions of the samples stored under pressure, except for the pressure conditions.

Table 7 - Storage period, temperature and pressure conditions applied on the laboratorial storage experiments of whey cheese.

| Storage Period | Temperature | Pressure |
|---|---|---|
| **4 hours** | 4 ºC | 0.1 MPa |
| | 25 ºC | 0.1, 100 and 150 MPa |
| | 30 ºC | 0.1, 100 and 150 MPa |
| | 37 ºC | 0.1 and 100 MPa |
| **8 hours** | 4 ºC | 0.1 MPa |
| | 25 ºC | 0.1, 100 and 150 MPa |
| | 30 ºC | 0.1 and 100 MPa |

## 2. Experiments in an industrial equipment

With the recent acquisition of a high pressure industrial-scale equipment, Hyperbaric 55 (**Figure 1**), a storage experiment using this equipment was conducted. Hyperbaric 55 enable throughputs of more than 255 kg per hour, has a 200 mm diameter vessel inside with 55 litre vessel capacity reaching approximately 600 MPa.

*Requeijão* samples with the same size (≈170 g) as those sold commercially, were stored at room temperature (≈21 °C) under 100 MPa for 12 and 24 hours, with respective control samples at atmospheric pressure at room temperature and under refrigeration (4 ºC) for the same time periods.

After the 24 hours of storage, samples under pressure and atmospheric pressure were stored at refrigeration for 3 days at 0.1 MPa (post-hyperbaric storage).

## 3. *Requeijão* samples

*Requeijão* was produced from *Bordaleira* and *Churra Mondegueira* ewe's milk whey, used to produce *Serra da Estrela* cheese and were obtained on the production day. For the laboratorial experiment, this whey cheeses where aseptically mixed in one mass, and then aliquots (≈10 g) were individually packed into low permeability polyamide/polyethylene bags (Albipack-Packaging Solutions, Águeda, Portugal), previously sterilized by UV light (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) and manually heat-sealed under vacuum (Packman, Albipack - Packaging Solutions, Águeda, Portugal), avoiding leaving air inside the bags. *Requeijão* samples (≈170 g per unit) used in the experiment in an industrial equipment, were also obtained on the production day, and packaged as mentioned above. All samples were then frozen and stored at -80 ºC until used and thawed at 4 ºC before each experiment.

## 4. Microbial analyses

Samples of laboratorial and industrial equipment experiment were analysed for total aerobic mesophiles microorganisms (TAM), *Enterobacteriaceae* (ENT), lactic acid bacteria (LAB) and yeast and moulds (YM).

### 4.1. Sample preparation and dilutions

In the laboratorial experiments, two grams of each sample, obtained aseptically were homogenized with 18.0 ml of Ringer's solution for 240 seconds using a Stomacher at high-speed. As for the large scale experiment, instead of two grams, ten grams were obtained aseptically and were homogenized with 90.0 ml of Ringer's solution for 240 seconds using a Stomacher at low-speed. Decimal dilutions were made with the same diluent and triplicates of dilutions were plated on the appropriate media.

### 4.2. Total aerobic mesophiles microorganisms counts

Total aerobic mesophiles microorganism were enumerated using 1,0 ml of diluted solution sample, on plate count agar (PCA; Merck) incubated at 30 ± 1 °C for 72 ± 3 hours (ISO 4833:2003) under aerobic conditions.

### 4.3. *Enterobacteriaceae* counts

*Enterobacteriaceae* counts were determined on violet red bile glucose agar (VRBDA; Merck), by pour-plated method using 1,0 ml of diluted solution sample, aerobically incubated at 37 °C ± 1 °C for 24 hours (ISO 8523:1991).

### 4.4. Lactic acid bacteria counts

Lactic acid bacteria counts were determined on man rogosa sharpe agar (MRS; Merck) by pour-plated method using 1,0 ml of diluted solution sample, after aerobically incubation at 30 ºC for 5 days (ISO 15214:1998).

### 4.5. Yeasts and moulds counts

Yeasts and moulds were enumerated using rose bengal chloramphenicol agar (RBCA; Merck) at 25 ± 1 °C for 5 days (ISO 7954:1987). These microorganisms were plated using the spread-plate method with 200 µL of diluted solution sample in five plates per sample.

### 4.6. Microbial counts

Microbial counts were considered countable for all microorganisms in plates containing 15 to 300 colonies, with the exception of yeasts and moulds, where there colonies were considered countable between 15-150 colonies. Results were expressed as $Log_{10}$ CFU units per gram of whey cheese ($Log_{10}$ CFU/g). Microbial counts were calculated using the following equation (equation 1):

$$\boldsymbol{Log_{10}(N)} = \frac{\sum \boldsymbol{Caracteristic\ colonies}}{\boldsymbol{V}\,[(\boldsymbol{n_1}+\boldsymbol{0.1}\times\boldsymbol{n_2})\times\boldsymbol{d}]} \qquad \text{(equation 1)}$$

**N** - Colony forming units per gram of sample (CFU/g)

**V** - Sample volume (ml)

$\mathbf{n_1}$ - Number of plates in the 1st dilution

$\mathbf{n_2}$ - Number of plates in the 2nd dilution

**d** - 1st dilution

## 5. Physicochemical analysis

### 5.1. pH analysis

After samples homogenization with distilled water using an Ultraturrax T25 homogeniser (Janke & Kunkel IKA-Labortechnik) 1:10 (w/v), pH value was measured at room temperature with a properly calibrated pH meter (Titromatic 1S, Crison Instruments, S.A., Barcelona, Spain). Each sample was read three times.

### 5.2. Water activity analysis

Samples were measured three times with a water activity analyser (Novasina Lab Swift-aw, Lachen, Switzerland) through direct reading at room temperature, after proper stabilization.

### 5.3. Lipid oxidation analysis

Lipid oxidation was determinate by malondialdehyde (MDA) quantification, using 2-thiobarbituric acid (TBA) reactive substances method (TBARS) adapted from King (1962) method. MDA is a three-carbon dialdehyde with carbonyl groups at the C-1 and C-3 positions, as a result of the secondary oxidation of hydroperoxides.

Standard solutions of MDA in 7.5% trichloroacetic acid (TCA) were prepared from 1,1,3,3-tetrametoxipropano (TMP) and calibration curves were prepared at a concentration ranging from 0.2 to 10 μM. The standard curve obtained has the equation 2, as shown in Figure 26, in Appendix C.

$$ABS(532) = 6.28 \times 10^{-2} \times MDA + 6.7 \times 10^{-3} \qquad R^2 = 0.999 \text{ (equation 2)}$$

For the MDA extraction, five grams of sample were homogenized with 10.0 ml of 7.5% TCA solution at room temperature, using an Ultraturrax T25 homogeniser (Janke & Kunkel IKA-Labortechnik). Then it was centrifuged in falcon tubes during 10 minutes at 3500 rpm at room temperature. The resulted supernatant was filtered with Whatman no.40 and used for the TBA reaction.

In a test-tube, previously wrapped in aluminum foil, 1 ml of the previous MDA extract clear supernatant, were added to 1 ml of TBA reagent (46 mM), and placed in boiling water during 40 minutes. After cooling, 300 μL were pipette in triplicate to a microplate, which was measured, using a micro-plate spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, USA) with Brand plate of 96 wells, at 532 nm. 1 mL of the extracting solution and 1 mL of TBA reagent were mixed and used as blank.

## 6. Colour analysis

Colour was measured three times per sample using the CIELAB system by a single operator. It was determined using the Minolta Konica CM 2300d (Konica MinoltaCM 2300d, Osaka, Japan) which is an on-bench reference spectrophotometer (the accuracy is 99.7% and the repeatability is 99.4% as stated by the manufacturer) and has a d/8 geometry (diffuse illumination of the object and 8 degree viewing angle). This spectrophotometer was calibrated before the colour measurement of each sample. The

colour parameters were recorded in CIELAB system and directly computed through the original SpectraMagic NX software (Konica Minolta, Osaka, Japan), according to the International Commission on Illumination regulations: a psychometric index of lightness $L^*$, a chromatic measure taking values ranging from 0 (black) to 100 (white), and two colour coordinates $a^*$, a green-red continuum (takes positive values for reddish colours and negative values for greenish ones), and $b^*$, a blue-yellow continuum (positive values for yellowish colours and negative for the bluish ones). The samples colour parameters $L^*$, $a^*$, and $b^*$ were measured and the total colour difference (ΔE) calculated by Equation 3.

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad \text{(equation 3)}$$

where $\Delta E^*$ is the total colour difference between a sample and the control (initial values), $L^*$ and $L_0^*$ are the lightness of the sample and respective control, $a^*$ and $a_0^*$ are the redness of sample and control, respectively, and $b^*$ and $b_0^*$ are the yellowness of sample and control, respectively.

## 7. Statistical analysis

The results obtained for each treatment were carried out in duplicate and all analyses were done in triplicate. Treatment groups were compared using Analyses of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD Test, at a 5% level of significance, using STATISTICA 7 for Windows.

# CHAPTER IV - Results and Discussion

## PART I - Laboratorial experiment

At the laboratorial experiment, several temperatures (4, 25, 30, and 37 ºC), pressure levels (0.1, 100 and 150 MPa) and different storage periods (4 and 8 hours) were tested and analysed at a microbiological, physicochemical and colour level.

### 1. Microbial analysis

Total aerobic mesophiles, *Enterobacteriaceae*, yeast and moulds and lactic acid bacteria microbial counts were calculated through all the storage conditions tested in the laboratorial scale experiment.

### 1.1. Total Aerobic Mesophiles analysis

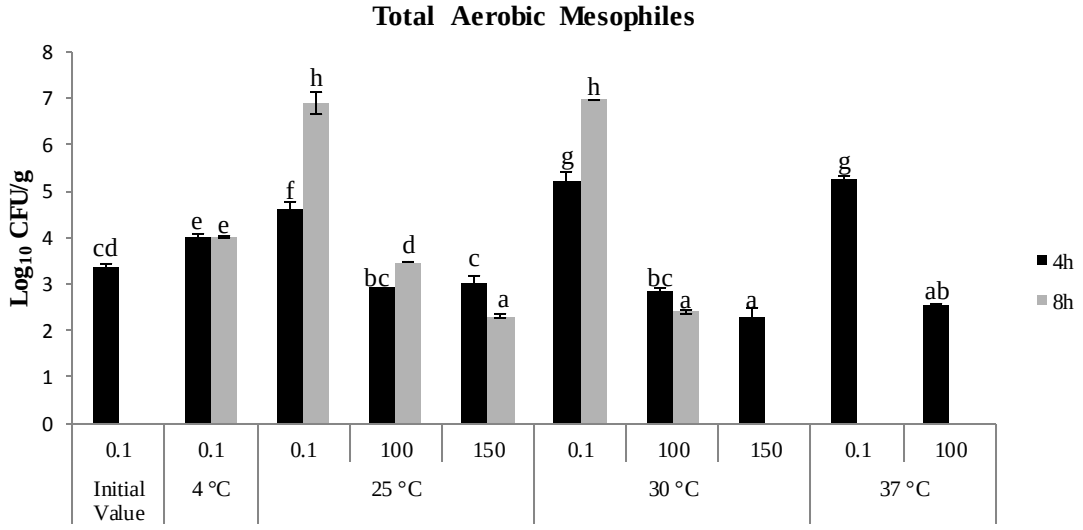


Figure 4 - Total aerobic mesophiles counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 4 and 8 hours at different pressure (0.1, 100, and 150 MPa) and temperature (4, 25, 30, and 37 ºC) condition. Different letters (a-h) indicate significant differences ($p < 0.05$) between the different conditions.

The total aerobic mesophiles are usually used as an indicator of general bacterial populations in foods that does not differentiate types of bacteria, giving only general information on the sanitary quality of products, manufacturing practices, processing conditions, handling practices and shelf life (Aycicek et al., 2006).

The initial load of the whey cheese for total aerobic mesophiles was 3.38 ± 0.06 $Log_{10}$ CFU/g (**Figure 4**). Samples stored at 4 ºC increased significantly ($p < 0.05$) TAM counts for 4 and 8 hours of storage (4.03 ± 0.04 and 4.01 ± 0.01 $Log_{10}$ CFU/g, respectively), when compared to the initial TAM load. Even under refrigeration, whey cheeses are reported to achieve microbial counts increments after few days of storage (Pintado et al., 2001). This behaviour is related to the high water activity (0.987) and pH (6.49) values of this whey cheese that pose no considerable restrains to microbial proliferation. In the present case, samples stored at or above room temperature at 0.1 MPa, quickly reached high microbial count values during the 4 hours (more than 1.5 Log units) and 8 hours of study (an increment of more than 3.5 Log units), showing the perishable nature of this dairy food. The maximum microbial load was observed for 8 hours at 25 and 30 ºC, reaching 6.88 ± 0.25 and 6.97 ± 0.01 $Log_{10}$ CFU/g, respectively.

Compared with the other microorganisms studied, TAM were the least affected by HS conditions. For the three temperatures studied, during 4 and 8 hours at both 100 and 150 MPa, hyperbaric storage was able to inhibit TAM growth and to cause a small TAM inactivation, resulting in microbial counts similar to lower compared to the initial microbial load. At 25 ºC, HP was able to inhibit TAM microbial growth, reducing TAM counts when samples were stored for 8 hours under 150 MPa (2.29 ± 0.04 $Log_{10}$ CFU/g). While at 30 ºC, HS was able to significantly ($p < 0.05$) reduce TAM counts under 100 MPa for 8 hours and 150 MPa during 4 hours (2.31 ± 0.19 and 2.40 ± 0.03 $Log_{10}$ CFU/g, respectively). Even at 37 ºC, under 100 MPa for 4 hours a reduction in TAM counts was observed, 2.53 ± 0.05 $Log_{10}$ CFU/g. A greater TAM growth inhibition was observed for all temperatures at hyperbaric conditions when compared to refrigeration ($p < 0.05$), resulting in all cases in TAM values lower than preservation under refrigeration (**Figure 4**).

### 1.2. *Enterobacteriaceae* analysis

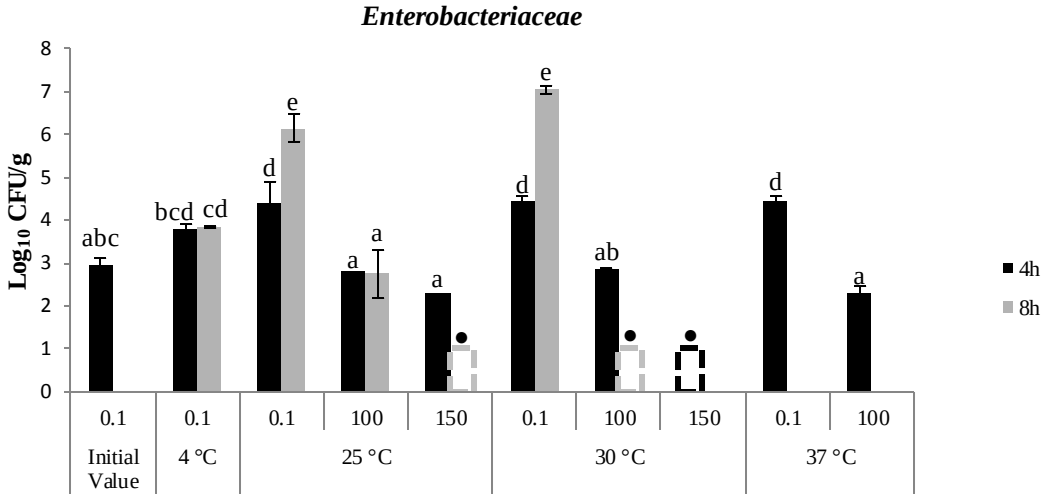


Figure 5 - *Enterobacteriaceae*, counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 4 and 8 hours at different pressure (0.1, 100, and 150 MPa) and temperature (4, 25, 30, and 37 ºC) condition. Bars with ● represent microbial loads below the detection limit (lower than 1 Log unit). Different letters (a-e) indicate significant differences ($p < 0.05$) between the different conditions.

The *Enterobacteriaceae* family is constituted by a large group of Gram-negative non-spore-forming bacteria and are mostly facultative anaerobes. Many members of this family are responsible for spoilage of a variety of foods, such as, vegetables and fruits, meats, eggs, milk and dairy products (Lehner and Stephan, 2004). *Enterobacteriaceae* family also includes a number of important foodborne pathogens like *Salmonella*, pathogenic *Escherichia coli* (including *E. coli* O157:H7), *Shigella spp*. and *Cronobacter spp*. (Schofield, 1992). Nowadays, *Enterobactericeae* and coliforms are isolated from foods as evidence of poor hygiene or inadequate processing (especially heat-treatment), process failure and post-process contamination of foods. However, *Enterobacteriaceae* growth is dependent on extrinsic factors like temperatures and relative humidity, implicit conditions like possible interactions with microbial populations and intrinsic factors like pH, water activity and intrinsic antimicrobial substances (Nazarowec-White et al., 1999). *E. coli* can be used as an index microorganism for the presence of pathogens like *Salmonella*, and is generally used to provide evidence of potential faecal contamination in certain foods.

The initial *Enterobacteriaceae* load in the whey cheese was 2.97 ± 0.15 $Log_{10}$ CFU/g, as it can be seen in **Figure 5**. When stored at all three temperatures under atmospheric pressure a significant ($p < 0.05$) increment of approximately 1.5 Log units was observed for 4 hours, while during 8 hours at both 25 and 30 ºC initial ENT load doubled (6.14 ± 0.33 and 7.04 ± 0.10 $Log_{10}$ CFU/g, respectively). Under refrigeration, ENT counts remained similar to the initial load during 4 and 8 hours, being reported an increment of ≈3 Log units for ENT and YM after 6 days under refrigeration by Pintado et al. (2001).

ENT under HS at 25 ºC, at 100 MPa for 4 and 8 hours, suffered microbial growth inhibition when compared to the initial load ($p > 0.05$), with the values being significantly lower ($p < 0.05$) comparatively to refrigeration. Similar results were verified for HS at 150 MPa at 25 ºC for 4 hours, and at 100 MPa at 30 and 37 ºC for 4 hours. Under 150 MPa at 25 ºC for 4 hours, at 100 MPa at 30 ºC for 8 hours, and at 150 MPa at 30 ºC for 4 hours, ENT inactivation was observed, resulting in values below the detection limit. Globally, and comparatively to refrigeration, HS at all tested conditions performed significantly ($p < 0.05$) better, resulting in lower microbial counts that reached in some cases even values below the detection limit. Gram-negative bacteria are more susceptible to high hydrostatic pressure than gram-positive bacteria, the thinner peptidoglycan constitution in gram-negative bacteria confers lower protection against high pressure (Wuytack et al., 2002).

### 1.3. Yeasts and Moulds analysis

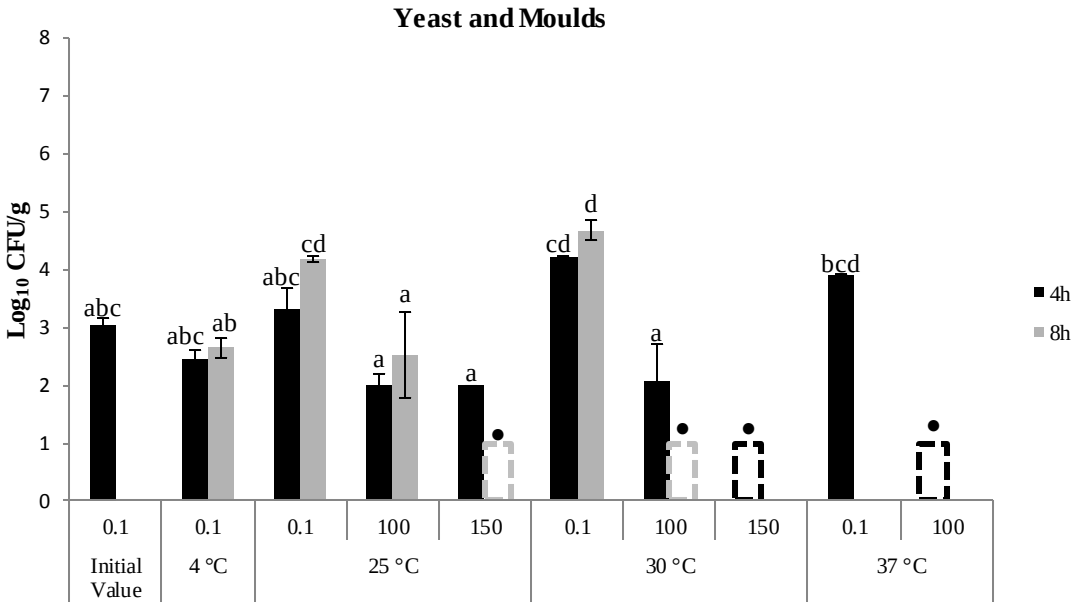


Figure 6 - Yeast and moulds counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 4 and 8 hours at different pressure (0.1, 100, and 150 MPa) and temperature (4, 25, 30, and 37 ºC) condition. Bars with ● represent microbial loads below the detection limit (lower than 1 Log unit). Different letters (a-d) indicate significant differences ($p < 0.05$) between the different conditions.

Yeasts and molds can commonly contaminate foods and grow under a wide range of environmental conditions like acidity (pH 2–9), temperature (5–35 °C), with some species capable of growth above or below this range, and water activity ($a_w$ >0.85), with many foodborne yeasts and molds able to growth outside the limited conditions mentioned before, like *Zygosaccharomyces*, *Eurotium*, and *Xeromyces* species. This allows yeasts and molds to invade and growth a wide range of foods, like crops such grains nuts, beans, and fruits in fields before harvesting and during storage, causing several degrees of deterioration and decomposition of foods (Tournas et al., 2006). In addition to spoilage of foods, many fungal species are known to produce mycotoxins that may pose a greater concern for public health.

YM initial load was 3.04 ± 0.13 $Log_{10}$ CFU/g, which was maintained when whey cheese was stored under refrigeration ($p < 0.05$) for 4 and 8 hours, as **Figure 6** shows. Under atmospheric pressure YM counts increased, reaching higher counts at 30 ºC for 4 and 8 hours, 4.22 ± 0.03 and 4.68 ± 0.18 $Log_{10}$ CFU/g, respectively. YM

showed a similar behaviour to ENT under hyperbaric conditions. At 25 ºC under HS conditions, microbial growth inhibition occurred for all conditions, resulting in similar values ($p > 0.05$) when compared to the initial load and refrigeration counts. For samples stored for 8 hours at 150 MPa, also at 25 ºC, YM counts were reduced ($p < 0.05$) below the detection limits, showing an inactivation effect at these storage conditions. At 30 and 37 ºC and 0.1 MPa, YM counts increased, reaching values of at least 4 $Log_{10}$ CFU/g. Contrarily, at these temperatures HS resulted in YM inactivation ($p < 0.05$) to below the detection limits at all conditions, with the exception of storage at 100 MPa for 4 hours at 30 ºC, where YM growth inhibition was observed, yielding values similar the initial load and to storage under refrigeration. Smelt (1998) reported that even moderated hydrostatic pressure (30-100 MPa) can cause changes on gene expression, protein synthesis and nuclear membrane of yeasts, reaching inactivation at higher pressures (250 MPa), however, the application of low pressure for long periods at different temperatures in foods is poorly documented.

### 1.4. Lactic Acid Bacteria analyses

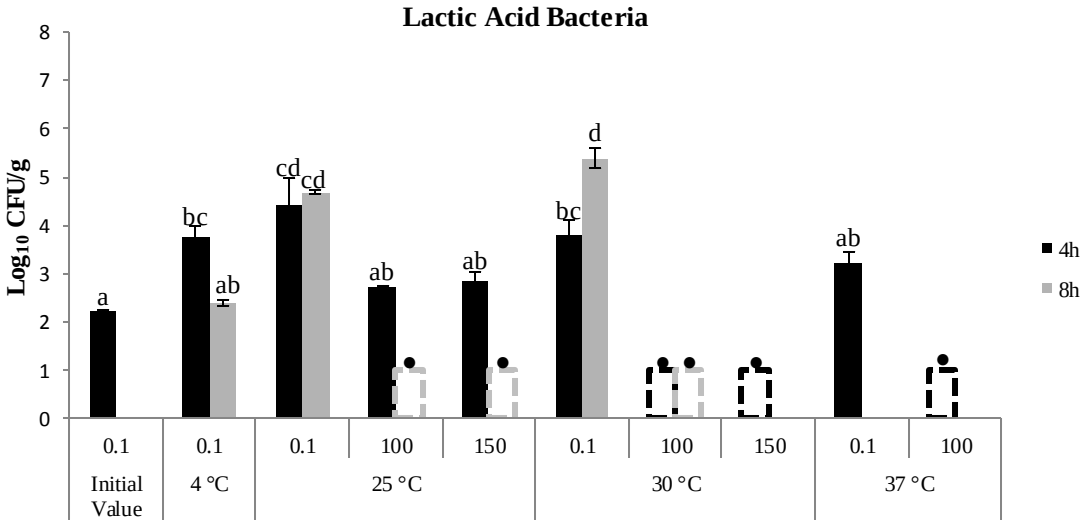


Figure 7 - Lactic acid bacteria counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 4 and 8 hours at different pressure (0.1, 100, and 150 MPa) and temperature (4, 25, 30, and 37 ºC) condition. Bars with ● represent microbial loads below the detection limit (lower than 1 Log unit). Different letters (a-d) indicate significant differences ($p < 0.05$) between the different conditions.

Lactic acid bacteria are a very heterogeneous group, Gram positive, nonmotile, nonsporeforming and aerotolerant bacteria that ferment hexoxes to lactid acid, being used for the production of fermented dairy products and in meat, vegetable and silage fermentations (Adamberg et al., 2003).

Lactic acid bacteria initial load of *Requeijão* sample was 2.22 ± 0.05 $Log_{10}$ CFU/g, shown in **Figure 4**. The results obtained for storage at 0.1 MPa showed considerable LAB growth, reaching a maximum value higher than 5 Log units at 30 ºC. By the contrary, LAB were found to be more susceptible to HS conditions, being reduced to undetectable counts at all HS conditions, with the exception of 25 ºC for 4 hours at both pressures studied (100 and 150 MPa, 2.74 ± 0.01 and 2.84 ± 0.18 $Log_{10}$ CFU/g, respectively), where LAB microbial growth were inhibited, resulting in counts similar ($p > 0.05$) to the initial load. Considering these results, HS at all tested conditions resulted in lower to equal microbial LAB counts, comparatively to samples stored at refrigeration. These results are according to Molina-Höppner et al. (2003), who studied the pressure effect from 0.1 to 100 MPa, for 20 and 48 hours at 30 ºC, on two mesophile LAB, *Lactococcus lactis* and *Lactobacillus sanfranciscensis* growth rate, concluding that 50 MPa were able to inhibit *Lb. sanfranciscensis* growth, and reduce *Lc. lactis* growth rate to less than 30 %, comparatively to its growth rate at atmospheric pressure.

### 1.5. General analysis

This is an innovating study that uses pressure as a preservation technique at and above room temperatures for a dairy food storage. Queirós et al. (2014) for melon juice (pH near to 6), obtained a microbial growth inhibition similar to refrigeration at pressures of 50 and 75 MPa at room temperature for 8 hours, reaching microbial inactivation to ENT and YM at 100 MPa. While a more significant microbial inactivation effect was observed at 150 MPa for all tested microorganisms at and above room temperature (25, 30 and 37 ºC). Also, TAM were the most resistant microorganisms on this study, being ENT and YM reduced to undetectable counts, when stored for 8 hours under 150 MPa for all temperatures tested, which was similar to the results obtained for hyperbaric storage of whey cheese.

Overall, for this product and for the studied conditions it was possible to achieve a similar to better microbial stability comparatively to refrigeration with HS at and

above room temperatures (25-37 ºC), independently of the temperature used. These results indicate the feasibility of this promising food preservation methodology, by storing food at variable room temperatures, as a promising lower energetic cost alternative to refrigeration. This fact is of great importance, for possible application to all foods preserved by refrigeration, since HS would allow food storage at uncontrolled, naturally variable room temperature, with no energy costs associated to temperature control, with energy being only required to reach the desired pressure and decompress at the end of the storage period.

## 2. Physicochemical Analyses

### 2.1. pH analysis

Initial whey cheese pH was 6.49 ± 0.17 (**Table 8**), this value is similar to what is found in literature, however, it may vary according to whey acidity used to produce *Requeijão* (Pintado et al., 2001). For 4 and 8 hours storage, pH value did not significantly ($p > 0.05$) change comparatively to refrigeration, with the exception of samples stored for 4 hours at 100 MPa and 30 ºC which was significantly ($p < 0.05$) lower (6.22 ± 0.03).

### 2.2. Water activity analysis

Water activity is one of the major factors for microbial growth, influencing also lipid oxidation, enzymatic reactions, degradation of vitamins and other nutrients. As expected, initial water activity value for *Requeijão* used in this experiment was high, 0.987 ± 0.002, allowing this way microbial spoilage by the majority of microorganisms. Water activity did not significantly ($p > 0.05$) changed throughout the storage period comparatively to the initial and refrigeration value. No significant changes were observed among storage periods for the same conditions (**Table 8**).

**Table 8 -** Physicochemical properties of stored whey cheese at different pressure (MPa) levels and temperature (ºC) conditions. Different letters in a row between conditions (a-c) and storage times (A-B) indicate significant differences ($p < 0.05$). Mean values were obtained from duplicated samples, each analysed in triplicate.

| | | pH | | Water activity | | Lipid oxidation | |
|---|---|---|---|---|---|---|---|
| **Conditions** | | **4 h** | **8 h** | **4 h** | **8 h** | **4 h** | **8 h** |
| **Initial** | **0.1** | 6.49 ±0.17 a | 6.49 ±0.17 a | 0.987 ±0.002 abc | 0.987 ±0.002 a | 0.22 ±0.04 a | 0.22 ±0.04 bc |
| **4 ºC** | **0.1** | 6.60 ±0.16 abcA | 6.52 ±0.11 aA | 0.986 ±0.001 aA | 0.986 ±0.002 aA | 0.13 ±0.02 aA | 0.37 ±0.02 abB |
| **25 ºC** | **0.1** | 6.53 ±0.31 abA | 6.60 ±0.04 aA | 0.986 ±0.001 abA | 0.986 ±0.002 aA | 0.41 ±0.02 bA | 0.34 ±0.04 abA |
| | **100** | 6.42 ±0.04 aA | 6.46 ±0.11 aA | 0.987 ±0.001 abA | 0.985 ±0.001 aA | 0.14 ±0.07 aA | 0.40±0.03 aB |
| | **150** | 6.76 ±0.04 cA | 6.25 ±0.22 aA | 0.986 ±0.001 abA | 0.985 ±0.001 aA | 0.20±0.10 aA | 0.35 ±0.06 abA |
| **30 ºC** | **0.1** | 6.54 ±0.07 abA | 6.63 ±0.02 aA | 0.987 ±0.001 abA | 0.986 ±0.001 aA | 0.45 ±0.01 bA | 0.37 ±0.02 aB |
| | **100** | 6.22 ±0.03 dA | 6.64 ±0.02 aB | 0.986 ±0.001 aA | 0.989 ±0.001 aA | 0.30±0.02 abA | 0.13 ±0.01 cB |
| | **150** | 6.43 ±0.08 a | * | 0.989 ±0.001 c | * | 0.30±0.004 ab | * |
| **37 ºC** | **0.1** | 6.72 ±0.01cd | * | 0.988 ±0.001 bc | * | 0.47 ±0.01 b | * |
| | **100** | 6.62 ±0.03 abc | * | 0.988 ±0.001 bc | * | 0.30±0.01 ab | * |

* – Experiments were not carried out in these conditions.

### 2.3. Lipid oxidation analysis

This product has a strong fatty acids content in its constitution (≈30 %), that can oxidise and cause unpleasant odours and flavours to the product. Initial MDA content of whey cheese was 0.228 ± 0.035 mg/g (**Table 8**). This value maintained stable for 4 and 8 hours at 4 ºC and no significant ($p > 0.05$) changes were observed for HS for 4 hours of storage comparatively to refrigeration. On the other hand, samples stored for 4 hours at atmospheric pressure at 25, 30 and 37 ºC significantly ($p < 0.05$) reach higher levels of MDA. Hyperbaric samples stored for 8 hours showed higher levels of MDA ($p < 0.05$) comparatively to the initial value, but not different ($p > 0.05$) when compared to refrigeration, with the exception of samples stored at 30 ºC under 100 MPa, which was significantly ($p < 0.05$) low. Some studies report that the use of high pressures (200–600 MPa) may increase the primary oxidation compounds (peroxides) (Ohshima et al., 1993), and raise secondary and tertiary lipid oxidation compounds at lower pressures (170-200 MPa) for short time treatments (Aubourg et al., 2010). However, the

information regarding lipid oxidation for long storage periods at low pressure is scarce and deserves further studies. At the different hyperbaric conditions, overall whey cheese MDA content did not significantly ($p > 0.05$) changed when compared to control samples, showing that for this product, those conditions were unable to accelerate lipid oxidation. Such changes would diminish the product acceptability, due to rancidity and the formation of undesirable compounds, which would limit the product shelf life.

## 3. **Colour analysis**

**Table 9 -** Colour parameters of stored whey cheese at different pressure (MPa) levels and temperature (ºC) conditions. Different letters in a row between conditions (a-d) and storage times (A-B) indicate significant differences ($p < 0.05$). Mean values were obtained from duplicated samples, each analysed in triplicate.

| | | **Colour** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | L* | | *a** | | *b** | | *ΔE* | |
| **Conditions** | | **4 h** | **8 h** | **4 h** | **8 h** | **4 h** | **8 h** | **4 h** | **8h** |
| **Initial** | **0.1** | 92.07 ±0.06b | 92.07 ±0.06 ab | -2.40 ±0.02b | -2.40 ±0.02a | 9.16 ±0.10 ab | 9.16 ±0.10 a | * | * |
| **4 ºC** | **0.1** | 91.54 ±0.63 abcA | 91.62 ±0.41 aA | -2.39 ±0.09 bcA | -2.39 ±0.05 aA | 8.98 ±0.02 abA | 9.17 ±0.30 aA | 0.61 ±0.05 abA | 0.51 ±0.36 aA |
| **25 ºC** | **0.1** | 91.93 ±0.20 abA | 92.74 ±0.16 bB | -2.41 ±0.04 bA | -2.30 ±0.20 aA | 8.90 ±0.36 abA | 9.35 ±0.43 aA | 0.40 ±0.16 abA | 0.77 ±0.28 aA |
| | **100** | 91.72 ±0.15 abcA | 91.78 ±0.48 abA | -2.51 ±0.04 abcA | -2.49 ±0.05 aA | 9.56 ±0.27 aA | 9.22 ±0.25 aA | 0.60 ±0.09 abA | 0.45 ±0.39 aA |
| | **150** | 92.02 ±0.18 abA | 91.98 ±0.29 abA | -2.66 ±0.03 aA | -2.39 ±0.05 aB | 9.02 ±0.19 abA | 9.98 ±0.12 aB | 0.30 ±0.01 bA | 0.83 ±0.01 aB |
| **30 ºC** | **0.1** | 90.92 ±0.10 cA | 92.31 ±0.12 abB | -2.52 ±0.04 abcA | -2.37 ±0.02 aB | 9.20 ±0.24 aA | 8.99 ±0.30 aA | 1.17 ±0.11 aA | 0.34 ±0.23 aB |
| | **100** | 91.11 ±0.23 acA | 91.64 ±0.34 abA | -2.53 ±0.06 abcA | -2.59 ±0.09 aA | 8.43 ±0.05 bA | 9.05 ±0.14 aB | 1.21 ±0.15 aA | 0.51 ±0.28 aA |
| | **150** | 91.78 ±0.10 abc | * | -2.61 ±0.01 ac | * | 9.00 ±0.01 ab | * | 0.39 ±0.07 ab | * |
| **37 ºC** | **0.1** | 93.17 ±0.20d | * | -2.65 ±0.09a | * | 8.99 ±0.30 a | * | 1.17 ±0.14 a | * |
| | **100** | 92.06 ±0.10ab | * | -2.46 ±0.02 abc | * | 9.05 ±0.14 a | * | 0.29 ±0.11 b | * |

* – Experiments were not carried out in these conditions.

The colour parameters values of whey cheese, *L**, *a** and *b**, were measured for all whey cheeses samples used in this study, and used to calculate total colour change (*ΔE**). Initial *L** value for whey cheese was 92.07 ± 0.06, and in general, a significant variation ($p < 0.05$) was observed for storage at above room temperature conditions (30 and 37 ºC) and atmospheric pressure for 4 hours (90.92 ± 0.10 and 93.17 ± 0.20, respectively), when compared to the initial *L** value, as it can be seen in **Table 9**. When compared to refrigeration, hyperbaric conditions at both 4 and 8 hours didn't significantly change ($p > 0.05$) the *L** value.

Initial *a** value for whey cheese was -2.40 ± 0.02, that significantly changed ($p < 0.05$) at 25 and 30 ºC for the highest pressure (150 MPa) during storage for 4 hours, -2.66 ±0.03 and -2.61 ±0.01 respectively. No significant ($p > 0.05$) differences were observed, for storage during 8 hours, comparatively to the initial value and refrigeration, between the different conditions. *b** initial value was 9.16 ± 0.10, with no significant ($p > 0.05$) differences founded, in samples stored for 4 and 8 hours, comparatively to the initial and refrigeration value.

Considering total colour change (*ΔE**), the most significant variations occurred at atmospheric conditions for 4 hours and 30 ºC (1.17 ± 0.11), at 30 ºC for 4 hours at 100 MPa (1.21 ± 0.15) and at 37 ºC for 4 hours (1.17 ± 0.14) which was a result of a higher increase of *L** value, with no significantly variations ($p > 0.05$) when compared to *ΔE** of refrigeration (0.61 ±0.05). However, according to Drlange (1994) the colour differences observed for all hyperbaric conditions can be classified as "small difference", *ΔE** between 0.5 and 1.5, being this values similar to refrigeration ($p > 0.05$).

## 4. General analysis

The physicochemical analyses performed in this study showed that, hyperbaric storage caused lower changes, when compared to the corresponding temperature treatments at atmospheric pressure and refrigeration.

# PART II – Experiment in an industrial equipment

Considering the result obtained in the laboratorial experiment, an experiment in an industrial equipment was conducted using an Hyperbaric55. This experiment was performed in the last part of this Thesis, using commercially samples of *Requeijão* for a better analysis of the hyperbaric storage on real size products for an extended storage period.

In this study, samples were stored under 100 MPa at room temperature (≈21 ºC) for 12 and 24 hours, with respective controls under refrigeration and room temperature under atmospheric pressure for the same respective periods. After 24 hours at hyperbaric storage, a post-hyperbaric storage (PHS) was conducted, with samples from hyperbaric storage, under refrigeration for 24 hours, and samples stored at room temperature at 0.1 MPa for 24 hours, being those samples stored under refrigeration for three days.

## 1. Microbial analysis

Total aerobic mesophiles, *Enterobacteriaceae*, lactic acid bacteria, yeasts and moulds microbial were performed and analysis for each storage mentioned above.

### 1.1. Total Aerobic Mesophiles analysis

Total aerobic mesophiles initial load, 2.70 ± 0.09 $Log_{10}$ CFU/g, was lower when compared to samples used in the laboratorial experiment. TAM counts under refrigeration at 0.1 MPa reached higher counts ($p < 0.05$) at both 12 and 24 hours of storage, 4.50 ± 0.15 and 5.39 ± 0.32 $Log_{10}$ CFU/g, respectively. At room temperature under atmospheric pressure for 12 and 24 hours, TAM counts surpass the 6 Log units, as it was expectable considering the results obtained in the previous study and taking in consideration the highly perishable nature of this dairy product. Hyperbaric storage maintained TAM counts similar ($p > 0.05$) to the initial load at 12 and 24 hours, 2.88 ± 0.24 and 2.85 ± 0.33 $Log_{10}$ CFU/g, respectively, and significantly lower ($p < 0.05$) when compared with refrigeration for both 12 and 24 hours of storage.

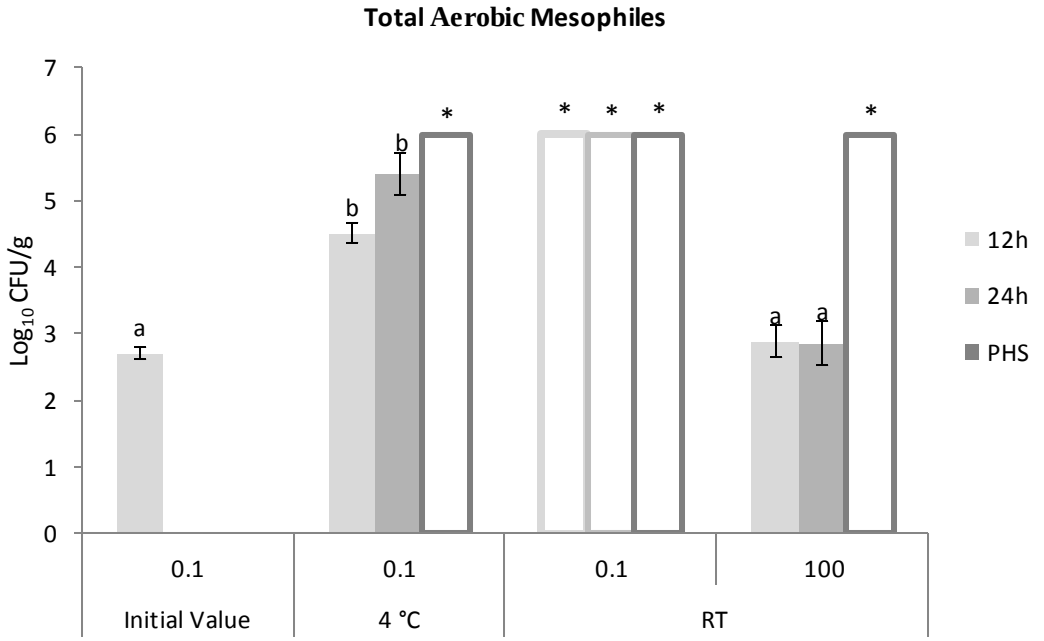


Figure 8 - Total aerobic mesophiles counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 12 and 24 hours at 0.1 and 100 MPa, at 4 ºC and room temperature (≈21 ºC) condition. Different letters (a-b) indicate significant differences ($p < 0.05$) between the different conditions. Bars with * represent microbial loads above 6 Log units.

### 1.2. *Enterobacteriaceae* analysis

Initial *Enterobacteriaceae* load in whey cheese was 3.69 ± 0.01 $Log_{10}$ CFU/g. ENT counts slightly decrease ($p > 0.05$) when stored under hyperbaric conditions, for both 12 and 24 hours (2.95 ± 0.25 and 2.92 ± 0.33 $Log_{10}$ CFU/g, respectively) maintaining ENT counts similar to the initial load. These values are similar to those obtained at the laboratorial experiment, for samples at 100 MPa for 8 hours at 25 ºC, in which a microbial growth inhibition was observed, being required possibly higher pressures at the experiment in an industrial equipment to observe some ENT growth inactivation. At refrigeration under atmospheric pressure during 12 hours, ENT counts (4.21 ± 0.19 $Log_{10}$ CFU/g) remained similar ($p > 0.05$) to the initial value, reaching higher counts at 24 hours (5.26 ± 0.32 $Log_{10}$ CFU/g) of storage ($p < 0.05$). HS during 12 and 24 hours was able to restrains ENT microbial growth, resulting in lower counts ($p < 0.05$), comparatively to samples under refrigeration. Storage at atmospheric pressure at

room temperature, as it was expected, at both 12 and 24 hours, ENT counts reached values above 6 Log units.

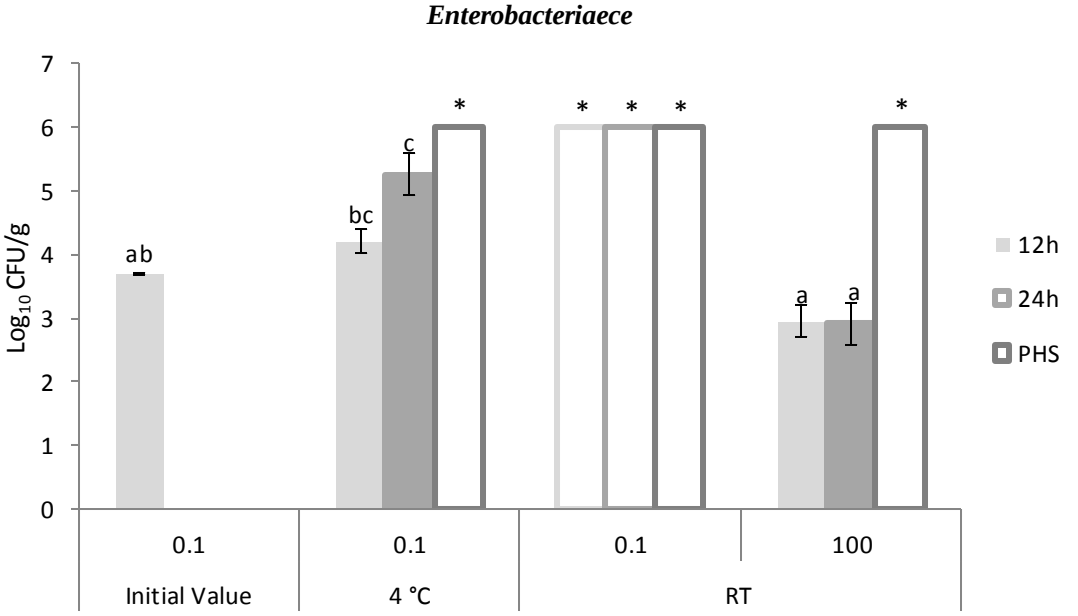


Figure 9 - *Enterobacteriaceae* counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 12 and 24 hours at 0.1 and 100 MPa, at 4 ºC and room temperature (≈21 ºC) condition. Different letters (a-c) indicate significant differences ($p < 0.05$) between the different conditions. Bars with * represent microbial loads above 6 Log units.

### 1.3. Lactic acid bacteria analysis

Lactic acid bacteria initial load was 2.48 ± 0.02 $Log_{10}$ CFU/g, which was similar to the one observed in the sample used in the laboratorial experience. Refrigeration at atmospheric pressure for 12 hours maintained LAB counts similar to the initial value ($p > 0.05$), rising during 24 hours of storage to 3.34 ± 0.09 $Log_{10}$ CFU/g ($p < 0.05$). Samples at room temperature under atmospheric pressure, quickly reached higher counts ($p < 0.05$) after 12 hours, 3.39 ± 0.15 $Log_{10}$ CFU/g, and counts above the 6 Log units after 24 hours of storage. Results in hyperbaric storage were in accordance to those obtained in the laboratorial experiment (storage under 100 MPa for 8 hours at 25 ºC), where LAB were inactivated to counts, below the detection limit.

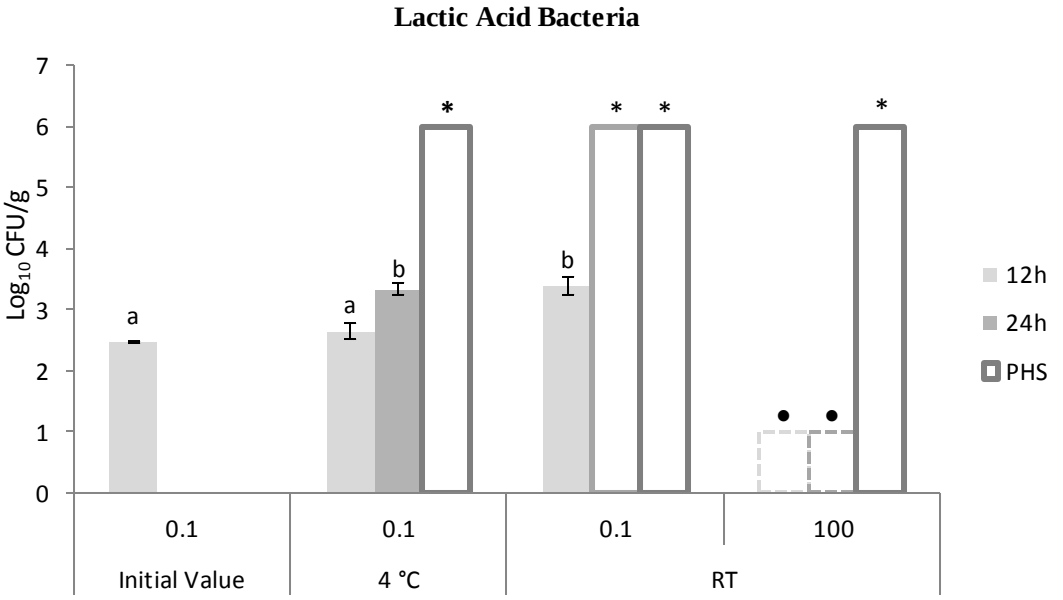


Figure 10 – Lactic acid bacteria counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 12 and 24 hours at 0.1 and 100 MPa, at 4 ºC and room temperature (≈21 ºC) condition. Different letters (a-b) indicate significant differences ($p < 0.05$) between the different conditions. Bars with * and with ● represent microbial loads above 6 Log units and microbial loads below the detection limit (lower than 1 Log unit), respectively.

### 1.4. Yeasts and Moulds analysis

The yeasts and moulds initial load of this product was 4.53 ± 0.01 $Log_{10}$ CFU/g, which was 1 Log unit higher than those used in the laboratorial experiment. However, at 100 MPa for 8 hours under 25 ºC, only an inhibitory effect was observed in the laboratorial experiment, by increasing the storage period to 12 and 24 hours using the same pressure level, YM counts were reduced to undetectable counts at both storage periods. Storage at atmospheric pressure at room temperature and refrigeration, despite the small increment observed, YM counts remained similar ($p > 0.05$) to the initial load.

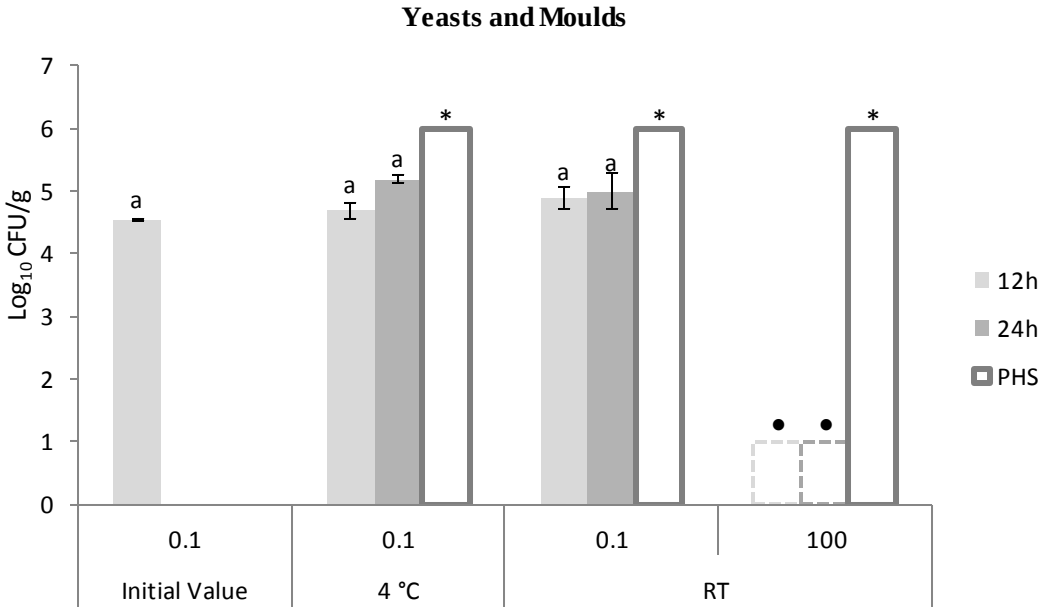


Figure 11 – Yeast and moulds counts (expressed in $Log_{10}$ CFU/g) initially presented in whey cheese and stored during 12 and 24 hours at 0.1 and 100 MPa, at 4 ºC and room temperature (≈21 ºC) condition. Different letters indicate significant differences ($p < 0.05$) between the different conditions. Bars with * and with ● represent microbial loads above 6 Log units and microbial loads below the detection limit (lower than 1 Log unit), respectively.

### 1.5. Post-hyperbaric storage analysis

The 3 days period of post-storage at refrigeration, after hyperbaric storage, was selected due to the highly perishable nature of *Requeijão* (shelf life of 2 days under refrigeration). 3 days were estimated, having in consideration the microbial inhibitory and inactivation effect observed at hyperbaric storage, which could allow an increase of *Requeijão* shelf life. However, even those samples stored for 24 hours at hyperbaric conditions, reached counts superior to 6 Log units, after three days under refrigeration. This means that despite the microbial inhibition observed during the 24 hours storage at 100 MPa, the remaining microorganisms were able to growth during the PHS at refrigeration.

### 1.6. General analysis

Overall, a similar tendency was observed between the results obtained in the laboratorial and in the industrial equipment experiment for almost all microorganisms. Whereas samples stored at refrigeration and room temperature at atmospheric pressure, reached higher counts for all microorganisms comparatively to the initial load. Hyperbaric storage successfully inhibit TAM and ENT microbial growth, inactivating LAB and YM counts to levels below the detection limit. It was clear that hyperbaric storage was able to restrain the microbial growth, performing by far better than refrigeration, as the storage period increase.

Fidalgo et al. (2013) stored raw watermelon juice (pH > 4.6) during 60 hours at 100 MPa, at uncontrolled, naturally variable room temperature conditions (18–21 ºC). TAM, ENT and YM where inactivated in the first 8 hours of storage, maintaining similar values at 24 hours of storage. ENT and YM were inactivated to counts below the detection limit just in the first 8 hours of storage, while for TAM a reduction of ≈1 Log unit was observed in the first day of storage, and ≈1.5 Log unit reduction at 60 hours. It was verified that storage at 100 MPa for 60 hours was capable to reduce the juice initial microbial counts and avoid microbial growth, thus yielding better results than refrigeration.

## 2. Physicochemical Analyses

### 2.1. pH analysis

Initial pH value for the Requeijão sample used in this study was 6.47 ± 0.02. Under refrigeration pH value was stable at all storage periods, with small decrease ($p$ > 0.05) observed as the storage period increased (**Figure 19**). At room temperature under atmospheric pressure, pH value remained stable during 12 and 24 hours, decreasing significantly ($p$ < 0.05) after 3 days under refrigeration (PHS) to 5.30 ± 0.09. The decrease observed for the pH value at room temperature and refrigeration at atmospheric pressure, is related to the high microbial growth observed for those storage conditions, through the production of organic acids. Hyperbaric storage maintained pH value similar to the initial, at all hyperbaric storage conditions.

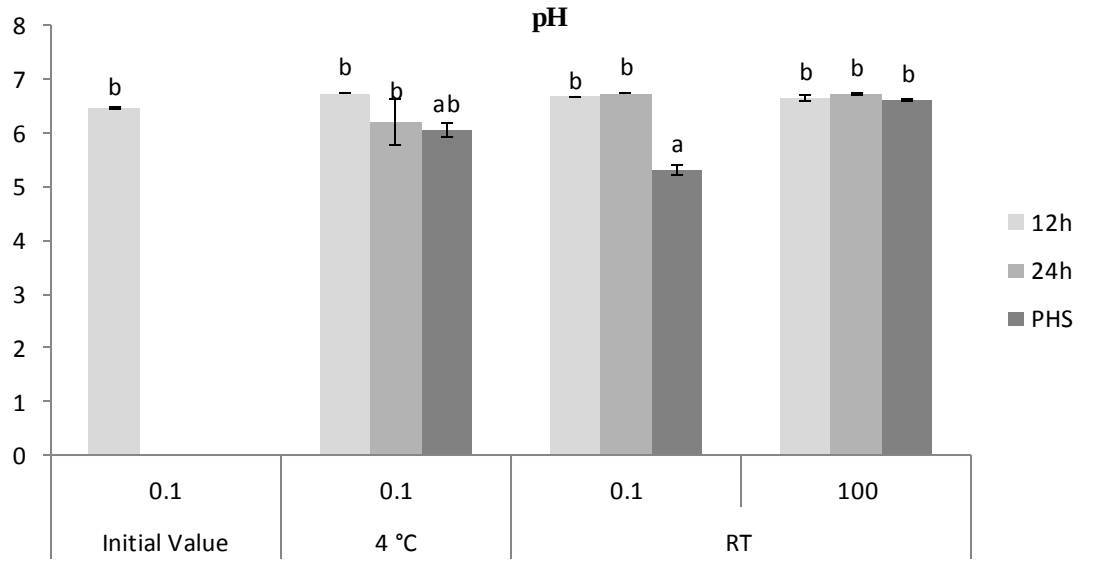


Figure 12 - pH value of stored whey cheese used in the industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters (a-b) indicate significant differences ($p < 0.05$) between the different conditions.

### 2.2. Water activity analysis

Water activity for *Requeijão* used in this experiment was still high, 0.982 ± 0.001 (**Figure 20**). Under refrigeration at atmospheric pressure storage this value remained stable at all conditions, with the exception ($p < 0.05$) of storage for 24 hours, 0.986 ± 0.001. The same tendency was observed for storage at room temperature at atmospheric pressure and hyperbaric storage conditions, being the values obtained at 24 hours, for both conditions (0.986 ± 0.001 and 0.986 ± 0.002, respectively), similar to the one obtained at refrigeration for the same period ($p > 0.05$).

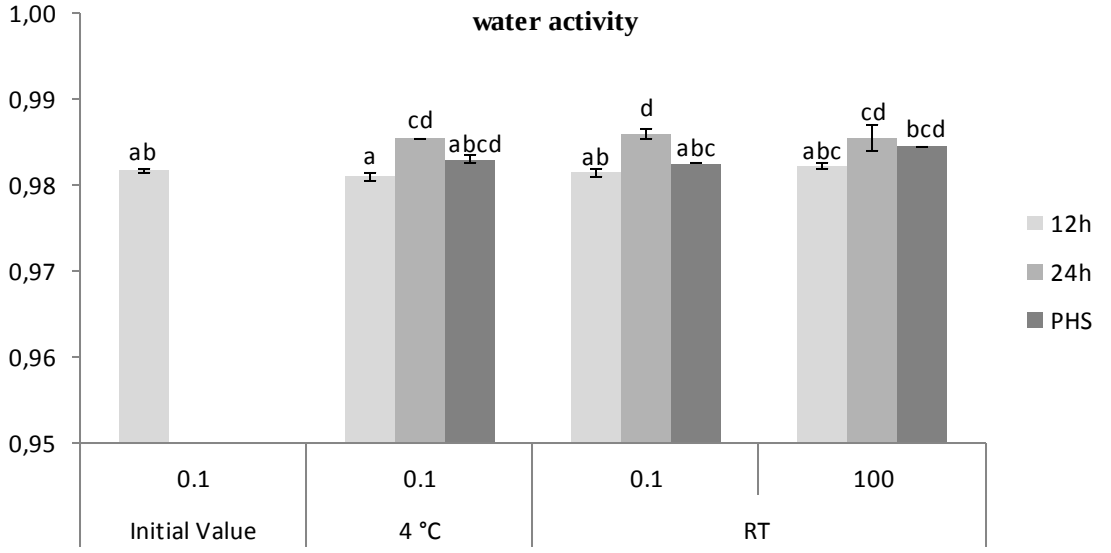


Figure 13 – Water activity value of stored whey cheese used in the industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters (a-d) indicate significant differences ($p < 0.05$) between the different conditions.

### 2.3. Lipid oxidation analysis

Initial MDA value observed for *Requeijão* used in this experiment was 0.191 ± 0.013 mg/g, which was similar to the one used in the laboratorial experiment. Statistically no significant changes were observed in the MDA levels comparatively to the all different storage conditions under refrigeration and room temperature at atmospheric pressure, as well as for hyperbaric storage samples (**Figure 21**).

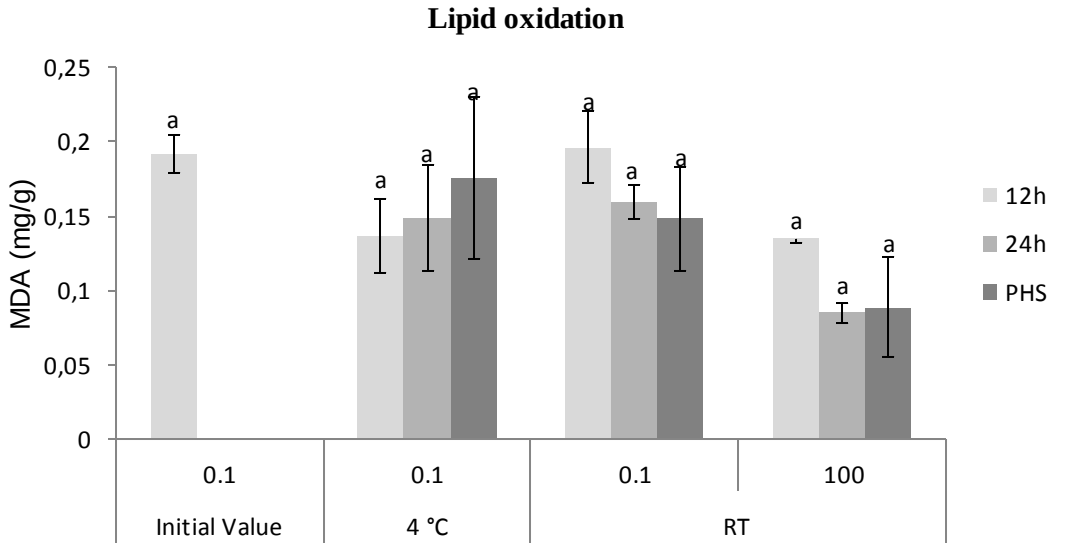


Figure 14 – MDA values (mg/g) in the stored whey cheese used in the industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters indicate significant differences ($p$ < 0.05) between the different conditions.

### 3. Colour analysis

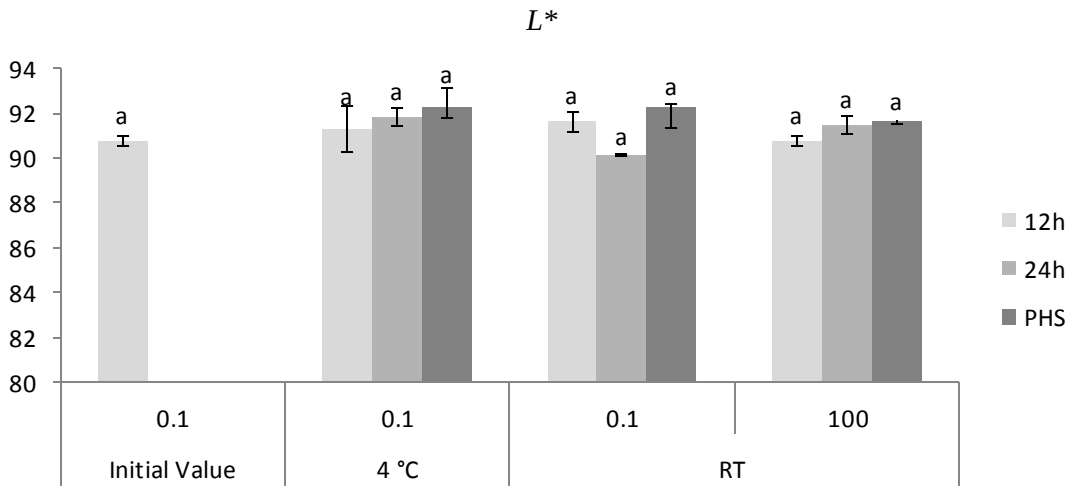


Figure 15 – $L^*$ values in the stored whey cheese used in an industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters indicate significant differences ($p$ < 0.05) between the different conditions.

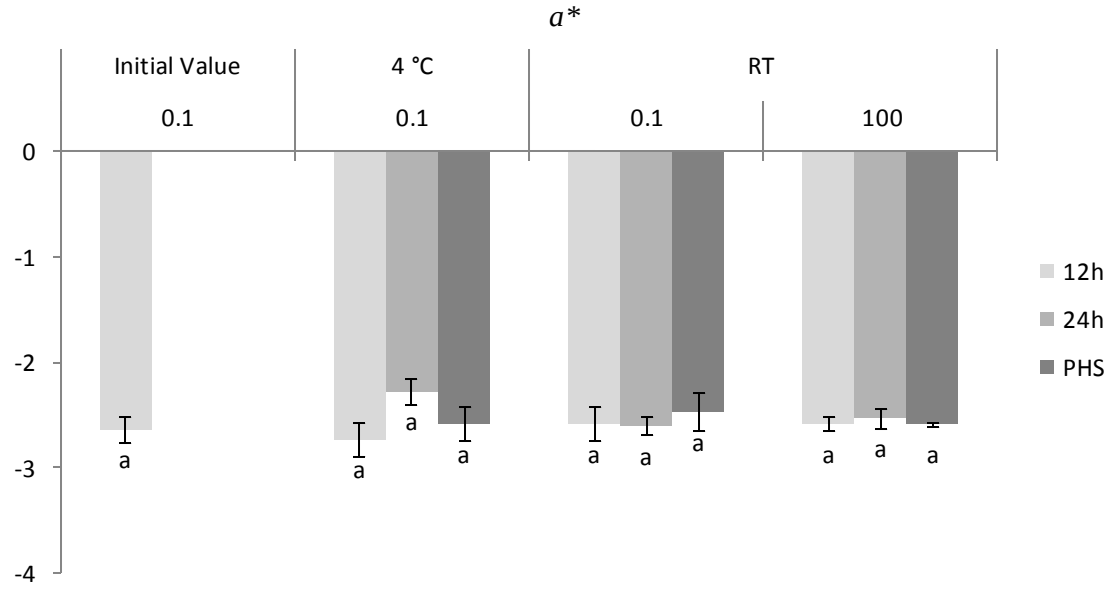


Figure 16 – *a** values in the stored whey cheese used in an industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters indicate significant differences ($p < 0.05$) between the different conditions.

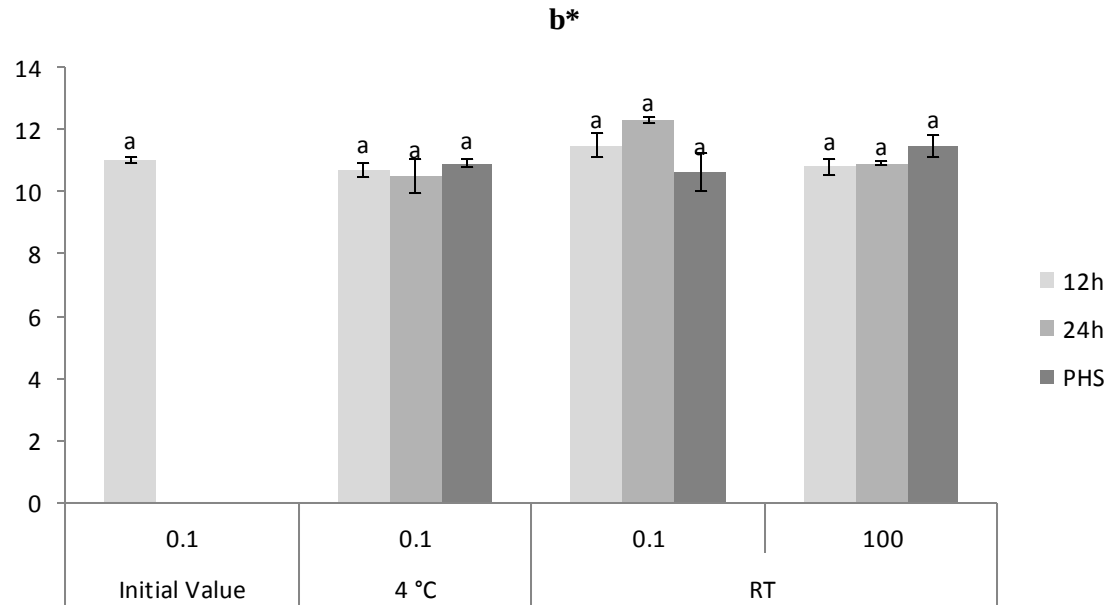


Figure 17 - *b** values in the stored whey cheese used in an industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters indicate significant differences ($p < 0.05$) between the different conditions.

The initial values of *L**, *a** and *b** of the *Requeijão* used in this study were 90.72 ± 0.22, -2.64 ± 0.12 and 11.02 ± 0.08, respectively. The *L** value remained stable ($p > 0.05$) at all different storage conditions used (**Figure 22**), although a small increment was observed for all storage conditions, with the exception of storage under atmospheric pressure at room temperature during 24 hours (90.45 ± 0.07) and storage at 100 MPa for 12 hours at room temperature (90.75 ± 0.21), that retain *L** values similar ($p > 0.05$) to the initial one.

a* and b* values also remained stable at all different storage conditions, with no significant variations ($p > 0.05$), as **Figures 23** and **24** shows. As for the total colour change ($\Delta E$), statistically no differences were found, although considering Drlange (1994), the $\Delta E$ value for samples at hyperbaric storage for 12 hours at room temperatures are considered to have a "very small difference" ($0.2 < \Delta E < 0.5$), with the exception of PHS at atmospheric pressure, the remaining storage conditions are considered to have a "small difference" ($0.5 < \Delta E < 1.5$).

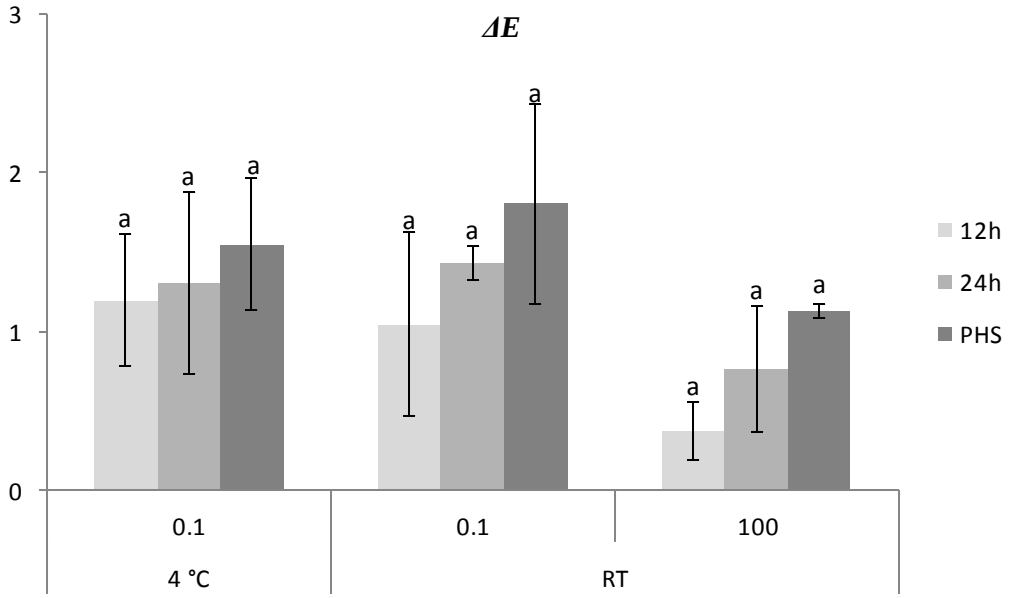


Figure 18 – Total colour change ($\Delta E$) values in the stored whey cheese used in an industrial equipment experiment, stored at different pressure (0.1 and 100 MPa) levels, temperature (4 and ≈21 ºC) and storage period, 12 and 24 hours. PHS stands for post-hyperbaric storage. Different letters indicate significant differences ($p < 0.05$) between the different conditions.

## 4. General analysis

Considering the physicochemical analyses performed in the large scale experiment, it was observed that hyperbaric storage retained the pH, water activity and MDA level, similar to the initial ones, maintaining also, the colour properties of *Requeijão* used in this study.

# CHAPTER V – CONCLUSIONS

This is the first study that uses pressure as a preservation technique at and above room temperatures for a dairy food storage. This study showed that, at a laboratorial scale, storage of *Requeijão* up to 8 hours, using 100 MPa at room temperature, was able to at least maintain the microbial load (TAM, ENT, LAB and YM), and in some cases microbial inactivation occurred. Treatments at 150 MPa, showed a significant lower microbial load comparatively to refrigeration and, in general, an inactivation effect occurred for all conditions at this pressure and room temperature. Above room temperature, at 30 ºC, hyperbaric storage at 100 and 150 MPa was able to achieve better results when compared with refrigeration, reaching microbial inactivation for all microorganisms. Even at 37 ºC, ENT optimal growth temperature, 100 MPa was able to restrain its microbial growth, inactivating TAM and reducing LAB and YM counts to values below the detection limit. The hyperbaric storage effect on the microbial load was considerably more evident as pressure and the storage period increases. The physicochemical parameters analysed, showed that overall, for the hyperbaric storage conditions, pressure maintained whey cheese colour, pH, MDA and water activity values similar to refrigeration.

The results obtained at the experiment in an industrial equipment, were similar to those obtained at the laboratorial scale experiments. YM growth was inhibited at 8 hours of storage at 100 MPa in the laboratorial experiments, while, by increasing the storage period in the industrial equipment to, 12 and 24 hours, YM were inactivated to counts below the detection limit. On the other hand, ENT growth was inhibited for 12 and 24 hours of storage. Hyperbaric storage was able to significantly perform better than refrigeration using 100 MPa under room temperature.

Hyperbaric storage at and above room temperatures seems to be a good and very promising food preservation methodology that would allow ensuring food safety, with energy being only required for the compression/decompression phases and so without basically no energetic costs.

# CHAPTER VI - FUTURE WORK

Consumers demand for safer products with high quality with minimal changes in the nutritional value of foods is of major importance in food processing and storage. On the other hand industries try to reduce the cost production and losses during storage to maximize their profit. Despite the high initial investment that the industries need to made, HHP as well as hyperbaric storage seem to respond in a positive way to both those demands. In this work, hyperbaric storage of a highly perishable food was obtained with success, comparatively to refrigeration, under mild pressure, from 4 to 24 hours.

However, the information regarding hyperbaric storage at and above room temperature is rather scarce, and more studies need to be conducted, to understand for example, the impact of longer storage periods on *Requeijão* shape, firmness, whey loss and on its chewiness.

Considering our results pressures at and above 150 MPa are enough to cause microbial inactivation for the majority of the microorganisms studied in this work, it would be interesting to find the minimal pressure where microbial inhibition could occur.

The effect of mild pressure in possible pathogenic microorganisms, like *Bacillus cereus* and *Listeria monocytogenes* should also be studied, as well as the enzymatic behaviour under low pressures.

If possible, to a better knowledge of hyperbaric pressure effect on *Requeijão*, other physicochemical analysis can be performed, like the lactic acid, lactose, protein and free fatty acids content.

The presence of bioactive peptides has been described in *Requeijão* (Appendix D), and its potential effect in ACE-inhibition activity is very interesting, as well as the possible effect of pressure in this bioactive peptides and its potential acquired activity. *Requeijão* has also been pointed as good matrix for probiotics (Appendix E), and so, the microbial inhibitor effect of hyperbaric storage observed in this work should be accessed, for these pointed health benefits.

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# APPENDICES

## Appendix A – Industrial equipment experiment physicochemical and colour results with statistical analysis

Table 10 - Initial physicochemical and colour properties of *Requeijão* used in the industrial equipment experiment.

| Physicochemical properties | Mean ±SD* |
|---|---|
| **pH** | 6.47 ± 0.02 |
| **Water activity** | 0.982 ± 0.01 |
| **Colour** | |
| ***L**** | 90.72 ± 0.22 |
| ***a**** | -2.73 ± 0.12 |
| ***b**** | 11.02 ± 0.08 |
| **Lipid oxidation (mg MDA/g)** | 0.191 ± 0.013 |

Table 11 – Physicochemical and colour analysis of the different storage conditions, at the experiment in an industrial equipment. RT means room temperature (≈21 ºC) and PHS means post-hyperbaric storage. Different letters (a-d) represent statistically differences ( $p < 0.05$) between storage conditions.

| Parameters | Storage period | Temperature | Pressure | |
|---|---|---|---|---|
| | | | 0.1 MPa | 100 MPa |
| pH | 12 | 4 ºC | 6.74 ± 0.01 b | * |
| | | RT | 6.68 ± 0.02 b | 6.65 ± 0.04 b |
| | 24 | 4 ºC | 6.20 ± 0.42 b | * |
| | | RT | 6.73 ± 0.01b | 6.72 ± 0.01 b |
| | PHS | 4 ºC | 6.06 ± 0.14 ab | * |
| | | RT | 5.30 ± 0.09 a | 6.62 ± 0.01 b |
| Water activity | 12 | 4 ºC | 0.981 ± 0.001 a | * |
| | | RT | 0.982 ± 0.001 ab | 0.982 ± 0.001 abc |
| | 24 | 4 ºC | 0.986 ± 0.001 cd | * |
| | | RT | 0.986 ± 0.001 d | 0.986 ± 0.001 cd |
| | PHS | 4 ºC | 0.983 ± 0.001 abcd | * |
| | | RT | 0.983 ± 0.001 abc | 0.985 ± 0.001 bcd |
| Lipid oxidation (mg MDA/g) | 12 | 4 ºC | 0.137 ± 0.024 a | * |
| | | RT | 0.196 ± 0.024 a | 0.135 ± 0.002 a |
| | 24 | 4 ºC | 0.149 ± 0.035 a | * |
| | | RT | 0.159 ± 0.011 a | 0.085 ± 0.001 a |
| | PHS | 4 ºC | 0.176 ± 0.054 a | * |
| | | RT | 0.148 ± 0.035 a | 0.088 ± 0.034 a |
| Colour | 12 | 4 ºC | 91.28 ± 1.04 a | * |
| | | RT | 91.62 ± 0.46 a | 90.75 ± 0.21 a |
| *L** | 24 | 4 ºC | 91.81 ± 0.40 a | * |
| | | RT | 90.13 ± 0.07 a | 91.45 ± 0.42 a |
| | PHS | 4 ºC | 92.25 ± 0.43 a | * |
| | | RT | 92.23 ± 0.89 a | 91.66 ± 0.13 a |
| *a** | 12 | 4 ºC | -2.73 ± 0.17 a | * |
| | | RT | -2.59 ± 0.16 a | -2.59 ± 0.06 a |
| | 24 | 4 ºC | -2.27 ± 0.12 a | * |
| | | RT | -2.61 ± 0.09 a | -2.53 ± 0.10 a |
| | PHS | 4 ºC | -2.59 ± 0,16 a | * |
| | | RT | -2.47 ± 0.18 a | -2.59 ± 0.02 a |
| *b** | 12 | 4 ºC | 10.70 ± 0.25 a | * |
| | | RT | 11.49 ± 0.39 a | 10.80 ± 0.28 a |
| | 24 | 4 ºC | 10.51 ± 0.55 a | * |
| | | RT | 12.32 ± 0.09 a | 10.92 ± 0.07 a |
| | PHS | 4 ºC | 10.92 ± 0.13 a | * |
| | | RT | 10.63 ± 0.62 a | 11.50 ± 0.36 a |
| *ΔE* | 12 | 4 ºC | 1.20 ± 0.41 a | * |
| | | RT | 1.05 ± 0.58 a | 0.37 ± 0.18 a |
| | 24 | 4 ºC | 1.31 ± 0.57 a | * |
| | | RT | 1.43 ± 0.11 a | 0.76 ± 0.40 a |
| | PHS | 4 ºC | 1.55 ± 0.42 a | * |
| | | RT | 1.80 ± 0.63 a | 1.13 ± 0.04 a |

* – Experiments were not carried out in these conditions.

## Appendix B - Standard curves of malondialdehyde

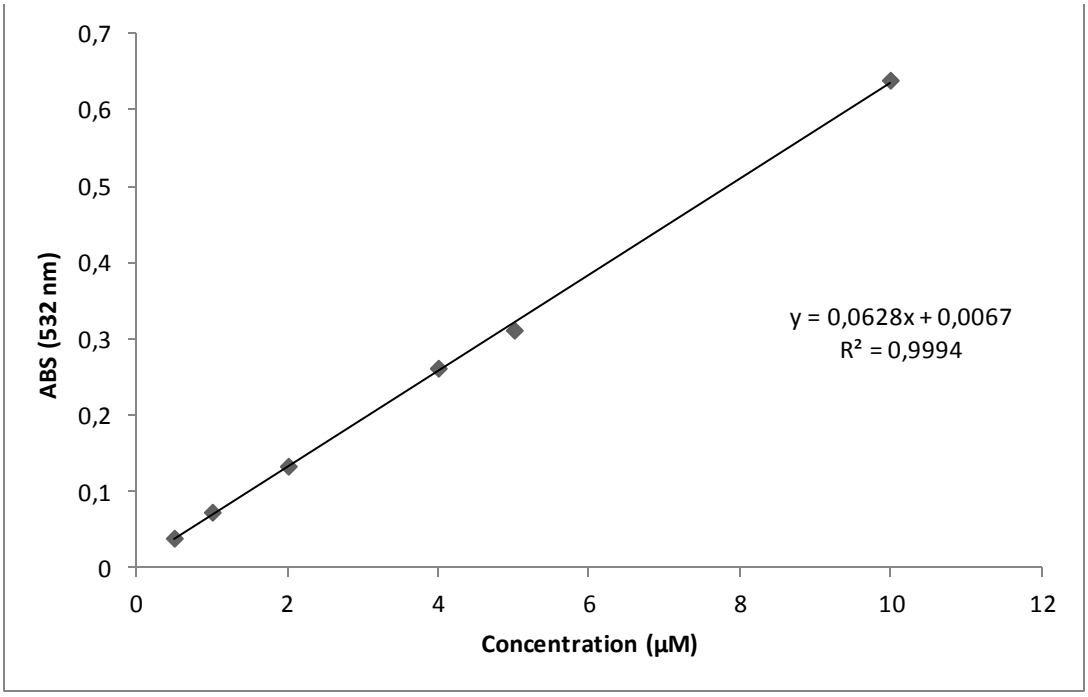


**Figure 19** - Standard curve of malondialdehyde content by TBARS method used in the experiment.

## Appendix C – Possible bioactive peptides in *Requeijão* as a probiotic matrix

### Bioactive Peptides

Bioactive peptides are of particular interest in food science and nutrition, because they can play several physiological roles. Important therapeutic properties have been claimed for whey proteins, including antioxidant activity, anticarcinogenic effects, immunomodulation, passive immunity, opioid functions, antithrombotic role, mineral-carrying capacity, antibacterial, antimicrobial and antiviral effects, toxin-binding, cell growth promoter, platelet binding, and anti-inflammatory and antihypertensive actions (Madureira et al., 2010). Bioactive peptides are originating from food protein including those in milk and whey, eggs, soy, maize, fish, meat and can be released through enzyme-mediated hydrolysis (Silva and Malcata, 2005). Proteins present in whey cheeses may go through enzyme hydrolysis present is the gastrointestinal tract.

In Portugal extracts of *Cynara cardunculus* flowers are used as coagulant in cheesemaking. *C. cardunculus* milk-clotting activity is caused by 2 aspartic proteases, cardosins A and B, which resemble chymosin and pepsin, respectively, in activity and specificity. Cardosin A is a proteolytic enzyme that is specific toward the Phe105-Met106 bond of κ-CN, whereas cardosin B is a nonspecific, highly proteolytic enzyme. Part of these proteases end up in cheese whey, and act on some soluble proteins present there, producing potential bioactive peptides (Pintado et al., 2001).

Hypertension is a cardiovascular disease, characterized for elevated blood pressure, which constitutes a dominant health worldwide problem. Angiotensin I-converting enzyme (ACE), occurs in many tissues and biological fluids, playing a crucial role in blood pressure regulation, because it promotes angiotensin I conversion to angiotensin II, a potent vasoconstrictor and inactivates the vasodilator bradykinin (Figure 1). Tavares and Xavier Malcata (2012) studied the antihypertensive properties of several oligopeptides released from glycomacropeptide (originated in κ-casein) and α-lactalbumin and one surprisingly from β-lactoglobulin, by *C. cardunculus* proteases. DKVGINYW, DAQSAPLRVY and KGYGGVSLPEW oligopeptides showed IC50 values of 25.2 ± 1.0, 13.0 ± 1.0 and 0.8 ± 0.1 lg/ml, respectively. All of them underwent simulated gastrointestinal digestion, however the activities of the latter two were not significantly affected, despite undergoing partial hydrolysis.

Antimicrobial and immunostimulatory activities of four peptide fragments [f(15–20), f(25–40), f(78–83), f(92–100)] released from β-Lg proteolytic digestion by trypsin was examined by Biziulevičius et al. (2006). These peptides showed bactericidal activity against Gram-positive bacteria. El-Zahar et al. (2004) found a peptide hydrolyzed from ovine β-La and α-Lg capable to inhibit the growth of *Escherichia coli* HB101, *Bacillus subtilis* Cip5262 and *Staphylococcus aureus* 9973 in a dose dependent manner.

Three peptides with the sequences KDQDK f(112–116), TAQVTSTEV f(163–171) and QVTSTEV f(165–171), resulted from ovine CMP hydrolysing with Trypsin, completely inhibited thrombin-induced human platelet aggregation (Clare and Swaisgood, 2000). According to Manso et al. (2002) bovine, ovine and caprine CMPs, hydrolyzed with Trypsin, are inhibitors of human platelet aggregation, having the ovine CMP hydrolyzate the strongest effect.

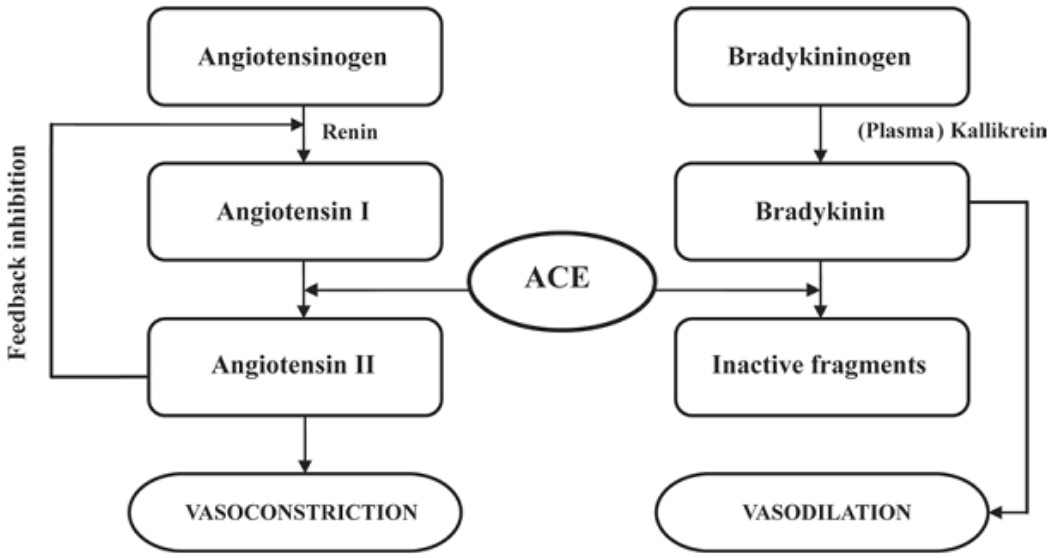


**Figure 20 -** Schematic representation of renin-angiotensin system, demonstrating the role of Angiotensin-I-converting enzyme (ACE) in angiotensin I and bradykinin. Adapted from Madureira et al. (2010)

## Appendix D - *Requeijão* as a probiotic matrix

### Requeijão as a good probiotic matrix

Probiotics typically belong to *Lactobacillus* and *Bifidobacterium* genera and several therapeutic properties are attributed to them: including antimicrobial activity, hypocholesterolaemic activity, improved lactose utilisation, maintenance of gastrointestinal balance and anticarcinogenic activity (Madureira et al., 2005a). Bacterial strains need to remain viable, to sufficiently high numbers, from inoculation, throughout processing and storage, pass through the gastrointestinal tract, survive to gastric juice, digestive enzymes and bile salts and adhere and colonise the intestine, to be considered probiotics. *Requeijão* proved to be a good matrix for probiotic bacteria incorporation and survival. Madureira et al. (2005b) studied if it was possible to incorporate probiotic bacterial strains, *Lactobacillus* and *Bifidobacterium* genera, in whey cheese with different matrices (plain or added with sugar or salt after protein coagulation). Madureira et al. (2005b) found that it was possible to maintain the viability of all bacteria tested in *Requeijão* through 28 days at refrigerated storage within acceptable limits. *L. acidophilus* and *L. paracasei ssp. Paracasei* were able to increase viable number during storage time ($10^7$ CFU/g). *Requeijão* whey proteins provide bifidobacteria with a nutritious medium bearing a low redox potential. This lower redox potential is contributed by whey proteins that are rich in sulphur-containing amino acid residues. The most important factor that influence the viability profiles of bacterial strains was matrix type, being plain and sugar-added matrices better for microbial growth than the salt-added one, in the case of *B. animalis* strain Bo, *L. acidophilus* strain Ki, and *B. animalis* strain BLC-1.

The capability to transit through the gastrointestinal tract during digestion is a major challenge for bacterial strains, because they are exposed to gastric juice, digestive enzymes and bile salts. Madureira et al. (2005a) tested different probiotics strains that were incorporated in *Requeijão* and survived through the exposure period with combination of artificial gastric juice and bile salts. *L. brevis*, *B. animalis* Bo and *B. animalis* Bb-12 were the best viable strains that survived through the artificial period of exposure. However *Lactobacillus paracasei ssp. paracasei* LCS-1 and *B. animalis* BLC-1 resisted less to artificial gastric juice plus bile salts. Probiotic resistance to

passage through gastrointestinal tract, when incorporated in *Requeijão*, was strain-dependent.