Universidade de Aveiro
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RICARDO VALENTE DUARTE

ARMAZENAMENTO SOB PRESSÃO DE PRODUTOS LÁCTEOS EM ALTERNATIVA À REFRIGERAÇÃO

STORAGE UNDER PRESSURE OF DAIRY FOODS AS AN ALTERNATIVE TO REFRIGERATION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica do Professor Doutor Jorge Manuel Alexandre Saraiva, Professor Associado do Departamento de Química da Universidade de Aveiro, da Professora Doutora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro e da Professora Doutora Ana Maria Pereira Gomes, Professora Associada da Universidade Católica Portuguesa

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Dedico este trabalho aos meus pais, irmãos e à Gabriela.

o júri

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Doutor António Augusto Martins de Oliveira Soares Vicente
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Doutora Paula Cristina Maia Teixeira,
Professora Associada com Agregação, Universidade Católica Portuguesa

Doutor Jorge Manuel Alexandre Saraiva
Professor Associado, Universidade de Aveiro (orientador)

Doutora Maria Filipa Vinagre Marques da Silva Oliveira
Professora Auxiliar, Universidade de Lisboa

Doutora Susana Maria e Almeida Cardoso
Investigadora Doutorada, Universidade de Aveiro

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palavras-chave

Armazenamento hiperbárico, armazenamento sob pressão, leite cru, queijo fresco, tempo de vida útil, conservação, refrigeração, microbiologia, atividade enzimática, fisico-química, oxidação lipídica, perfil proteico, aminoácidos livres, perfil de ácidos gordos, compostos voláteis, viscosidade, textura.

resumo

O armazenamento hiperbárico (AH) à temperatura ambiente (TA) tem despoletado recentemente grande interesse científico, com um número crescente de publicações. O AH à TA surge como uma possível metodologia de conservação alimentar alternativa à refrigeração (RF), com menor gasto energético e pegada de carbono, dado que não é necessário utilizar energia de forma constante para manter a temperatura durante o AH, resultando também no aumento do prazo de validade.

Neste trabalho a viabilidade do armazenamento AH/TA foi avaliada usando leite cru de vaca e queijo fresco de vaca e de cabra e comparado com o armazenamento à pressão atmosférica (PA) sob TA e RF, durante 60 dias. Observou-se que para o leite, com carga microbiana dentro dos limites legais, que AH/TA permitiu a conservação de leite de vaca cru, levando a uma redução da carga microbiana naturalmente presente no leite (≥62 MPa), bem como nos microrganismos inoculados *Escherichia coli*, *Salmonella senftenberg* e *Listeria innocua* (≥50 MPa) e de endósporos de *Bacillus subtilis,* sendo a redução mais rápida sob 75 e 100 MPa. Numa segunda experiência com leite cru, com uma carga microbiana acima do limite legal, AH/TA resultou numa redução gradual da sua carga microbiana (≥75 MPa), bem como uma maior estabilidade quando o leite que foi armazenado sob AH/TA foi subsequentemente colocado a PA/RF. O AH/TA de leite, permitiu ainda manter a qualidade dos diversos parâmetros estudados, como os físico-químicos, enzimáticos, reológicos, a oxidação lípica, proteína total e perfil de ácidos gordos e compostos voláteis, semelhantes aos originais antes do armazenamento, tendo um desempenho bastante superior que PA/RF, para períodos de armazenamento consideravelmente mais longos. Contudo durante AH/TA, deverá ter ocorrido atividade proteolítica superior ao longo do armazenamento, resultando num aumento de proteína solúvel e de aminoácidos livres ao fim dos 60 dias.

Para os dois tipos de queijos frescos (produzido com leite pasteurizado de vaca ou de cabra) sob AH/TA, observou-se uma redução da carga microbiana ao longo do armazenamento, especialmente a 75 e 100 MPa. Mais uma vez, a maior parte dos parâmetros físico-químicos mantiveram-se ao longo do AH, observando-se inicialmente uma compressão da matriz do queijo, resultando num aumento da dureza e do soro expelido e uma diminuição do teor de humidade, que ao longo do armazenamento se foi revertendo, aproximando-se dos valores iniciais. A condição de armazenamento 100MPa/TA permitiu uma redução da taxa oxidação lipídica, evitou a formação de compostos voláteis indesejáveis, mantendo o perfil de ácidos gordos e do valor de proteína total. Semelhante ao observado no leite, deverá ter ocorrido um efeito proteolítico superior, resultando num aumento de aminoácidos livres nos períodos de armazenamento mais longos.

Apesar de algumas diferenças terem sido observadas, o AH a TA permitiu conservar os produtos lácteos estudados por um período consideravelmente mais longo (pelo menos até 60 dias) comparativamente com AP/RF, resultando num possível aumento do tempo de vida útil e de segurança microbiológica destes produtos.

keywords

Hyperbaric storage, storage under pressure, raw milk, fresh cheese, shelf-life, conservation, refrigeration, microbiology, enzymatic activity, physicochemical, lipid oxidation, protein profile, free amino acids, fatty acids profile, volatile compounds, viscosity, texture.

abstract

Hyperbaric storage (HS) at room temperature (RT) has recently sparked the scientific interest, with an increasing number of publications. HS at RT appears to be a possible alternative food preservation methodology to refrigeration (RF), with lower energy consumption and lower carbon footprint, since it is unnecessary to use energy constantly to maintain the temperature during HS, resulting also in an increase of foods shelf-life.

In this work, the feasibility of HS/RT was evaluated using cow's raw milk and cow's and goat's fresh cheeses and compared with storage at atmospheric pressure (AP) at RT and at RF for 60 days.

It was observed that for milk, with a microbial load within the legal limits, that HS/RT allowed the preservation of raw cow's milk, leading to a reduction in the microbial load naturally present in the milk (≥62 MPa), as well as in inoculated microorganisms, *Escherichia coli*, *Salmonella senftenberg* and *Listeria innocua* (≥50 MPa), and *Bacillus subtilis* endospores, with the fastest reductions being observed under 75 and 100 MPa. In a second set of experiments with raw milk, with a microbial load above the legal limits, HS/RT resulted in a gradual reduction of its microbial load (≥75 MPa), as well as in a greater stability after HS, when the milk stored previously at HS/RT was then subsequently placed at AP/RF. HS/RT of milk also allowed to maintain the quality of the studied parameters, such as physicochemical, enzymatic, rheological, lipid oxidation, total protein, fatty acids, and volatile organic compounds profile, similar to the ones prior storage, outperforming AP/RF for considerably longer storage periods. However, in HS/RT samples, a higher proteolytic activity may have occurred during storage, resulting in an increase in soluble protein and free amino acids after 60 days of storage.

For the two types of fresh cheeses (made with pasteurized cow's or goat's milk) stored under HS/RT, a reduction in the microbial load during storage was observed, especially under 75 and 100 MPa. Once again, most of the physicochemical parameters were maintained throughout HS, initially being observed a compression effect of the cheeses matrix, resulting in an increase in hardness and whey loss and a decrease in the moisture content, reversing to values similar to the initial ones throughout the storage. Storage at 100 MPa/RT allowed a reduction in lipid oxidation rate, avoided the formation of undesirable volatile organic compounds and maintained better the fatty acid profile and total protein. Similarly, to raw milk, a higher proteolytic effect seemed to take place, resulting in an increase in free amino acids, especially in the longer storage periods.

Despite some differences observed during HS at variable RT, it allowed the dairy products studied to be preserved for a considerably longer period (at least up to 60 days) comparatively to AP/RF, resulting in a possible extended shelf-life and microbial safety of these products.

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List of Abbreviations

AP	Atmospheric pressure
ALP	Alkaline phosphatase
COL	Coliform bacteria
ENT	Enterobacteriaceae
FA	Fatty acids
FAA	Free amino acids
FC	Fresh cheese
FFA	Free fatty acids
HP	High pressure
HPP	High pressure processing
HS	Hyperbaric storage
LAB	Lactic acid bacteria
LPO	Lactoperoxidase
LT	Low temperature
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acids
PHS	Post-hyperbaric storage
PSY	Psychrophilic bacteria
PUFA	Polyunsaturated fatty acids
RF	Refrigeration
RT	Room temperature
SFA	Saturated fatty acids
SP	Soluble protein
ST	Sub-zero temperature
TA	Titratable acidity
TAM	Total aerobic mesophiles
TBARS	Thiobarbituric acid-reactive substances
TEL	Total endospores load
TPA	Textural profile analysis
VOC	Volatile organic compounds
YM	Yeasts and moulds

List of Publications

Papers related to the PhD work submitted in peer reviewed journals

1- **Duarte, R. V.**, Gomes, A. M., Delgadillo, I., Saraiva, J. A. (2021). Hyperbaric storage at room temperature with several short intermittent interruption periods at atmospheric pressure results in similar microbial growth inhibition and inactivation as without interruption. Submitted as a short communication.

2- Duarte, R. V., Pinto, C. A., Gomes, A. M., Delgadillo, I., Saraiva, J. A. (2021). A microbiological perspective of raw milk preserved at room temperature under hyperbaric storage comparatively to refrigeration. Submitted.

3- **Duarte, R. V.**, Casal, S., Lopes-da-Silva, J. A., Gomes, A. M., Delgadillo, I., Saraiva, J. A. (2021). Nutritional, physicochemical, and endogenous enzymes assessment of raw milk preserved under hyperbaric storage at variable room temperature. Submitted.

4- **Duarte, R. V.**, Gomes, A. M., Delgadillo, I., Barba, F. J., Saraiva, J. A. (2021). Hyperbaric storage fresh cheese extended shelf-life at room temperature compared to refrigeration. Submitted.

5- **Duarte, R. V.**, Casal, S., Lopes-da-Silva, J. A., Gomes, A. M., Delgadillo, I., Saraiva, J. A. (2021). Evaluation of hyperbaric storage at room temperature for preservation of two fresh cheeses on nutritional, textural and physicochemical quality parameters versus refrigeration. Submitted.

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1- Santos, M. D., Fidalgo, L. G., Pinto, C. A., **Duarte, R. V**., Lemos, A. T., Delgadillo, I., & Saraiva, J. A. (2020). Hyperbaric storage at room like temperatures as a possible alternative to refrigeration: evolution and recent advances. *Critical Reviews in Food Science and Nutrition*, 12. doi:10.1080/10408398.2020.1770687.

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1- Santos, M., Inácio, R., Fidalgo, L., Queirós, R., Moreira, S., **Duarte**, **R.**, Gomes, A., Ivonne Delgadillo, I. and Saraiva, J. (2019). Chapter 4: "*Impact of High-Pressure Processing on Food Quality*" in Effect of Emerging Processing Methods on the Food Quality, Springer, Switzerland: Editor Shahin Roohinejad.

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1 – **Duarte, R. V.**, Casal, S., Gomes, A. M., Delgadillo, I., Saraiva, J. A. (2021). Fresh cheese shelf-life extension at variable room temperature comparatively to refrigeration. Microbiotec 2021, (November, 23-26, 2021) Nova University, Lisbon, Portugal.

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CHAPTER 1

General Introduction, Objectives and Thesis Structure

1.1 General introduction and objectives

Milk and dairy products have a rich nutrient profile, being an important part of the human diet, contributing to a healthy growth and development, being available in a great variety of products, widely consumed all over the world. However, generally these products are characterized by a near neutral pH, high water activity and high nutrient composition, creating a propitious environment for microbial proliferation and spoilage. This is especially true for raw milk and fresh cheese, among other dairy products, which require refrigerated storage in order to slowdown microbial spoilage, as well as minimize other quality losses, resulting for instance from lipolysis, proteolysis, oxidation, and off-flavour formation, among others, even so, resulting in a rather short shelf-life.

Recently, a new food preservation methodology has been investigated due to its potentiality to substitute conventional refrigeration, denominated Hyperbaric Storage (HS). The first studies evaluated HS capacity in several foods at refrigerated temperatures, with positive outcomes, while more recently, several food products, from juices, meats, ready-to-eat meals, and to fish products been evaluated under HS at and above room temperature (RT). All studies pointed to shelf-life extension under HS, mainly due the inhibition, and at higher pressures, inactivation of microbial population, with generally minimal impairment of the physicochemical properties, although most of the studies were performed during short storage periods. Besides the significant shelf-life extension achievable under HS/RT, it can also contribute to a more sustainable preservation methodology, as no energy is applied continuously during storage to maintain the temperature, contrary to refrigeration, being energy only used during the pressurization/depressurization of the vessel, where food is stored.

In this work, the feasibility of HS at RT was studied regarding the storage of cow's raw milk and two fresh cheeses (from cow's and goat's milk) and compared with storage at atmospheric pressure (AP) at RT and at refrigeration (4 ºC, RF). In order to assess HS potential, several parameters were evaluated, namely microbiological (endogenous and inoculated), physicochemical, rheological, textural, enzymatic, and nutritional, being also compared with the initial values before storage.

1.2 Thesis structure

In order to achieve the proposed objectives, this thesis was divided into two main sections, organized by the respective dairy product, milk or fresh cheese, as it can be seen in **Figure 1.1**, organized by the submitted scientific papers. Initially an overview of the studies regarding hyperbaric storage, milk and fresh cheese composition and preservation, as well as the possible effects of high pressure on the constituents and properties of these dairy products available in the literature were summarized and described in **Chapter 2**.

To optimize HS studies, generally a high-pressure vessel is filled with samples, with sampling taking place over time, requiring several compression and decompression (C/D) cycles. In order to evaluate the possible effects of several C/D cycles on microbial behaviour, a preliminary study was performed (**Chapter 3**). In this chapter raw milk endogenous microbiota (total aerobic mesophiles and Enterobacteriaceae) behaviour was monitored throughout HS/RT (75 MPa) for 31 days, in three different conditions. In Condition 1, samples were only (C/D) when a sample was removed from the vessel for microbiological evaluation at the specific sampling period (total of 5 C/D cycles), and in Conditions 2 and 3, samples were intentionally C/D once (total of 31 C/D cycles) or three times (total of 93 C/D cycles) a day, respectively.

In **Chapter 4**, raw milk was stored under a wide range of HS conditions and globally evaluated in terms of microbiological evolution during storage. This chapter was divided in several sets of experiments; in the first one, raw milk with a microbiota level within the ones legally acceptable for further pasteurization was stored under 50, 62, 75 and 100 MPa at RT, and the endogenous microbiota composition was evaluated during 60 days; on the second set of experiments, in order to simulate a worst case-scenario, raw milk with a higher microbial spoilage, was evaluated regarding the endogenous microbiota evolution during 130 days of storage (50, 75 and 100 MPa at RT); a post-hyperbaric storage experiment was also conducted with samples from that experiment being stored under refrigeration (RF) at atmospheric pressure (AP). The effect of HS (50, 75 and 100 MPa at RT) on inoculated pathogenic surrogate vegetative bacteria (*Escherichia coli* and *Listeria innocua*), pathogenic *Salmonella senftenberg* and on a bacterial spore (*Bacillus subtilis* endospores) microorganisms in raw milk were also assessed. Overall D_p- and Z_p-values were calculated, when possible, for a particular microorganism or microbiological group under HS conditions.

Afterwards, a further evaluation on the physicochemical, nutritional parameters, and of milk endogenous enzymes was carried out in raw milk (from the first set of experiments) stored under HS/RT for 60 days and compared with the ones under AP/RT and RF (**Chapter 5)**.

In **Chapter 6**, two kinds of fresh cheese, made with pasteurized cow's or goat's milk, were stored under HS conditions (50, 75 and 100 MPa at RT) during 60 days, and evaluated microbiologically (endogenous microbiota), physiochemically (whey and cheese pH, whey loss, moisture content and lipid oxidation) and in the colour properties (L^*, a^*, b^* and total colour change - ΔE^*).

In the next chapter, (**Chapter 7**), several parameters regarding the textural profile and nutritional and quality parameters (total protein, free amino acids, protein digestibility, fatty acids and volatile organic profile) of fresh cheeses were evaluated under the previous HS conditions studied, in order to attain a better assessment of the best storage conditions and possible achievable shelf-life.

Lastly, in **Chapter 8**, the main results from this work are discussed, and suggestions regarding possibly future work are presented.

Chapter 2

Hyperbaric storage / Dairy Products / Effect of high pressure in dairy products

Chapter 3

Evaluation of the effect of several compression and decompression cycles during hyperbaric storage

Storage Conditions:
- 75 MPa at RT
- 0, 1 and 3 C/D per day
- 31 days

Microbiota evaluated:
- Total aerobic mesophiles
- Enterobacteriaceae

Chapter 4

Cow's raw milk microbial evaluation under hyperbaric storage at room temperature

Storage Conditions:
- HS/RT (50, 62, 75 and 100 MPa)
- 60 days
- AP/RF
- AP/RT

Raw cow's milk

Microbiota evaluated:
- Endogenous microbiota
- Inoculated microorganisms:

Pathogens surrogates: *L. innocua* and *E. coli and an endospore* (*B. subtilis*)
Pathogens: *S. senftenberg*

Chapter 5

Physicochemical, nutritional and endogenous enzymatic evaluation of raw milk under hyperbaric storage at room temperature

Storage Conditions:
- HS/RT (50, 75 and 100 MPa)
- 60 days
- AP/RF
- AP/RT

Parameters evaluated:
- pH, titratable acidity, total solids, density, lipid oxidation, colour
- Alkaline phosphatase and lactoperoxidase
- Viscosity
- Volatile organic profile
- Fatty acids profile
- Total and soluble protein, free amino acids

Chapter 6

Microbial and physicochemical evaluation of hyperbaric storage at room temperature of fresh cheeses

Storage Conditions:
- HS/RT (50, 75 and 100 MPa)
- 60 days
- AP/RF
- AP/RT

Cow's and goat's fresh cheese

Parameters evaluated:
- Endogenous microbiota
- pH of cheese and whey
- Whey loss and moisture content
- Colour
- Lipid oxidation

Chapter 7

Evaluation of hyperbaric storage at room temperature in nutritional, textural and physicochemical properties of fresh cheeses

Storage Conditions:
- HS/RT (50, 75 and 100 MPa)
- 60 days
- AP/RF
- AP/RT

Parameters evaluated:
- Textural profile analysis
- Total protein, free amino acids, digestibility
- Fatty acids profile
- Volatile organic compounds

Chapter 8

Concluding Remarks and Future Work

Figure 1.1 - Thesis structure overview.

CHAPTER 2

Bibliographic Review

2.1 Hyperbaric storage

Hyperbaric storage (HS) is a novel food preservation methodology that has been receiving more attention by the scientific community recently. HS relies on the use of constant moderate pressure throughout the storage, however at a considerably lower level (25-150 MPa) when compared to high pressure processing (up to 600 MPa), and has been investigated at sub-zero (ST), refrigerated (RF), low (LT) and at and above room temperature (RT) (Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). Detailed information of the studies available regarding HS at the different temperatures can be assessed in **Table 2.1-2.6**.

2.1.1 Discovery and first studies on hyperbaric storage

After the recovery of a sunken submersible, sandwiches, bouillon, and apples that were at 1540 m (~15 MPa) at 3-4 ºC for a year, upon taken to the surface, were apparently in consumable conditions, retaining most of its organoleptic characteristics, as texture, taste, odour, without signs of microbial spoilage, and with reduced enzymatic activity (Jannasch, Eimhjellen, Wirsen, & Farmanfarmalan, 1971). Jannasch et al. (1971) conducted a follow up study, mimicking similar conditions of those recovered foods (at a higher pressure, 53 MPa), observing a great decreasing in decomposition rate of ^{14}C-labeled substrates (acetate, mannitol, amino acids), ranging from 8-666 times slower in HS/RF when compared to same carbon source stored at RF and atmospheric pressure (AP). Also, the same authors, inoculated pure and mixed cultures of mesophilic and psychrophilic strains in a rich substrate (starch, galactose, albumin), reporting microbial growth inhibition during two months under HS/RF, contrary to controls kept at AP/RF (**Table 2.1**).

In order to reduce post-harvest losses of seeds, Mitsuda, Kawai, Yamamoto, and Omura (1971) kept brown, polished and white rice submersed underwater at 8.5-13 ºC, inside waterproof containers for one year. Rice stored at HS/RF presented lower changes in moisture, fatty acids, water soluble nitrogen and acidity, vitamin B_1 and reducing sugars when compared with the ones under AP/RF, with the authors reporting also a higher biological activity of HS/RF rice, with higher germinative capacity, catalase, and peroxidase activities and with lower development of alcohol volatile compounds over prolonged storage. When cooked, HS/RF rice maintained a textural profile and organoleptic score similar to control, evaluated with a texturometer and by a trained panel.

Cod fish and pollock were stored under 24.12 MPa at 1 ºC by Charm, Longmaid, and Carver (1977), being evaluated microbiologically during storage, and organoleptically after cooked. Microbiological analysis reported no significant growth under HS/RF (constant counts around 4.5 log units), with the expert panel evaluating the pollock stored under HS/RF for 12 days as having apparently 6.7 days, and codfish with 21 days as having 8.2 days.

Table 2.1 - Fist hyperbaric storage studies performed at refrigerated temperatures.

Food product	HS conditions	Main results	References
Bouillon, sandwiches, and apples	15/53 MPa 2-10 months 3-4 °C	Recovered foods under pressure presented lower deterioration signs, comparatively to AP/RF. Under HS, slower decomposition rate measured in $_{14}C$-labeled substrates.	Jannasch et al. (1971)
Rice: brown, polished, and white	3 MPa 1 year 8.5-13 °C	Reduction in biochemical parameters monitored, comparatively to storage under AP/RF.	Mitsuda et al. (1971)
Cod fish, pollock	24.12 MPa at 12/21 days 1 ºC	When cooked both food products were still organoleptically acceptable for consumption, opposingly to controls at AP.	Charm et al. (1977)

2.1.2 Hyperbaric storage performed at sub-zero temperatures

The first study regarding HS at sub-zero temperatures (ST) was performed by Charm et al. (1977) on meat and fish products under 22.8 MPa/-3 ºC during 36 days. HS/ST restrained not only enzymatic activity (peroxidase and trypsin), but also inhibited endogenous microbial growth and other inoculated microorganisms (*Bacillus subtilis*, *Clostridium sporogenes* and *Salmonella typhimurium*) throughout the storage (**Table 2.2**). Organoleptically tests of cod fish performed by a trained panel revealed the good preservation state of this product after 36 days under HS/LT, while controls stored at 1 ºC under AP were considered unacceptable for consumption after 9 days.

Deuchi and Hayashi (1990) stored uncooked fruits (strawberries and tomatoes) under 50-200 MPa at -5 and -20 ºC, with these fruits retaining the colour, texture and flavour during 8 days without the characteristic losses in texture resulting from freezing/thawing processes. The same authors (Hayashi & Deuchi, 1991), tested later the possibility to preserve ground beef under HS/ST up to 200 MPa (170/-20 ºC and 195/5 ºC) during 9 days. Meat stored under AP at 5 ºC showed clear signs of microbial spoilage, with increased counts in all studied microorganisms, with a pronounced change in colour and strong odours after 9 days. While storage at 195/5 ºC and 170/-20 ºC resulted in microbial growth inhibition and inactivation, especially for the last storage condition, with yeasts completely inactivated after

9 days, retaining the texture, colour and reduced drip loss. HS/ST also retarded the formation of volatile nitrogen compounds, and reduced catalase, β-amylase, cathepsin and lactate dehydrogenase activities.

In the following years a fish and meat product were also stored under HS/ST (110 MPa/-8 and 170 MPa/-15 ºC) by Ooide et al. (1994) during 50 days. Under HS/ST, carp and chicken meat showed better signs of preservation, without extensive protein denaturation and similar texture with the ones stored under AP/RF and AP/-15 ºC, although some enzyme activity was noticeable in foods stored at HS/ST. However, enzymatic degradation of nucleic acid-related substances (ATP, ADP, AMP, and IMP) was slightly slower under HS/ST when compared to storage under AP/RF, but significantly reduced at AP/-15 ºC.

The feasibility of HS/ST was applied to fruits and several fish and meat products, in order to prevents damage caused by freezing/thawing processes, while also successfully inhibiting microbial growth and reducing enzymatic activity, which could be the main factor limiting the products quality for longer storage periods. Still, HS/ST appears to be a potentially useful methodology to prolong the shelf-life of certain foods under these conditions.

Table 2.2 - Main results of hyperbaric storage at sub-zero temperatures applied to several food products.

Food product	HS conditions	Main results	References
Cod fish, beef and chicken	22.8 MPa at 36 days -3 ºC	Foods under HS/ST were stable during storage, with reduced enzymatic (peroxidase and trypsin) and microbial activity.	Charm et al. (1977)
Strawberries and tomatoes	50-200 MPa 8 days -20/-5 ºC	Fruits retained colour, texture and flavour during 8 days at HS/ST.	Deuchi & Hayashi (1990)
Beef	170/195 MPa 9 days -20/5 ºC	Reductions observed regarding drip loss, and no microbial growth development. Activity inhibition of several studied enzymes.	Hayashi & Deuchi (1991)
Muscle of carp and chicken	110/170 MPa 50 days -15 to -8 ºC	Most of the parameters were stable under HS/ST, although at AP/-15 ºC enzymes were greater inhibited comparatively to HS/ST, where enzymes activity was slightly reduced.	Ooide et al. (1994)

2.1.3 Hyperbaric Storage at room and low temperature performed in several food products

2.1.3.1 Fruit juices

In the past decade, the interest in HS re-emerged with special interest at RT, evaluated initially in fruit juices as case studies, from more acid (strawberry juice) to low acidity juices, and so more perishable like watermelon and melon juice, with a more complete assessment on HS/RT feasibility, as detailed in **Table 2.3**.

An initial study was performed in strawberry juice by Segovia-Bravo et al. (2012), under HS (25, 100 or 220 MPa) at 20 °C for 15 days and compared to raw and thermally pasteurized juices kept at AP/RF. The authors observed that the combination of HS/RT with the low pH of strawberry juice, restrained microbial growth even under 25 MPa, while with higher pressures microbial inactivation was achieved, without significant variations for pH, titratable acidity, total soluble solids, browning degree and cloudiness, attenuating also viscosity losses during storage. A post-hyperbaric storage (PHS) was also carried out, with samples that were under HS/RT conditions after 15 days, been then placed at AP/RF for another 15 days. After this additional period at AP/RF, no detectable counts were observed in all microbiological groups, with no considerable colour alteration, while a greater decay in viscosity was reported. However, in general the authors concluded that raw strawberry juice was highly sable during and after HS/RT.

In another study conducted by the same research group in strawberry juice, Bermejo-Prada, Segovia-Bravo, Guignon, and Otero (2015) evaluated the HS/RT effect on pectin methylesterase activity and serum viscosity. HS/RT under 50 and 200 MPa did not affect pectin methylesterase catalytic activity throughout the storage period nor promoted its inactivation. On the other hand, losses in viscosity were reported, especially in the first couple days under HS/RT, which remained constant until the end of the studied storage period. As pectin methylesterase activity was stable during HS/RT, the authors hypothesize that other chemical reactions or other endogenous pectinases enzymatic activity, other than pectin methylesterase, could be pressure enhanced and affect pectin characteristics and serum viscosity, as these changes were more pronounced in HS/RT juices under 200 MPa, which presented at the end of storage a slight cloud destabilization. Under the same HS/RT conditions, the volatile profile of strawberry juice was analysed by Bermejo-Prada, Vega, Pérez-Mateos, and Otero (2015). Microbial inhibition of juices under HS/RT avoided juice

spoilage, maintaining the strawberry volatile profile similar to the one prior storage, with no significant losses in the juice key aroma compounds during HS/RT, namely for methyl and ethyl butanoates, methyl hexanoate, trans-2-hexenyl acetate, and linalool. Juices at HS/RT presented a lower decrease in total colour change when compared to AP/RF, and a similar reduction in total phenolic and anthocyanin content over the storage period, with polyphenoloxidase activity tending to increase over storage for all conditions, while peroxidase tended to decrease, especially under 200 MPa (reduction of 15%) (Bermejo-Prada & Otero, 2016).

A more detailed effect of HS/RT in the endogenous microflora of strawberry juice was performed under 25, 50, 100 and 200 MPa at constant RT (20 ºC). After 1 day, at the lowest pressure Bermejo-Prada, López-Caballero, and Otero (2016) reported microbial counts reduction for lactic acid bacteria (LAB) and yeasts and mounds (YM), whereas total aerobic mesophiles (TAM) growth was inhibited. Pressure increase during HS/RT resulted in microbial inactivation for all studied microbiological groups. After 3 days at PHS at AP/20 ºC, microbial recovery occurred, which was affected by the duration and pressure intensity during HS/RT. Sensorial evaluation conducted by a semi-trained panel of panellists in Bermejo-Prada, Colmant, Otero, and Guignon (2017) work, showed that raw juices stored under HS/RT and pasteurized juices under AP/RF were distinguished from the raw juice prior storage, while the panellists found no differences between pasteurized juices stored under HS/RT and AP/RF, thus suggesting the possibility to store processed juice at HS/RT, without noticeable organoleptic changes.

Our research group studied HS, especially at and above RT, testing the possibility to store more perishable juices, watermelon and melon juice (low acidity), under a combination of different pressures (25-150 MPa) at and above RT (20-37 ºC), from 8 h to 365 days (Lemos, Ribeiro, Fidalgo, Delgadillo, & Saraiva, 2017; Queirós et al., 2014). The first study was published by Fidalgo et al. (2014) focusing on HS possible application at naturally variable uncontrolled RT (18-21 ºC) and at 30 ºC, differently to the previous studies performed at constant RT (20 ºC). Storage of watermelon juice under 100 MPa after 8 h at RT and 30 ºC, showed no significant effect of storage temperatures, promoting equally similar microbial counts reduction in all studied microbiological groups. Reduced counts were achieved by increasing the storage period up to 60 h, with Enterobacteriaceae (ENT) and YM reduced to undetectable counts, while total TAM were reduced around 1 log unit.

Most of the physicochemical parameters were stable during HS, but increases in total colour change were more pronounced with increasing of the storage period, with some slight variations reported for pH, titratable acidity and browning degree. A new set of HS conditions (25-150 MPa) was further tested with watermelon juice at temperatures ranging from 20-37 ºC in Santos et al. (2015) study, observing consistently that a minimal of 50 MPa was required to inhibit microbial growth, and storage under ≥75 MPa resulted in microbial inactivation even during short periods of time at and above RT. Similar results were obtained by Queirós et al. (2014), where melon juice was kept under the same conditions described in the previous study. Regarding physicochemical parameters, Santos et al. (2015) reported a decrease in cloudiness and browning degree of HS juices, which were more pronounced at the higher temperature (37 ºC), while in general, the physicochemical variations observed in melon juice under HS conditions, were within the ones observed in juice at AP/RF (Queirós et al., 2014).

Possible shelf-life extension of watermelon juice at variable RT was analysed by Carlos Pinto et al. (2016) during 7 days under 100 MPa, resulting in microbial counts reduction, with HS/RT performing equally to better than AP/RF in most of the physicochemical parameters, however slightly more noticeable changes in colour were reported under HS/RT. The first study regarding the sensitivity of possible pathogenic microorganisms on HS/RT was assessed by Pinto et al. (2017), with two non-pathogenic surrogated strains. *L. innocua* ATCC 33090 and *E. coli* ATCC 25922 were inoculated (3-4 log CFU/mL) in watermelon juice and stored during 10 days under 50-100 MPa at RT. Both endogenous and inoculated loads required pressures ≥75 MPa, in order to have a more stable and safe juice, with a greater inactivation effect observed under these pressures. After 10 days at 100/RT, juices presented lower colour changes, stable pH, total solids, titratable acidity, and cloudiness comparatively to AP/RF, while decreases in total phenolics, browning degree, peroxidase and pectin methylesterase activity more noticeable under HS/RT. Lemos et al. (2017) studied the possible shelf-life extension of watermelon juice when HS was combined with lower temperature (15 ºC) for up to 58 days. After 7 days of storage at AP/RF juices were unacceptable for consumption, as microbial spoilage surpassed the microbiological acceptable limit, while HS at pressures ≥62.5 MPa restraining microbial growth, retaining the pH, and reduced colour losses throughout the storage, especially under 75 MPa. These results pointed to a possible juice shelf-life extension under HS/LT, however

further investigation is necessary to fully verify this possibility, such as analyses of the possible effects in other nutritional and physicochemical parameters, as well as sensorial evaluation. Similar results were obtained in watermelon juice stored during 1 year under 75/RT (25 ºC), without microbial development (reaching undetectable counts at day 62), stable pH and ºBrix values, while cloudiness and *a** tended to decrease and *L** and *b** increased throughout the storage, resulting in significant colour changes from the 21st day of storage onwards (Lemos, Ribeiro, Delgadillo, & Saraiva, 2020).

In another study Pinto, Santos, Fidalgo, Delgadillo, and Saraiva (2018) evaluated the effect of HS/RT in *B. subtilis* endospores formation/germination using three different matrices, selected through nutrient availability: McIlvaine buffer < carrot juice < brain-heart infusion broth. HS and nutrients triggered endospores germination into the lower resistant form (vegetative), resulting in endospores inactivation at pressures ≥50 MPa in all matrices. The authors hypothesize a two steps endospores inactivation under HS: 1- stimulus that activates endospores germination; 2- outgrowth inhibition due to HS. The same research group conducted another HS/RT study on the heat-resistant endospores of *Alicyclobacillus acidoterrestris* in pasteurized apple juice (low pH) stored for 60 days. In this juice HS/RT at 25 MPa was sufficient to gradually inactivate both the vegetative and endospores microbial load, while at higher pressures (50 MPa) the inactivation was faster, with storage at 100/RT resulting in both microbial populations reduction to undetectable counts already in the first couple days of storage.

Table 2.3 - Main results of hyperbaric storage applied in fruit juices.

Food product	HS conditions	Main results	References
Strawberry juice	25-220 MPa 15 days 20 ºC	25 MPa successfully inhibit microbial growth, with no changes observed in physicochemical parameters, with a slight decrease in viscosity.	Segovia-Bravo et al. (2012)
	50/200 MPa 15 days 20 ºC	Pectin methylesterase catalytic activity was not affected by HS. Viscosity decreased significantly in the first days.	Bermejo-Prada, Segovia-Bravo, et al. (2015)
		Strawberry juices at HS had a similar volatile profile to the one prior storage.	Bermejo-Prada, Vega, et al. (2015)
		Although some changes were reported in individual colour parameters, total colour difference was generally reduced under HS. After 15 days, peroxidase activity was reduced around 15% under 200 MPa.	Bermejo-Prada & Otero (2016)
	25-200 MPa 15 days 20 ºC	Microbial growth inhibition under 25 MPa of the endogenous microflora, while inactivation occurred at higher pressures.	Bermejo-Prada et al. (2016)
	25-50 MPa 15 days 20 ºC	Panellists were able to distinguish raw juice under HS from raw juice prior storage, while no differences were detected between pasteurized juice under HS/RT and AP/RF.	Bermejo-Prada et al. (2017)
Watermelon juice	100 MPa 60 h 18-30 ºC	Juice in HS was better preserved than at AP/RF, without microbial development. Colour loss was more pronounced under HS, with increasing storage period.	Fidalgo et al. (2014)
	25-150 MPa 8 h 20-37 ºC	Microbial response was directly affected by pressure intensity. Changes in cloudiness and browning degree for HS juices, were especially higher at 37 ºC.	Santos et al. (2015)
	100 MPa 7 days RT 18-21 ºC	Reduction in endogenous microbial load at HS/RT. At the end of the storage, colour of HS juices presented instrumentally higher values.	Carlos Pinto et al. (2016)
	50-100 MPa 10 days RT 18-23 ºC	Better reduction in inoculated and endogenous microbial load above 50 MPa. Noticeable changes were only obtained for total phenolics, browning degree, pectin methylesterase and peroxidase activity at the end of storage under 100 MPa.	Pinto et al. (2017)
	50-75 MPa 58 days 10-25 ºC	Possible shelf-life extension up to 58 days. HS of ≥62.5 MPa reduced microbial load, resulting in stable pH, while under 75 MPa at 15 ºC, colour was less affected.	Lemos et al. (2017)
	75 MPa 365 days RT 25 ºC	Undetectable microbial counts from the 60 day of HS onforward. Increases in cloudiness and colour observed, while pH and ºBrix remained stable.	Lemos et al. (2020)
Melon juice	25-150 MPa 8 h 20-37 ºC	25 MPa allowed microbial proliferation, while at 50 MPa and ≥75 MPa resulted in growth inhibition and inactivation, respectively.	Queirós et al. (2014)
Carrot juice	25–100 MPa 60 days RT 18–23 ºC	*B. subtilis* endospores reduction in juices at ≥50 MPa. HS at 25/RT and AP/RF allowed vegetative growth of *B. subtilis*.	Pinto et al. (2018)
Apple juice	25–100 MPa 60 days RT 18–23 ºC	*A. acidoterrestris* vegetative and endospores load successfully inactivation at ≥25 MPa, throughout the entire storage at variable RT.	Pinto et al. (2019)

2.1.3.2 Whey cheese and ready-to-eat meals

Fresh dairy products have high water activity and almost neutral pH, that limits these products shelf-life to only a few weeks, even at AP/RF. *Requeijão* is a traditional Portuguese whey cheese, studied by Duarte et al. (2014), as the first dairy product case study under HS (100-150 MPa) at and above RT (25-37 ºC), for short periods of time (up to 8 h). Generally, no pronounced changes in colour, pH, lipid oxidation, and water activity were denoted during HS, with 100 MPa allowing a better microbial population control, resulting in microbial inactivation to values bellow the detection limit for LAB, ENT and YM, with TAM load, reduced around 1 log unit (**Table 2.4**). In a second study, the same dairy product was stored for longer periods of time, 10 days at variable RT, with part of the experiment performed in an industrial size HP equipment (for 24 hours). Whey cheese under HS/RT showed higher stability, retaining the pH, water activity, and fatty acid profile, and presenting fewer colour losses comparatively to AP/RF, with an additional microbial inactivation effect in all the studied microbiological groups to counts bellow the detection limit (≤1 log CFU/g) from the 3rd day of storage on forward (Duarte et al., 2017).

Moreira, Fernandes, et al. (2015) also reported a better preservation of carrot soup under HS at and above RT (25 and 30 ºC), comparatively to AP/RF. Similarly to what was reported above, ENT and YM were highly susceptible to HS, especially under 150 MPa, with a higher sensibility to HS observed at 30 ºC for these microbiological groups. No variations were found in carrot soup under HS, regarding colour, pH, titratable acidity and reducing sugars. Prolonged storage (up to 12 h) on a soup (*caldo verde*) and ready-to-eat *Portuguese* meal (*bacalhau com natas*) were carried out by the same authors, also analysing the stability of these food products at AP/RF after HS/RT of 12 h (Moreira, Duarte, et al., 2015). During HS/RT, 50 MPa allowed TAM proliferation in both food products, while under 100 MPa the authors observed microbial growth inhibition, while at 150 MPa all microbial groups were inactivated to counts below the detection limit, expect for TAM in *bacalhau com natas* RTE meal that was reduced around 1 log unit after 12 h. No considerable changes were observed for pH, titratable acidity, colour and fatty acid content in both products under HS/RT, comparatively to AP/RF. After 12 h under 100 MPa at RT, both products were placed at AP/RF for another 3 days of storage (PHS). After PHS, both products presented stable values in all physicochemical parameters, with a generally slower microbial development when compared to the respective control at AP/RF for the same time.

Table 2.4 - Main results of hyperbaric storage applied to whey cheese and ready-to-eat meals.

Food product	HS conditions	Main results	References
Requeijão (Portuguese whey cheese)	100/150 MPa 8 h 25-37 ºC	HS maintained the physicochemical parameters of whey cheese similar to AP/RF, while restraining microbial growth.	Duarte et al. (2014)
	100 MPa 10 days RT 17-21 ºC	HS at variable RT resulted in microbial inactivation of all studied microbiological groups, promoting fewer physicochemical losses.	Duarte et al. (2017)
Carrot soup	100/150 MPa 8 h 25/30 ºC	No physicochemical changes were observed during HS, with a clear microbial inactivation especially under 150 MPa.	Moreira, Fernandes, et al. (2015)
Caldo verde soup RTE *bacalhau com natas*	50-150 MPa 12 h RT 17-21 ºC	Physicochemical parameters remained stable under HS/RT. Reduction in microbial counts observed from HS/RT ≥100 MPa.	Moreira, Duarte, et al. (2015)

2.1.3.3 Raw and processed meat products

At the moment only five studies have been published regarding HS of raw meat and meat products above RF temperatures, ranging from a period of 8 h to 60 days, detailed in **Table 2.5**.

Cooked ham is a rather highly consumed product, with high water activity and high pH (around 6), which limit its shelf-life. HS (25, 50, 100 and 150 MPa) of this product was carried out by Fernandes et al. (2015) at and above RT (25-37 ºC) for up to 8 h, and compared with storage at AP/RF. Storage under 25 MPa allowed microbial proliferation, on the other hand 50 MPa inhibited microbial growth similarly to AP/RF, while an additional significant decrease in microbial counts occurred at higher pressures. During the short storage period analysed in this study, generally no changes were reported for pH, water holding capacity, lipid oxidation and colour parameters.

Freitas et al. (2016) stored raw bovine meat initially under 50-150 MPa during 12 h at RT (21 ºC), performing a second storage experiment for 10 days under 50 MPa at RT. In the first part of the study, no pronounced changes in pH, colour and fatty acid profile were reported under HS/RT, and similar to what was previously reported, inhibition of microbial growth of TAM, ENT, YM and psychrophilic bacteria (PSY) was achieved under 50 MPa, with a significant reduction in coliform bacteria counts, while higher pressures resulted in an additional inactivation effect (reduction ~1 log unit). After 12 h under 150 MPa, a post-hyperbaric storage period of 6 days at AP/RF was conducted. Microbial proliferation was

still slower for HS/RT raw meat samples placed at AP/RF conditions, when compared with control raw meat at AP/RF for the same period duration, 3.3 ± 0.1 and 4.8 ± 0.2 log CFU/g for TAM, respectively, without deterioration of the analysed quality parameters. In the second experiment, 50 MPa restrained TAM and PSY growth until day 3 of storage, with an increase in microbial counts of both microbiological groups by the 7th day, reaching counts above the acceptable limit (6 log CFU/g) on the 10th day of storage. The pH was not affected throughout the 10 days of HS/RT, with reduced variations in L^*, a^* and b^* colour parameters, resulting in fewer overall total colour losses.

In another work, Fernandes et al. (2018) stored raw minced pork meat, under 100 MPa for 24 h at RT, where pH, colour, lipid oxidation and fatty acid composition were monitored. In concordance with the aforementioned studies, TAM were more resistant to this level of pressure, while ENT and YM had its counts reduced under HS, opposingly to AP/RF just after 12 h, where TAM counts had surpassed the acceptable microbiological limit. After 24 h under 100 MPa, a slightly higher pH value was reported, with a higher rate in lipid oxidation products development also observed, while the fatty acid content was not affected. During the PHS period evaluated for HS samples, microbial proliferation reached the acceptable limit, after 3 days at AP/RF.

Further studies regarding storage under pressure of other meat products, minced and bovine and pork meat in pieces were performed by Santos et al. (2020) at variable RT (25 ºC) for a duration of 60 days. Microbial population was unable to grow under HS/RT of ≥75 MPa, decreasing over the storage time, while under 50 MPa, TAM and LAB succeeded to grow. During the 60 days of storage, pH, drip loss, and colour changes tended to increase in both raw beef minced and in pieces, with a more pronounced effect in minced meat at 100/RT. In order to minimize these changes during HS/RT, Santos, Castro, Delgadillo, and Saraiva (2020) conducted a new HS preservation study of minced bovine and pork pieces, at low temperature 60/10 ºC during 60 days and compared with 75/25 ºC and AP/RF. Gradual increases in the microbial load in both meat products at AP/RF led to unacceptable counts after 15 days of storage, while HS conditions promoted a progressive counts reduction of all microbiological groups, resulting in an inactivation effect, over the 60 days of storage, except for bovine raw meat stored at 60/10 ºC, where TAM growth was inhibited. The authors found that a better control of the physicochemical parameters was achieved at lower temperatures

(10 ºC) when combined with HS, attenuating losses in the moisture content, drip loss, colour and lipid oxidation, comparatively to HS at 25 ºC.

Table 2.5 - Main results of hyperbaric storage applied to raw and processed meat products.

Food product	HS conditions	Main results	References
Sliced cooked ham	25-150 MPa 8 h 25-37 ºC	25 MPa resulted in microbial proliferation. 50 MPa restrained microbial growth, with higher pressure reducing microbial counts.	Fernandes et al. (2015)
Raw bovine meat	50-150 MPa 10 days RT 21 ºC	Better microbial stability resulted from HS ≥50 MPa. No noticeable changes reported in colour, pH and fatty acid profile during HS.	Freitas et al. (2016)
Raw pork minced meat	100 MPa 24 h RT 20 ºC	HS inhibited microbial proliferation. With increases in pH and lipid oxidation values under HS for 24 h.	Fernandes et al. (2018)
Raw beef and pork, minced and in pieces	50/75/100 MPa 60 days RT 18-23 ºC	HS/RT (≥75 MPa) extended raw meat products microbiological shelf-life, although generally increases in the physicochemical parameters were found.	Santos et al. (2020)
Minced bovine and pork meat in pieces	60/75 MPa 60 days 10/25 ºC	Both HS conditions prolonged the microbiological shelf-life of meat products. HS at a lower temperature reduced the detriment in the physicochemical parameters.	Santos et al. (2020)

2.1.3.4 Fish and other seafood products

The first fish product studied at HS/RT was conducted in tilapia by Ko and Hsu (2001), where HS (≥203 MPa) yielded better microbial results, inhibiting TAM and PSY growth, while also scoring more acceptable K-values (freshness quality index) at and above this pressure than at lower ones (**Table 2.6**), with K-values for HS tilapia of 51, 44, 33, and 28%, under 51, 101, 203, and 304 MPa, respectively, while fish at AP/RT had an unacceptable K-value of 92% (K-value above 60% indicates putrefaction). After 12 h under HS conditions, tilapia fillets were stored for another additional 12 h at AP/RT (PHS), with reduced microbial growth in HS/RT samples previously under ≥203 MPa, for both TAM and PSY, with K-values increasing to 60 and 52%, from samples originally kept under 203 and 304 MPa, respectively.

Okazaki, Shigeta, and Aoyama (2007) stored sea cucumber guts (traditional Japanese meal) under 60 MPa at 30 ºC to possibly induce autolysis, in order to reduce the salt that is usually added to this product. In fact, autolysis was enhanced during HS, with the panel test preferring cucumbers (seasoned and cooked) from HS than the controls (with regular salt content), to which was attributed to enhanced activity of thermolysin and aminopeptidase

activity and increases in free amino acids. Also, HS after 24 h resulted in 1 log unit reduction of PSY counts, while TAM increased around 1.2 log units.

Hake loins were maintained at HS combined with RF temperatures for 7 days in Otero, Pérez-Mateos, and López-Caballero (2017) study. After 7 days, fish stored at AP/RF was spoiled, while under 50 MPa no increase in TAM counts was observed, while ENT counts were below the detection limit, with generally no changes noticeable in the physicochemical parameters. Some parameters were however affected by the compression effect of pressure during HS, leading to a slight decrease in water content (-1%), and increase in whiteness (+4.72) and shear resistance (+1.81 N/g), comparatively to samples prior storage. Cooking of these samples resulted in an attenuation of these changes, nevertheless fish stored under HS still had a higher shear resistance that was noticeable also in the sensory analysis, although generally only moderate differences were reported.

In a more fatty fish (Atlantic mackerel) HS also at RF was studied by Otero, Pérez-Mateos, Holgado, Márquez-Ruiz, and López-Caballero (2019) during 12 days. HS/RF prevented microbial fish deterioration, reduced microbial counts, and attenuated losses in fish-quality indicators, such as pH, total volatile basic-nitrogen content, drip loss and water-holding capacity, with a slight increase in L^* and b^* colour parameters, preventing lipid degradation, but promoted changes in the protein profile. On the other hand, regular storage (AP/RF) was unable to restrain microbial growth, causing a more pronounced change in most of the fish-quality indicators. Once HS/RF fish was cooked, and similarly to the aforementioned study, the differences were attenuated, with similar values when compared to cooked control fish, with zero days of storage.

More recently the same group of authors compared the combination of lower pressure and refrigerated temperatures (50 MPa at 5 ºC) with higher pressures at RT (75 MPa at 20 ºC), in order to find the best storage condition that could extend Atlantic razor clams shelf-life (Laura Otero & Pérez-Mateos, 2021). After 7 days, a more pronounced reduction in endogenous microflora was observed in HS/RT versus HS/RF, although after 14 days, both conditions yielded similar counts reduction. Physicochemical parameters indicate a better maintenance of bivalves quality at HS/RF when compared with HS/RT, with bivalves under this condition for 14 days presenting higher pH, water content and weight, and even after cooking, these bivalves were two times firmer, with a higher b^* colour parameter. And thus,

HS at RF for some food products, may be more adequate, as comparatively, HS at RT produced greater quality losses.

Regarding Atlantic salmon, several studies were published with a more comprehensive evaluation in the quality parameters of this fatty fish, under HS conditions (40-75 MPa) at RF, LT and at and above RT (4-37 ºC), to possibly extend its shelf-life (detailed in **Table 2.6**). In the first study that Fidalgo, Lemos, Delgadillo, and Saraiva (2018) conducted, a more general overview of HS (50-75 MPa) at RT conditions, possible effects on the microflora, and on a few physicochemical parameters were reported. Storage at AP/RF and 50 MPa/RT allowed microbial growth; 60 MPa restrained microbial growth until the 6th day of storage; while storage at 75 MPa at 25 and 37 ºC reduced all microbial counts. However, at 75/25º C fresh salmon tended to present higher differences throughout the storage, mainly regarding increases in colour parameters as well as in primary and secondary lipid oxidation products. Other physicochemical parameters were analysed on the second study, such as enzymatic profile, protein quality indicators, with an additional HS condition performed at LT, 60/10 ºC (Fidalgo, Delgadillo, & Saraiva, 2020). In the first part of the experiment all enzymes tended to decrease its activity in all storage conditions under HS, with the exception for acid phosphatase under 60 MPa at 10 and 25 ºC and for cathepsin D at 60 MPa/25 ºC, presenting enhanced activity under these conditions. Protein quality parameters were more stable under 60/10 ºC, while a higher quality loss was noticeable with increasing temperature. On the second experiment of this study, low temperature (10 ºC) under 60 MPa was compared with storage at RT under 75 MPa, with the first condition rendering a better enzymatic and protein stability. Taking into consideration the previous results that indicate the requirement of low temperature in Atlantic salmon preservation, Fidalgo et al. (2019) studied the storage of this fish under 40-60 MPa at 5-15 ºC. Endogenous microflora was more sensitive during storage at 60/10 ºC, as microbial counts were gradually reduced, with the exception of anaerobic bacteria, that under this condition were unable to grow until the 10th day of storage, and thus this condition was used in the second part of the study. On the second experiment, prolonged storage (50 days) was conducted, with volatile base-nitrogen content surpassing the acceptable limit after 30 days, with trimethylamine-nitrogen, formaldehyde, secondary and tertiary lipid oxidation products increasing over storage. Overall, the authors stated a possible life extension of 30 days under HS/LT, which is significantly higher when compared to only 6 days at AP/RF. Later Fidalgo et al. (2020) evaluated this HS/LT

condition in the lipid stability, physical properties and on the volatile profile of vacuum-packaged Atlantic salmon. Under HS/LT the physical properties remained unaffected, with the textural profile analyses indicating some protein destabilization, as hardness and resilience decreased over storage, but the lipidic and volatile profile resembled those of fresh salmon prior to storage. Only primary lipid oxidation products increased slightly at the 30th day of storage at HS/LT, without significant increases observed in secondary and tertiary lipid oxidation, which seems be related to the low availability of oxygen in vacuum-packaged salmon. Also, HS/LT successfully led to possible shelf-life extension of Atlantic salmon, proving also its capability to reduce endogenous and inoculated vegetative microbial load (*E. coli* ATCC 25922, *L. innocua* ATCC 33090 and *B. subtilis* ATCC 6633), as well as inactivated inoculated *B. subtilis* endospores, improving the safety of fresh fish.

Table 2.6 - Main results of hyperbaric storage applied to raw fish and other seafood products.

Food product	HS conditions	Main results	References
Tilapia fillets	203 MPa 12 h 25 ºC	Storage under HS (≥203 MPa) yielded fewer freshness losses, resulting in a more stable product when placed at AP/RT.	Ko & Hsu (2001)
Sea cucumber guts	60 MPa 36 days 30 ºC	Autolysis induced during HS, resulting in preferable sea cucumber without added salt. After 24 h, TAM increased counts while TAP growth was inhibited.	Okazaki et al. (2007)
Cape hake loins	50 MPa 7 days 5 ºC	Microbiologically stable under HS, with some quality differences reported, manly textural, partially reduced after cooking.	Otero et al. (2017)
Atlantic mackerel	50 MPa 12 days 5 ºC	Reduction in microbial counts at HS/RF, retaining fish quality even after 12 days. Once cooked no noticeable differences were observed.	Otero et al. (2019)
Atlantic razor clams	25/50/75 MPa 14 days 5/20 ºC	Similar microbial reductions observed for 50 MPa at 5 ºC and under 75 MPa at 20ºC. Bivalves stored at 75/20 ºC had a higher quality decline after 14 days of storage.	Otero & Pérez-Mateos (2021)
Atlantic salmon	50/60/75 MPa 10 days 25/37 ºC	Better microbial preservation at 75 MPa, but detectable changes in most physicochemical parameters were observed.	Fidalgo et al. (2018)
	50/60/75 MPa 50 days 10/25/37 ºC	Enzymatic profile and protein quality parameters indicate a better preservation at low temperature under 60 MPa.	Fidalgo et al. (2020)
	40/50/60 MPa 10 days 5/10/15 ºC	60 MPa at 10 ºC performed better than the other HS conditions, regarding microbial control. Possible shelf-life extension of this product, up to 30 days.	Fidalgo et al. (2019)
	60 MPa 30 days 10 ºC	Salmon vacuum-packaged at HS/LT resulted in a more stable preservation, with only a slightly increase in primary lipid oxidation at the 30th day, while retaining the characteristic lipidic and volatile profile.	Fidalgo et al. (2020)
	60/75 MPa 30 days 10/25 ºC	Both HS conditions inactivated the endogenous and inoculated microbial load. Velocity of inactivation was pressure intensity related.	Fidalgo, Pinto, Delgadillo, & Saraiva (2021)

2.1.4 Hyperbaric storage possible industrial viability at room temperature

In the modern world, food and energy are essential for the human society sustainability, and are increasingly interlinked, with energy applied in every step of the food system, from production to processing, distribution, and consumption. Approximately 30% of the world's total energy consumption is attributed to the agri-food sector, accounting for over 20% of total greenhouse gas (GHG) emissions (Song, Reardon, Tian, & Lin, 2019). Conventional RF is extremely important in the food industry, as it minimizes microbiological growth in food, and is applied in several stages of today's food system, from packaging, distribution, logistics centers, supermarket store/display to home storage (Hundy, Trott, & Welch, 2016). Worldwide RF accounts for about 15% of electric energy consumption, while in Europe it is around 17%, and in the UK retail food, RF is responsible for 3% of total electrical energy consumption and 1% of total GHG emissions (Cascini, Gamberi, Mora, Rosano, & Bortolini, 2016). Emissions of GHG can derive from direct emissions related to RF high electrical energy consumption and CO_2 emissions from the power stations, and indirect emissions from refrigerants leakage into the atmosphere, like hydrochlorofluorocarbons and hydrofluorocarbons (Suamir, Tassou, & Marriott, 2012). Increasing population, creates a global challenge that needs to develop a sustainable food system capable not only to feed everyone safely, but at the same time use less energy and emit fewer GHG emissions.

As mentioned in this section, HS feasibility at RT has been studied in several food products, from juices to dairy, meat and fish products, with a very positive outcome. When HS is performed at variable RT no energy is needed to maintain a certain temperature within a specific range, unlike conventional RF. And thus, under HS at RT a considerable low level of energy would only be applied in the pressurization and depressurization processes, since once the desired pressure level is achieved, no more energy would be required to maintain it.

Bermejo-Prada et al. (2017) evaluated the industrial viability of HS/RT using strawberry juice as a case study, and the possible effects on consumers perception of the stored products, feasibility of the equipment design, storage cost, and environmental impact. No differences were denoted in a sensorial evaluation of pasteurized strawberry juice stored

under HS/RT and AP/RF, indicating the same level of acceptation between these two types of preservation methodologies. Also high pressure processing (HPP) for food pasteurization products is now a well stablished industrial reality, experiencing worldwide commercial growth, with a positive perspective and acceptance from the consumers that are now more experienced and familiarized with HPP technology (Bruhn, 2016), which also seems to be a good indicator for HS implementation. Regarding the equipment design, the same authors (Bermejo-Prada et al., 2017) did take into consideration the main components characteristic in a HPP equipment, such as the hydraulic pump, intensifier, and vessel, based on the product and pressure required for its storage. Theoretically the hydraulic pump and intensifier could the fixed and connected, in order to pressurize the vessel, that in turn should be portable. As typical forklifts can easily move loads up to 3000 kg, thereby the total weigh of the vessel plus the food product cannot exceed this total load. Also, the vessel should be made with stainless steel, based on the currently available, with high strength and hardness along with an excellent corrosion resistance and good fracture toughness. A hypothetical scenario was chosen, using the selected vessel for the storage of 200 kg of juice for 15 days at HS/RT and compared with AP/RF. In general, AP/RF has an associated significant lower cost, when compared to HS/RT, of 0.081 and 0.291 €/kg of juice for 15 days, respectively. The high investment in HPP equipment, is the major factor accounting for this variation (0.200 €/kg), followed by maintenance (0.090 €/kg), while as predicted, energy usage would be minimal (0.001 €/kg) under HS/RT when compared to AP/RF (0.026 €/kg). The high investment required for the HP equipment acquisition is the main factor limiting HS viability, however in this study it was based on the ones currently available for HPP, that are highly more complex and exigent. For instance, HPP equipment used in the food industry usually operate at a maximum pressure of 600 MPa, with reduced processing times (3-6 min) that are financially vital for HPP, which results in high performance intensifiers. On the other hand, HS usually has great food preservation results under 75-100 MPa, and the pressurization velocity does not represent a critical economic impact in HS as in HPP, pointing to significant costs reduction. Also, the maintenance costs in HPP are mostly associated with wear of valves and the intensifier system, resulting from the high levels of pressure used in this processing technology, that are noticeable higher than the ones mentioned for HS (Elamin, Endan, Yosuf, Shamsudin, & Ahmedov, 2015). Taking all into consideration, it is expected that manufactures would design equipment specifically for HS, resulting in a more

attractive investment, similarly to the initial steps taken by the HPP industry, resulting in the increase of manufacture companies and HP equipment/parts, which would considerably reduce costs. Bermejo-Prada et al. (2017) also estimated HS to have a considerably lower carbon footprint (CF - expressed in kg CO_2/kg juice) when compared to RF, of 0.0042 and 0.1085 CF, respectively. Regarding HS the vessel would account for most of the CF associated (0.0041 CF) but energy consumption would be negligible (0.00003 CF). While for RF, similar CF was attributed to cold chamber materials (0.0045 CF), however a considerable higher CF was associated with refrigerant leakage (0.0472 CF) and energy consumption (0.0554 CF). Bermejo-Prada et al. (2017) estimated an almost 26-fold lower carbon footprint for HS, resulting in a more sustainable preservation methodology that in return would account for negligible emission taxes when compared to RF, thus additionally contributing to costs reduction.

Storage under pressure without temperature control would be applicable for instance, on prolonged storages in maritime transportation where electricity supply is less accessible, or during road transportation, logistics center or even at the supermarket storage, while also reducing losses associated with breaks in the refrigeration chain from production, until the food product reaches the consumer. HS also has the potential to increase foods shelf-life, decreasing food waste, and enhance microbiological safety, since opposingly to RF, HS can inactivate vegetative pathogens and spoilage microorganisms as well as spores, to considerable levels, as described previously.

2.2 Milk general overview

Milk is defined as the secretion of the mammary gland that comes from female mammals, being the sole source of nutrition for the offspring, essential for young mammals. Thus, it is considered one of the most complete foods, rich in essential nutrients including protein, fat, carbohydrates, vitamins and various mineral needed for an healthy growth and development (Sharabi, Okun, & Shpigelman, 2018). Equally, dairy products are well known as added-value products, which includes low or lactose free products or enriched milk with calcium and vitamins, and dairy-based functional foods, with an additional health benefit, provided for instance from the added probiotics, omega-3, isoflavins or phytosterols, for example. Dairy products can be consumed as liquid milk, concentrated milk, fermented dairy products (like cheese and yoghurt), milk powder, butter, cream, ice cream or dairy beverages among others, or applied widely in great quantities by the food manufacturing industry (ÖZer & Kirmaci, 2010). The highly perishable nature of milk requires it to be sold as heat-treated product (like pasteurization) or transformed into dairy products with enhance shelf-life, like cheese, cultured milk, yoghurts, or milk powder.

The high nutritional value combined with the great diversity and variety of milk and dairy products make them widely consumed around the world by virtually all population groups, from infants, children, teenagers, middle-aged and the elderly, representing an important part of the human diet, culture, and economy. In 2020, the worldwide milk production reached more than 876 Megatons, with a significant contribution by the European Union, producing around 155 Mt. In 2021, this production is estimated to increase 1.6% worldwide, at a slower growth rate for the EU, of around 0.8% (OECD/FAO, 2020). As it can be seen in **Figure 2.1**, between 2017-19, India and the EU were the two major milk producers, with 184 and 152 Mt respectively, followed by the United States, Latin America and Pakistan, with 98, 81 and 46 Mt respectively, with a foreseen market expansion mainly for Pakistan, India and Africa, with a predicted production growth for 2029 of 28.4, 27.7 and 21.8%, respectively (OECD/FAO, 2020). Milk is consumed as fresh but also in the form of various products such as yogurt, butter, ice-cream and cheese, with the later one being manly produced in the EU, with an estimated growth of 44% by 2029. In Europe and North America the consumption of fresh dairy products is stable to declining, and due to milk highly perishable nature and high-water content, only around 8% of milk world production is traded internationally, with most exportations accounted for butter, cheese, skim and whole milk

powder (OECD/FAO, 2020). New markets where demand is expected to grow faster than production, such as Africa, Southeast Asian countries, and the Middle East and North Africa, place an economic opportunity in the dairy economy, possibly leading to a diversification in products, supporting the dairy sector's sustainability.

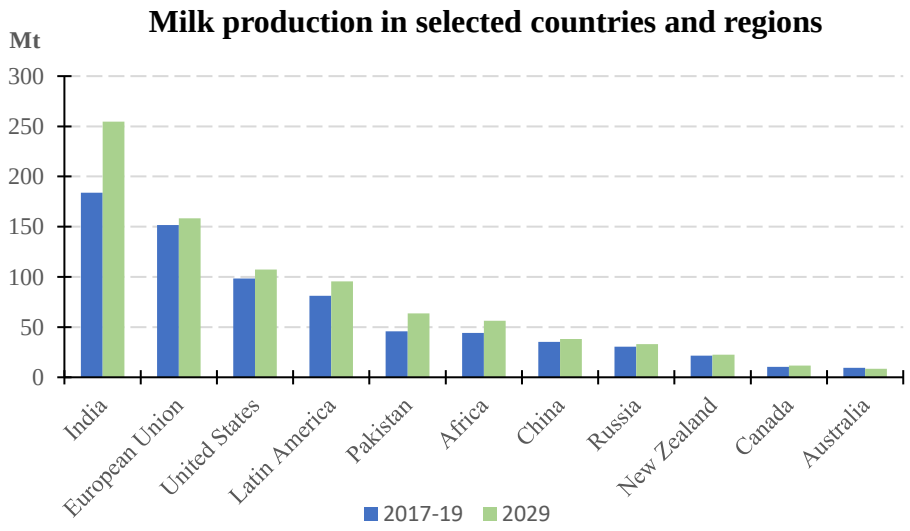

Figure 2.1 - Quantity of milk (in Megatons) produced in several countries and regions between 2017-2019, and predictions for 2029. Adapted from OECD/FAO (2020).

Portugal has an overall self-sufficient dairy production (93%), with in 2017, 33% of total milk production coming from Azores, from which mostly produced by cows, while the other 67% derived from Portugal Mainland, mostly again from cow's milk (96%) and the rest accounted to goat's and sheep's milk (4%) (CAM, 2019). The majority of this milk is processed into fresh dairy products (76%), mostly in the form of milk directly for consumption (59%), or processed into cream (2%), sour milk products (9%) and milk-based drinks (5%). More recent information from 2019, suggests that overall production stabilized, comparatively to the previous year, with only a negligible reduction reported for goat's milk of 0.05% (INE, 2020). Overall, the number of dairy cattle has settled around 244 000 in 2018, with an increase in the average number of 48.4 dairy cows per farm in 2019, while the number of dairy farms decreased also in 2019 to 36104 and the productivity have stabilized around 7500-7700 L per cow in the last 5 years (CAM, 2019). In 2019 the national production was mostly capable to meet the national market needs, with a positive supply observed for milk (105%), milk powder (200%), butter (136%), and other fresh dairy products, like cream (122%), while on the opposite side, importation of some dairy products

were required to attain market demands, like sour milk products (e.g., yogurts), milk-based drinks and cheese, with the national production reaching 53, 86 and 63% of consumers demand, respectively (CAM, 2019).

Changes in the dairy industry are inevitable, as some segments of society are moving towards the reduction of animal protein consumption (reduction in meat and dairy consumption) especially for the younger demographics and generations (Dos Santos & Ahmad, 2020). Also, public pressure to reduce livestock numbers is growing, due to its association with high greenhouse gas (GHG) emissions along with detrimental impacts on the environment, like excessive water consumption, land and biodiversity destruction (Shafiullah, Khalid, & Shahbaz, 2021). Although in some regions outside Europe, such as Asia, Africa and South America there is an increasing demand for meat and dairy products driven by income and population growth, the dairy sector needs to adapt to climate change demands and the sector's new place in modern society. A challenging effort that needs to take place between industry, consumers, policy makers and other stakeholders, to ensure long-term future of the sector while minimise environmental and animal welfare impacts.

2.2.1 Milk composition

Nutritional composition of milk can vary greatly depending on the specie, diet, breed, individual animal, season, stage of lactation, number of gestations, age and overall health status (Lindmark Månsson, 2008). Water is the most abundant element, 80.6-88.5% for sheep and goat's milk, respectively, with the other elements being dissolved, colloidally dispersed, and emulsified in water. Although it is mostly in its free form, water can also be bonded to other elements, such as proteins, lactose and minerals (Wijesinha-Bettoni & Burlingame, 2013). As detailed in **Table 2.7**, milk from different species show variations in composition and nutritional values, as for instance, sheep's milk is richer in fat (7.62%), protein (6.21%), and Cl (0.27%) than goat's and cow's milk. On the other hand, goat's milk is richer P (0.27%), Ca (0.19%), and K (0.18%), while cow's milk has a richer composition in lactose (4.78%). Differences in milk composition are responsible for variations in the physical properties, like viscosity, conductivity, density, etc, detailed in **Table 2.8**.

Table 2.7 - Composition comparison of milk from different species, sheep, goat and cow. Adapted from Jandal (1996), Wszolek, Tamime, Muir, and Barclay (2001) and Rasheed, Qazi, Ahmed, Durrani, and Azmat (2016).

Component	Sheep	Goat	Cow
Water (%)	80.6	88.5	87.5
Protein (%)	6.21	3.37	3.23
Casein (%)	4.20	2.4	2.6
Albumin, globulin (%)	1.00	0.60	0.60
Fat (%)	7.62	3.80	3.67
Lactose (%)	3.70	4.08	4.78
Total ash (%)	0.90	0.79	0.73
Ca (%)	0.16	0.19	0.18
P (%)	0.15	0.27	0.24
Cl (%)	0.27	0.15	0.11
K (%)	0.14	0.18	0.06

Table 2.8 - Some physical properties of sheep's, goat's and cow's milk. Adapted from Park, Juárez, Ramos, and Haenlein (2007).

Properties	Sheep	Goat	Cow
Specific gravity (density)	1.0347–1.0384	1.029–1.039	1.0231–1.0398
Viscosity	2.12	2.86–3.93	2.0
Conductivity (Ω^{-1} cm^{-1})	0.0038	0.0043–0.0139	0.0040–0.0055
Refractive index	1.3492–1.3497	1.450 ± 0.39	1.451 ± 0.35
Freezing point (°C)	0.570	0.540–0.573	0.530–0.570
Acidity (lactic acid %)	0.22–0.25	0.14–0.23	0.15–0.18
pH	6.51–6.85	6.50–6.80	6.65–6.71

2.2.1.1 Protein

Milk has a complete essential amino acid profile, being a significant source of protein, as it is considered as one of the most important nutrients for human growth and development (Sharabi et al., 2018). Also, milk protein has a high protein-digestibility-corrected amino

acid score, containing peptides and other bioactive factors that contribute to an additional health effect, that may help in preventing some diseases, such as like cardiovascular ones, blood pressure and diabetes (Michaelsen, Nielsen, Roos, Friis, & Mølgaard, 2011). Cow's milk total protein range around 3.0-3.5% (Horne, 2020), majorly in the form of casein and whey proteins, 78 and 17%, respectively. Caseins are produced in the epithelial cells of the mammary gland in the form of α_{s1}-, α_{s2}-, β-, and κ-casein, while whey proteins can be produced in the mammary gland (β-lactoglobulin e α-lactoalbumin) or in the blood (immunoglobulins and serum albumin) (Farrell et al., 2004). Caseins are usually separated through precipitation at pH 4.6 (isoelectric pH) at 30 ºC, while whey proteins are soluble under these conditions, and can also be referred as serum proteins or non-casein nitrogen (Park et al., 2007).

2.2.1.1.1 Caseins

Most of the caseins are present in the colloidal phase, in the association form of casein–calcium–transport complexes known as casein micelles, typically with a diameter of 200 nm (**Figure 2.2**), contributing to the physicochemical properties and to the stability of milk and resulting dairy products (Wang et al., 2017). Caseins (CN) are heat-stable, highly hydrated, with approximately 3.5 g of water per g of protein, while if dried, casein micelles consist majorly of 94% proteins and 6% of minerals (Qi, 2007). Caseins consist of thousands of individual casein molecules connected mostly by calcium phosphate to α_{s1}-CN, β-CN and α_{s2}-CN (**Figure 2.3**), with κ-CN playing a crucial role in the stabilization of the casein micelles, due to caseins hydrophobicity that is stabilized by the hydrophilic portion of κ-CN, known as the glycomacropeptide (de Kruif et al., 2012). γ-CN can also be naturally present, usually in trace amounts, produced by plasmin proteolytic activity in β-CN (Phadungath, 2005). Casein micelles high calcium and phosphate content serves as a delivery vehicle of this elements to the neonate, and also its unique structure are key constituents, determining milk functionality in traditional dairy processes, like rennet coagulation of cheese and acid coagulation of yoghurt (Qi, 2007). Today, caseins are produced and applied in several fields to prepare innovative products, from functional foods with bioactive casein-derived peptides to delivery vesicles for nutraceuticals (Phadungath, 2005).

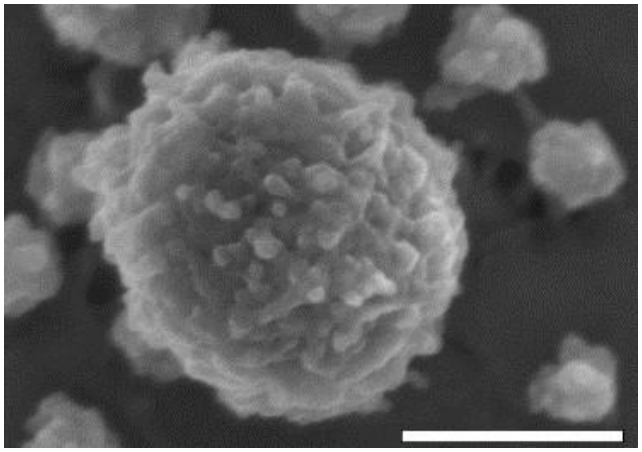

Figure 2.2 - Electron micrograph of an individual casein micelle. Scale bar =200 nm. Adapted from Dalgleish, Spagnuolo, and Douglas Goff (2004).

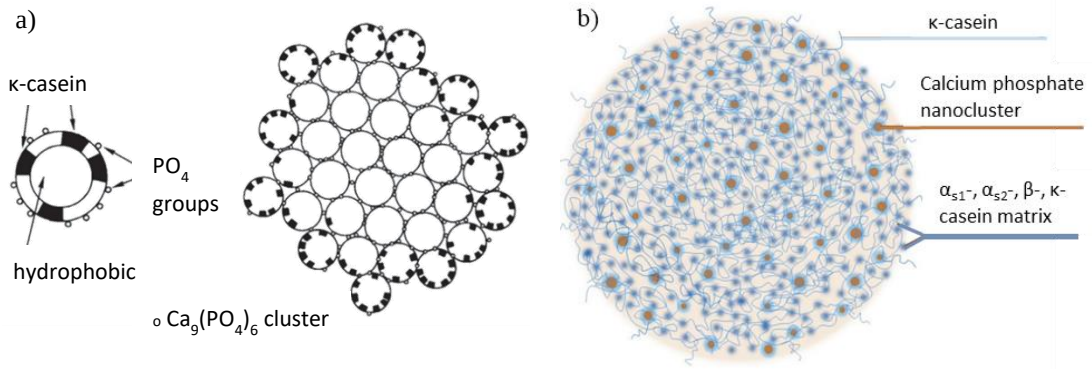

Figure 2.3 - (a) casein sub-micelle schematic representation and casein micelle composition of sub-micelles; (b) representation of a casein micelle internal structure, consisting of a matrix of α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN and calcium phosphate nanoclusters. Adapted from Qi (2007) and de Kruif, Huppertz, Urban, and Petukhov (2012).

2.2.1.1.2 Whey proteins

Whey proteins are constituted by several distinct proteins, having a globular shape with substantial contents of α-helix motifs, with acidic/basic and hydrophobic/hydrophilic amino acids balanced throughout their sequences (Pintado & Malcata, 2000). These proteins possess important nutritional and biological properties, particularly regarding to health promotion and prevention of diseases. Whey proteins include α-lactalbumin, β-lactoglobulin, immunoglobulins, serum albumin, lactoferrin, and lactoperoxidase (Farrell et al., 2004). α-Lactalbumin is the second most important protein in whey (20%), while β-lactoglobulin is quantitatively the dominant one in milk from cow, sheep, goat and other

ruminants (50%), with a high content of essential amino acids, acting as a transporter of several compounds (such as fatty acids, vitamin D, cholesterol, calcium, and triacylglycerols). β-Lactoglobulin is commonly used in the formulation of modern foods and beverages, due to its high nutritional and functional value (Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011). Immunoglobulins play a crucial postnatal role in neonate own immune system through colostrum, with lactoferrin responsible for iron transportation, and lactoperoxidase playing an important part of the natural host defence system in mammals (Hernández-Ledesma et al., 2011; Wakabayashi et al., 2007). Whey proteins can be obtained as a by-product of cheese production and added to ready-to-drink and powdered beverages, sport meals, nutrition bars, high protein cookies and in tablet form, as the consumption of whey proteins is associated with muscle mass gains with resistance exercise (Phillips, Tang, & Moore, 2009).

Other proteins can be found in milk, such as several enzymes, from lipases, proteases, and phosphatases. With the main ones represented by lipoprotein lipase, plasmin, alkaline and acid phosphatase, being responsible for rancidity (especially in homogenised milk), hydrolysis of proteins and hydrolysis of organic phosphates, respectively, assisting also the neonate digestive process (Huppertz, Fox, & Kelly, 2004b). Lysozyme is also present, produced in the mammary gland, acting as a defensive mechanism capable of lyses certain bacteria wall (LeJeune & Rajala-Schultz, 2009).

2.2.1.2 Fat

Lipids are considered, one of the most important components in milk, in terms of cost, nutrition, and physical and sensory characteristics that they provide to dairy products. Differences in lipid content vary between animal species, with generally sheep being the richest one, followed by goat's and cow's milk (**Table 2.7**), while usually after standardization, fat content decreases to around 3.5 g/100 g (Park et al., 2007). Most of the lipids are associated in the form of globules, made of a core of triglycerides surrounded by a membrane (milk fat globule membrane, MFGM), which prevents fat globules from lipolysis catalysed by lipase activity, mainly linked to the casein micelles (Hanuš, Samková, Křížová, Hasoňová, & Kala, 2018). MFGM size increases with increasing fat content in milk, playing a crucial role in its stability and technological properties (Lindmark Månsson, 2008). Other components are associated with the MFGM, such as proteins, phospholipids,

trace elements, enzymes, and carotene, with some components acting as emulsifiers and preventing individual globules from joining together. Fat globules vary in size, floating in the milk, with 98% of milk fat being a mixture of triacylglycerols, in a smaller concentration free fatty acids, mono and diacylglycerols, phospholipids, sterols, and hydrocarbons (Park et al., 2007). Milk fat also acts as a transporter for fat soluble vitamins, such A, D, E and K and supply also essential fatty acids, and pigments (like carotene) that are responsible for the yellow colour in butter (Lindmark Månsson, 2008).

Fatty acids (FA), vary in chain length and degree of unsaturation, position and orientation of double bonds, contributing to its unique physicochemical and biological properties with over 400 different FA being detected in milk (Djordjevic et al., 2019). In ruminant's milk, FA can be synthetised from two different ways, in the mammary gland (so-called *de novo* synthesis), with the precursors produced in the rumen from dietary polysaccharides, or synthesized from dietary lipids and adipose tissue reserves (Jensen, 2002). As detailed in **Table 2.9**, milk FA profile varies between mammals (sheep, goat and cow), with the most important factor within the same specie attributed to the diet (95%), followed by breed, season, lactation stage, lactation number, age of the dairy cows, and geographical location (Lindmark Månsson, 2008). Generally, the high content in 5 FA account for ≥70% (C10:0, C14:0, C16:0, C18:0, and C18:1) in sheep's, goat's and cow's milk (**Table 2.9**). Usually, goat's milk is richer than cow's milk in short- and medium-chain FA with 6–10 carbon atoms, and so, caproic (C6:0), caprylic (C8:0) and capric acids (C10:0) are named after goats. While sheep's milk FA composition regarding C10:0, C14:0, C16:0, C18:0 and C18:1 is more similar to goat's milk, the saturated fatty acids (SFA) content is comparable to that in cow's and goat's milks (Park et al., 2007).

Regarding human health, SFA (mainly C12:0, C14:0, C16:0, respectively) are the primary source of fat in the huma diet, however high consumption of SFA is associated with increased concentrations of low density lipoprotein (LDL) in blood (that can lead to the accumulation of cholesterol in the blood vessels), while other SFA can also increase high density lipoproteins (HDL) in blood (transporting cholesterol from blood vessel walls to the liver), neutralising their effect (Djordjevic et al., 2019). Consumption of monounsaturated fatty acids (MUFA) have a positive effect on the concentration of HDL, while reducing the concentration of LDL (Markiewicz-Kęszycka, Czyżak-Runowska, Lipińska, & Wójtowski, 2013). Polyunsaturated fatty acids (PUFAs) are regarded as beneficial for human health,

from the n-3 and n-6 FA families, regulating various physiological processes, such as development of the nervous system, in the vision process, in the development of premature babies and children, and also protection against the coronary heart disease and carcinogenic effect (Markiewicz-Kęszycka et al., 2013; Michaelsen et al., 2011).

Table 2.9 - Fatty acids main content (% in total fatty acid methyl esters) in goat's, sheep's and cow's milk fat. Adapted from Park et al. (2007), Jensen (2002) and Lindmark Månsson (2008).

Fatty acid (%)	**Sheep**	**Goat**	**Cow**
C4:0	3.51	2.18	4.40
C6:0	2.90	2.39	2.40
C8:0	2.64	2.73	1.40
C10:0	7.82	9.97	2.70
C10:1	0.26	0.24	0.30
C12:0	4.38	4.99	3.30
C12:1	0.04	0.19	0.03
C13:0	0.17	0.15	0.11
C14:0	10.4	9.81	10.9
i-C15:0	0.34	0.13	0.35
ai-C15:0	0.47	0.21	0.57
C14:1	0.28	0.18	0.80
C15:0	0.99	0.71	0.90
i-C16:0	0.21	0.24	0.26
C16:0	25.9	28.2	30.6
i-C17:0	0.53	0.35	0.70
ai-C17:0	0.30	0.42	0.54
C16:1	1.03	1.59	1.00
C17:0	0.63	0.72	0.40
C17:1	0.20	0.39	0.10
C18:0	9.57	8.88	12.20
C18:1 total	21.1	19.3	24.90
C18:2 total	3.21	3.19	2.17
C20:0	0.45	0.15	0.20
C18:3	0.80	0.42	0.51
C18:2 conjugated	0.74	0.70	0.54

2.2.1.3 Lactose

Lactose is the principal carbohydrate present in milk, it is a disaccharide formed from glucose and galactose. Cow's milk averages a lactose content around 4.7 to 4.9%, although milk from individual cows may vary more, and some factors can also influence it, such as

reduction in lactose secretion from animals with mastitis (Quigley et al., 2013). Some bacteria can breakdown lactose through the enzyme β-galactosidase, to glucose and galactose that can then be fermented to lactic acid, turning milk sour. Heat treatment of milk, especially above 100 ºC, may promote milk browning through Maillard reactions, causing lactose irreversible interlink with milk proteins, resulting in nutritional value losses (Van Boekel, 1998).

The main role of lactose is to provide energy, but also contributes (along with milk oligosaccharides) to growth, aids in softening of stools and enhances water, sodium and calcium absorption (Hernández-Ledesma et al., 2011). Some people are unable to metabolize lactose (lactose intolerance), caused by insufficient amounts or activity of lactase in the human intestine and suffer in varying degrees of abdominal discomfort, bloating, diarrhoea and flatulence (Jomanah Abduljalil, 2021). Lactase enzymatic pre-treatment of milk, breaks down lactose and helps to overcome this problem, with an increasingly demand, mainly in North America and Europe for lactose-free food products (Świąder, Kulawiak, & Chen, 2020).

Besides lactose, other carbohydrates can be found in milk, like glucose and galactose present in trace amounts or associated with protein and lipids (glycoproteins and glycolipids, respectively), or in the form of oligosaccharides and nucleotide sugars in smaller amounts (Park et al., 2007). Although this carbohydrates are not converted into energy, several biological activities are attributed to milk oligosaccharides, such as prebiotic activity, anti-inflammatory properties, anti-adhesion effects, and a role in brain development and growth-related characteristics of intestinal cells (Kunz, Rudloff, Baier, Klein, & Strobel, 2000).

2.2.1.4 Minerals

Milk is considered also an important source of growth-supporting minerals, comprising less than 1% of the milk, with some entirely soluble in the whey, while other are in a colloidal suspension of casein micelles (Park et al., 2007). The most abundant elements are calcium, phosphorus, potassium, sodium, and magnesium, while zinc, iron, copper and manganese are trace-elements (Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, & Chilliard, 2008). 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). As detailed in **Table 2.10**, cow's milk has overall the lowest mineral content, with sheep's milk presenting a more abundant content in calcium, phosphorus, chloride and magnesium, while goat's milk is richer in potassium, manganese, iodine and selenium.

Table 2.10 - Mineral composition of sheep's, goat's and cow's milk (per 100 g). Adapted from Park et al. (2007).

Mineral (mg)	**Sheep**	**Goat**	**Cow**
Calcium	193	134	122
Phosphorus	158	121	119
Potassium	136	181	152
Sodium	44	41	58
Chloride	160	150	100
Magnesium	18	16	12
Zinc	0.57	0.56	0.53
Iron	0.08	0.07	0.08
Copper	0.04	0.05	0.06
Manganese	0.007	0.032	0.02
Iodine	0.020	0.022	0.021
Selenium (µg)	1.00	1.33	0.96
Aluminium	0.05-0.18	-	-

2.2.1.5 Vitamins

Vitamins are important micronutrients available in milk, required in small quantities to sustain health and well-being. Part of these nutrients are fat soluble vitamins, A, D, E and K, associated with the fat globule, while water soluble vitamins, from the B complex and D are associated with the water phase. Concentration of vitamins in milk greatly depend on the animals' diet, and species, as for instance, goat's milk is richer in vitamin A, and several ones from the B complex (B_1, B_2, B_5, and B_{12}), vitamin C and D, while cow's milk has a higher content in vitamin B_9 (**Table 2.11**). Vitamins are sensible to heat processing of milk, which generally results in vitamin loss after pasteurization, while during processing fat-

soluble vitamins are retained by the cream, while the water-soluble vitamins remain in skim milk or whey (Michaelsen et al., 2011).

Table 2.11 - Vitamin content of sheep's, goat's and cow's raw whole milk (µg per 100 g). Adapted from Raynal-Ljutovac et al. (2008) and Park et al. (2007).

Vitamins (µg)	**Sheep**	**Goat**	**Cow**
Fat soluble			
Retinol (A)	40	80	40
Beta carotene (Vit.A precursor)	0	0	20
D	0.06	0.18	0.08
Tocopherol (E)	40	110	110
Water soluble			
Thiamin (B_1)	50	80	40
Riboflavin (B_2)	140	350	170
Niacin (B_3)	200	420	90
Pantothenic acid (B_5)	310	410	340
Pyridoxin (B_6)	50	80	40
Biotin (B_8)	2.00	1.50	2.00
Folic acid (B_9)	1.00	5.00	5.30
Cobalamin (B_{12})	0.06	0.71	0.35
Ascorbic acid (C)	1300	5000	1000

2.2.2 Sources of microbial contamination

After collection, raw milk temperature is around 38 ºC, and thus it needs be rapidly cooled and kept at refrigerated temperatures, as due to its high nutritional profile, near neutral pH and high-water activity makes it a highly prone environment for the growth of several microorganisms (Lundén, Tolvanen, & Korkeala, 2004). Microbial composition and diversity in raw milk can be associated to different types and origins of contaminations, occurring during pre- or post-harvest. Microorganisms can already be present when milk is excreted (pre-harvest), for example, if the mammary gland if infected (mastitis), which is the most common diseases associated with dairy cattle (Angulo, LeJeune, & Rajala-Schultz, 2009). This inflammation cannot always be visible and the source of infection ranges from bacteria, yeasts, mycoplasma and algae, that can subsequentially be excreted into milk

(Bradley, 2002). Also when the milk is being excreted it can become in contact with commensal microflora that live in the teat skin, or on the epithelial lining of the teat canal or via the lactiferous duct (Isaac et al., 2017). Thereby, by the time the milk leaves the animal, microbial contamination may occur even in a healthy animal. Post-harvest contamination can derive from dairy farm environment during production, collection, processing, distribution, and storage of milk. These contaminants may result from faecal, animal feed, mud, water, soil, human handling, farm utensils, distribution pipes, bulk or transport tanks (Damm, Holm, Blaabjerg, Bro, & Schwarz, 2017). *Staphylococcus*, *Campylobacter*, *Listeria*, *Escherichia*, *Salmonella*, *Micrococcus*, *Clostridium*, *Yersinia enterocolitica* and *Bacillus* (vegetative and spore cells) are commonly associated with milk contamination when unproper or poor sanitary conditions are in place (Papademas & Bintsis, 2010). Maintenance of low temperature (4-10 ºC) once the milk is collected or transported for further processing remains one of the most important factor for the overall quality in raw milk, since it slows chemical deterioration and microbial growth (Koutsoumanis, Pavlis, Nychas, & Xanthiakos, 2010). However, psychrophile microorganisms can proliferate under low temperatures, releasing lipases and proteases responsible for organoleptic alterations, like rancidity and bitter off-flavours (McClements, Patterson, & Linton, 2001). Heat pasteurization is also ineffective in the inactivation of spores commonly found in the farm environment, like *B. cereus* (Heyndrickx, 2011). Ultra High Temperature (UHT) (135 ºC for 2 sec) is a sterilization treatment that is applied in milk, which allows a shelf-life extension to up to 9 months, by targeting several microbial groups, destroying vegetative cells as well as most spore-forming pathogens (like *B. cereus* and *C. botulinum*), while the spores of some non-pathogenic are not inactivated (such as *B. sporothermodurans*) (Van Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004). However, contamination may even occur after pasteurization, since some microorganisms are able to create microbial biofilms in multiple reservoirs such as the distribution pipes, corners, cracks, crevices, gaskets, valves and the joints of stainless steel equipment, that require attention, proper/regular cleaning and maintenance (Borucki, Peppin, White, Loge, & Call, 2003). Which again, reinforces the importance of the incorporation of good hygiene codes and appropriate packaging technology in the dairy industry, to ensure adequate safety of milk and dairy products.

2.3 Fresh cheese

As previously mentioned, milk and dairy products have an excellent nutrient profile, rich in several vitamins and mineral, representing an important part of the human diet while contributing to a healthy growth and development (Sharabi et al., 2018). Fresh dairy products are widely consumed all over the world and it is estimated to increase 1% worldwide until 2029, playing an important role in the sustainability of the economies of many countries (OECD/FAO, 2020). Latin-style fresh cheese (non-ripened cheese) is a popular dairy product in some European and Latin American countries, made from pasteurized milk by acidic and enzymatic clotting, with milk from different animals, or even a mixture, that do not require a ripening period, resulting in cheese with a characteristic soft texture, mild flavour and low salt content, that is consumed fresh (Bleoancă et al., 2016). Fresh cheese production is estimated to account for around 30% of total cheese production, with factors such the soft texture, ingestible consistence, and mild flavour, contributing to its popularity among the very young and elderly population, while the healthy perception of these products attract diet-conscious consumers (Schulz-Collins & Senge, 2004). This kind of dairy product is very versatile and suitable for the preparations of various dishes or consumed solely as a snack.

2.3.1 Nutritional composition

The nutritional composition of fresh cheeses is directly related with milk source, as it can be seen in **Table 2.12**, regarding fresh cheese produced with goat's or cow's milk (the two most abundant ones), while also be produced with a mixture of both milks (Sant'Ana et al., 2013). Cheese production is an excellent form to concentrate the nutrients in milk, such as proteins, fat, mineral salts, and vitamins, through whey removal. Fresh cheeses still possess a higher water content, around 60%, with both types of cheeses characterized by a similar fat, vitamins and mineral content, with goat's cheese usually having a higher protein content than cow's cheese (**Table 2.12**).

Table 2.12 - General nutritional composition (%) of fresh cheese produced from goat's and cow's milk. Adapted from Van Nieuwenhove, Oliszewski, and González (2009) and Sant'Ana et al. (2013).

Component (%)	**Goat**	**Cow**
Water	60.7-64.0	59.9 -64.0
Protein	15.8-19.1	15.4-18.8
Fat	15.78-21.4	17.4-20.6
Lactose	1.26	1.60
Total ash	2.26	2.21

As it occurs in milk, fresh cheeses have a higher abundance of SFA, followed by MUFA and PUFA (**Table 2.13**). Fresh cheeses individual FA consist mostly of palmitic acid (C16:0), oleic acid (C18:1*c*9), stearic acid (C18:0), myristic acid (C14:0) and capric acid (C10:0), accounting to around 75% of total FA. The composition in FA is related to several factors, such as the individual animal, animal nutrition, seasonal feeding, farming type or stage of lactation, among other factors (Arnould & Soyeurt, 2009). Usually, goat's cheese offers a greater amount of conjugated linoleic acids (CLA) than cow's cheese, around 1.1 and 0.8, respectively, which is directly related to its content in raw milk.

Table 2.13 - Fatty acid composition (%) of fresh cheese produced from goat's and cow's milk. Adapted from Van Nieuwenhove et al. (2009).

Fatty acid (%)	**Goat**	**Cow**
C4:0	0.9	1.1
C6:0	3.2	2.0
C8:0	2.4	1.7
C10:0	5.2	3.5
C12:0	2.9	2.6
C14:0	11.7	12.0
C14:1	0.9	1.0
C15:0	2.5	3.0
C16:0	27.0	28.0
C16:1	1.1	1.2
C17:0	0.6	0.4
C18:0	11.8	12.3
C18:1*t*11	2.5	3.8
C18:1*c*9	20.6	21.0
C18:2	2.0	1.1
C18:3	0.8	0.7
CLA*c*9,*t*11	1.0	0.7
CLA*t*10,*c*12	0.1	0.1
C20:0	1.0	0.8
C20:1	0.4	0.3
C22:4	0.5	0.4
Saturated	69.1	67.6
Monounsaturated	25.5	27.3
Polyunsaturated	4.3	3.0

2.3.2 Sources of microbial contamination

Fresh cheese is made with pasteurized milk in order to eliminate vegetative microorganisms and increase its safety. However, the normal high moisture content and neutral pH level of fresh cheese, provide the ideal postprocessing conditions for the growth of contaminant microbiota, such as Enterobacteriaceae, moulds and yeasts, among others that are capable to proliferate even under low temperatures (Evert-Arriagada, Hernández-Herrero, Juan, Guamis, & Trujillo, 2012). Cross-contamination, especially during and after curd production can introduce such microbial populations, mainly through the manipulation of fresh cheese, limiting its shelf-life to a couple to four weeks even under refrigeration (Hnosko, San-Martin Gonzalez, & Clark, 2012). For instance, Evert-Arriagada, Hernández-Herrero, Guamis, and Trujillo (2014) reported initial microbial counts in fresh cheese made with pasteurized cow's milk, of 3.30, 2.66, 2.84, 2.57, 1.71 log CFU/g for aerobic mesophilic microorganisms, psychrotrophic bacteria, *Lactococci*, Lactobacili and spores, increasing after 14 days under refrigeration to counts of 7.76, 7.66, 7.25, 7.30 and 4.95 log CFU/g, respectively, surpassing the microbiological limit acceptable for human consumption, with the microbial counts reaching counts above 8 log units in those microbiological groups after 21 days at refrigeration. Yeasts and moulds, Enterobacteriaceae and *Pseudomonas* spp. that were initially undetected, achieved during refrigerated storage after 14 days, microbial counts of 5.20, 6.40 and 6.05 log CFU/g, respectively, denoting the highly cross contamination and microbial growth of this product and its perishability as other studies have also pointed (Dousset, Jaffrès, & Zagorec, 2016; Evert-Arriagada et al., 2012). Microbial spoilage is the main responsible agent that determines the shelf-life of fresh cheese, leading to increased syneresis, decrease in the pH, lipolysis, proteolysis, oxidation, and off-flavour formation, that crucially limits the shelf-life of this dairy product (Dousset et al., 2016).

As refrigeration alone only slows down microbial growth, other strategies have been evaluated in order to synergistically inhibit microbial proliferation, such as the application of nisin (natural antimicrobial agent), chemicals addition (as sorbates) or modified atmosphere packaging, among others (Boor & Fromm, 2006; Capellas, Mor-Mur, Gervilla, Yuste, & Guamis, 2000). As mentioned previously, HS acts primarily in microbial growth control, by microbial growth inhibition, at lower pressures, while at higher ones inactivation occurs, which represents an added advantage to reduce/eliminate antimicrobial agents in fresh cheeses.

2.3.3 Dairy outbreaks

From 1993 to 2006 a total 121 food disease outbreaks have been caused by contaminated dairy products, with 73 outbreak cases (60%) associated with non-pasteurized dairy products, 65 cases (54%) involved cheese and 56 (46%) involved fluid milk (Langer et al., 2012). From these 121 outbreaks, 4,413 resulted in cases of illnesses, resulting in 202 hospitalizations and 3 deaths (Langer et al., 2012). From the 73 outbreaks involving non-pasteurized dairy products, *Campylobacter* spp., *Salmonella* spp., Shiga toxin–producing *E. coli* (STEC), *Brucella* spp., *Listeria* spp. and *Shigella* spp. were the causative agents identified, being responsible for 54%, 22%, 13%, 4%, 4% and 3% of the outbreak cases, respectively. These microbial pathogens can contaminate milk from different sources, like from the blood into the milk, or from mastitis, faecal contamination or other environmental sources (Claeys et al., 2013). In the outbreaks involving pasteurized dairy products the main agents responsible were norovirus (44%), *Salmonella* spp. (20%), *Campylobacter* spp. (13%), *S. aureus* (10%) and *C. perfringens*, *B. cereus*, *Listeria* spp., and *Shigella* spp. (3% each). Overall, the data indicates that the reported outbreaks are more common in regions that allow raw milk sales than in areas where raw milk sales are banned (Langer et al., 2012).

In the following years, 2006 to 2012, an overall increase in the outbreaks related non-pasteurized dairy products in the US was reported, to 81, from which resulted 979 illnesses cases and 73 hospitalizations without losses of human lives (Mungai, Behravesh, & Gould, 2015). The increase in outbreaks cases from 30 during 2007–2009 to 51 during 2010–2012, may suggest an increase in the consumption of non-pasteurized dairy products, in fact, outbreaks associated with non-pasteurized milk increased from ≈2% to 5%, between the two periods. *Campylobacter* spp. remained the main responsible agent of these outbreaks (81%), followed by STEC (17%), *S. enterica* serotype Typhimurium (3%) and *Coxiella burnetii* (1%), while again most of the cases were reported in states that allowed the sale of non-pasteurized raw milk (81%) (Mungai et al., 2015).

In 2013 a total of 839 food related outbreaks were reported in the EU, with milk and cheese accounting for the most common dairy products responsible for these outbreaks, 1.3% each (van Asselt, van der Fels-Klerx, Marvin, van Bokhorst-van de Veen, & Groot, 2017). Between 2009-2014, 24% of the reported microbial contamination of dairy products were from spoilage microorganisms and the other 76% were related to pathogenic microorganisms, such as *L. monocytogenes* (52%), *E. coli* (11%), *Salmonella* spp. (10%),

Pseudomonas spp. (3%), and *Bacillus* spp. (2%), with milk and cheese commonly accounting again as the main contamination vehicles (van Asselt et al., 2017). In 2019, a total of 32 outbreaks were attributed to dairy products contamination, from which resulted 321 cases, 49 hospitalizations and 1 death (EFSA & ECDPC, 2021). From these dairy related outbreaks, 47.06% were strongly attributed to *Salmonella* spp., 17.65% to *Campylobacter* spp., 11.76% to Staphylococcal enterotoxins, 5.88% to STEC and 17.64% to other bacteria and virus (EFSA & ECDPC, 2021).

The consumption of raw milk, non-pasteurized, is an increasing trend observed in developed countries, such as the US (Angulo et al., 2009). Despite the fact that raw milk consumption has always been common among farm families, pro-raw milk defenders suggest that unpasteurized milk products are completely safe, while also preventing and treating a wide spectrum of diseases, including heart and kidney diseases, cancer, and lactose intolerance along with the additional bacteriostatic and antimicrobial properties provided by the native milk microflora (LeJeune & Rajala-Schultz, 2009). Despite the overall scientific consent regarding the benefits of milk pasteurization to be undisputable when compared to raw milk, pasteurization leads to negligible changes in the nutritional profile of milk (LeJeune & Rajala-Schultz, 2009; MacDonald et al., 2011). However, the demand for non-pasteurized milk has increased, with 30 states in the US allowing raw milk sales for human consumption, in retail stores, farmers markets, or on-the-farm-only sales, and some states also considered relaxing restrictions on the sale of non-pasteurized dairy products (Weisbecker, 2007). Such measures can result in increased health complications, especially for people most at risk such as the very young, elderly persons, pregnant women, and immune-compromised persons.

2.4 High pressure processing of milk and fresh cheese

2.4.1 High pressure processing

Food products quality and safety are among some of the most important factors influencing consumers demands in modern times, as well as being the most important considerations of food manufacturers and distributors (Cardello, Schutz, & Lesher, 2007; Ohlsson, 1994). And so, it is of utmost importance for the food industry to continuously seek more effective methods to reduce undesirable changes in foods associated with food processing. High pressure (HP) is a technology that has long been applied in several non-food industries, like in the production of plastics, ceramics, and metal-forming, etc. This technology is also applied in food processing, due to its unique advantages over thermal preservation methods (Mertens & Deplace, 1993). Thermal preservation (pasteurization and sterilisation) is the most used treatment for food preservation, however, these thermal treatments can cause undesired changes in foods, such as losses is texture, nutritional value, flavour and colour that overall lead to a reduction of final product quality (Tewari, Jayas, & Holley, 1999). Nowadays, consumers demand foods not only safe but also with high quality, fresher, and shelf-stable, with high pressure processing (HPP) possibly presenting a real solution to solve this problem. HPP is a nonthermal processing technology that can inactivate most microorganisms and enzymes, causing negligible impairment in foods sensory properties and nutritional qualities, as retention of flavour, colour and nutritional value. HP disrupts noncovalent bonds such as ionic and hydrophobic bonds but has little effect on covalent bonds. And so, consequently, large biomolecules, such as proteins and polysaccharides, are affected through alterations of their secondary, tertiary, and quaternary structures, but small molecules are generally not affected. Since colour and flavour compounds and vitamins are, in general, small molecules, HPP has little effect on these components in foods (Murchie et al., 2005).

This technology relies on two essential principles. The first one is Le Chatelier's principle, it 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 & Culioli, 1997). Isostatic principle is another basic principle governing HPP effect in foods,

stating that pressure is applied instantaneously and uniformly transmitted throughout the food, regardless of size, shape and composition, and 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 an effluent waste-free process, makes it also an environment-friendly technology (Huppertz, Smiddy, Upadhyay, & Kelly, 2006).

Over the last 20 years, significant research and advances in HPP technology have been made, in the form of semi-continuous to bulk systems, to the scaling up of laboratory/pilot units (**Figure 2.4)** to successful commercially viable processes (**Figure 2.5**) (Moreau, 1995). The first HP food processing equipment (**Figure 2.4)** was used to pressurize milk by Hite (1899), and in more recent years, HPP has been commercially applied to an increasing number of food products, such as jellies, juices, sauces, fish and meat products, and several studies have been conducted for a better knowledge of HPP effects on dairy foods, such as milk, cheese and yogurt (Ye, Anema, & Singh, 2004).

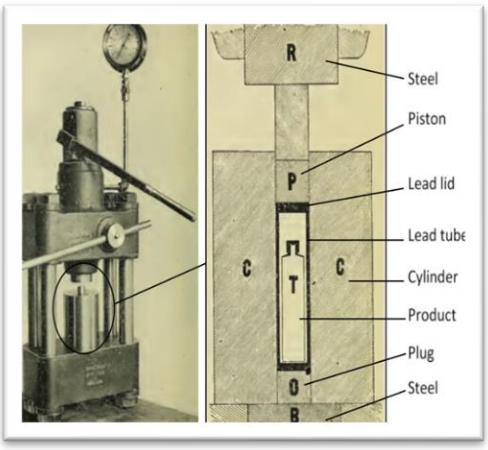

Figure 2.4 - First HPP equipment used to process milk. Adapted from Hite (1899).

Figure 2.5 - Schematic representation of a HPP bulk unit (adapted with permission from Hiperbaric, Spain).

2.4.2 HPP effect on the nutritional properties

2.4.2.1 HPP effect on the nutritional properties of milk

HPP has been applied in milk in order to prolong its shelf-life, as a nonthermal process, potentially avoiding the formation of undesirable flavours, colour and nutrient loss, while successfully reducing microbial counts (Georget et al., 2015). However, some studies have reported some changes on the light-scattering properties of milk (**Table 2.14**), leading to possible changes in its appearance, that can be attributed to casein micelles disintegration into smaller structures (López-Fandiño, De La Fuente, Ramos, & Olano, 1998). HPP had little effect on milk fat globule under HPP at 100-600 MPa, with also no significant protein aggregation in milk fat globules reported during HPP or after storage for 24 hours at 5 ºC, although a slightly increase in protein aggregation was observed immediately after HPP of 200 MPa (Huppertz, Fox, & Kelly, 2003). In another study, Ye et al. (2004) observed a slight increase in the fat globule size after processing under 700 MPa, which could result from *β*-lactoglobulin, κ-casein and *α*-lactalbumin, association with the milk fat globule, under pressures >100, 500 and ≥700 MPa, respectively. *α*-Lactalbumin has a more rigid molecular structure, being much more resistant than *β*-lactoglobulin to denaturation during HPP treatment. Under 500-600 MPa a slight increase in milk fat globules diameter was observed, rising significantly as pressure increased to 800 MPa (Kanno, Uchimura, Hagiwara, Ametani, & Azuma, 1998). Increases in micelle size were also reported by Garía-Risco, Olano, Ramos, and López-Fandiño (2000) after HPP of 400 MPa at higher temperatures (40-60 ºC), probably due to associations with denatured whey proteins with casein micelles. In a study where sheep's, goat's and cow's milk were processed using 100-400 MPa for 5-30 minutes, the release of micellar Ca, P and Mg into the serum was reported by López-Fandiño et al. (1998). Changes in the content of soluble Ca, P and Mg were more pronounced in sheep's milk processed by HP comparatively with cow's and goat's milk. Also increases in soluble protein were associated to HPP, promoted by caseins dissociation from the micelle, at different rates for each type of milk, with goat's and cow's milk having a maximum dissociation at 300 MPa, while sheep's milk at 400 MPa. Similarly Anema, Lowe, and Stockmann (2005) observed an increase in soluble protein in reconstituted milk from pressures ≥200 MPa, increasing also with pressurisation time and temperature, with no

variations observed under 100 MPa, independently of temperature and treatment duration. In another study, Zobrist, Huppertz, Uniacke, Fox, and Kelly (2005) reported that despite HP-induced casein micelles disruption resulting also in a slight increase in the pH and ionic Ca concentration, these changes were reversed upon warming HPP milk to 30 ºC.

Regarding free fatty acids, in Gervilla, Ferragut, and Guamis (2001) study, HPP treatments between 100–500 MPa did not change free fatty acids content, avoiding off-flavours development derived from lipolytic rancidity in milk, with Kanno et al. (1998) also observing a great lipid stability to lipase action in HPP treated milk.

In Lopez-Fandiño, Carrascosa, and Olano (1996) and Huppertz, Fox, and Kelly (2004a) studies no changes were observed regarding lactose degradation or hydrolysation under pressures up to 600 MPa, indicating also that no lactose isomerisation occurred, suggesting also that Maillard reactions did not took place.

Moltó-Puigmartí, Permanyer, Castellote, and López-Sabater (2011) suggested the possibility to process human milk using HP, since fatty acid proportions in milk, delta, gamma, and alpha-tocopherols, total vitamin C and ascorbic acid levels were preserved. Equivalent results were reported by Sierra, Vidal Valverde, and López Fandiño (2000) regarding vitamins B_1 and B_6, as covalent bonds are not affected or are affected very little by the pressure treatment. Also, regarding immunoglobulins (**Table 2.15**), IgM, IgA and IgG, after HPP all showed a greater stability under pressures up to 400 or at 600 MPa for short treatments, while at longer treatments or higher pressures, immunoglobulin losses were similar to the milk subjected to thermal treatment (Contador, Delgado-Adámez, Delgado, Cava, & Ramírez, 2013; Ramírez, Garrido, Rocha-Pimienta, García-Parra, & Delgado-Adámez, 2021; Sousa, Delgadillo, & Saraiva, 2014)**.**

The application of HPP in milk in the literature, report changes mainly regarding micelles size, due to micelle disintegration or association with milk proteins, that in some cases are denatured, especially under higher pressures, resulting also in protein aggregation. On the other side, HPP of milk maintained unchanged the content and quality of lipids, vitamins and lactose with some studies suggesting the viability to use HPP as a suitable alternative for the preservation of immunoglobulins in human milk.

Table 2.14 - Examples of the main effects of HPP on the nutritional properties of milk.

Milk	HPP conditions	Effect on nutritional properties	References
Cow	100-600 MPa 0-60 min 20 ºC	No changes in micellar size or protein aggregation observed after HPP or after 24 hours of storage at 5 ºC.	Huppertz et al. (2003)
Cow	100-800 MPa 0-60 min 20 ºC	Slight increase in fat globule size with increasing pressure up to 700 MPa. Serum proteins associated with micelle globe.	Ye et al. (2004)
Cow	100-800 MPa 10 min 37 ºC	Slight increase at 500 and 600 MPa, and a significantly rose at 800 MPa of milk fat globules diameter. Lipoprotein lipase was unable to act in milk fat globules pressurized.	Kanno et al. (1998)
Cow	400 MPa 15 min 25-60 °C	Decrease in micelle size under 400 MPa at 25 ºC, while at higher temperatures resulted in increased sizes. Decrease in soluble protein after 48 hours of storage at 37 ºC.	Garía-Risco et al. (2000)
Cow	100/200/300/ 400 MPa 5-30 min 20 ºC	Ca, P and Mg solubilization increased with ≤300 MPa, decreasing slightly at 400 MPa. Maximum dissociation was observed at 300 MPa.	López-Fandiño et al. (1998)
Sheep		Soluble Ca, P and Mg increased with pressure, but were smaller than those found in cow's milk. Milk dissociation increased with pressure up to 400 MPa.	
Goat		Soluble Ca, P and Mg increments were more pronounced in sheep's milk than in the milk from the other two species. Micelle dissociation was observed at a maximum at 300 MPa.	
Cow	100-600 MPa 30 min 20 ºC	HP-induced disruption of casein micelles and dissociation of micellar κ-casein. pH and calcium concentration changes were reversed after 15 min at 30 ºC, after HPP.	Zobrist et al. (2005)
Cow (Skim milk)	100-400 MPa 0-60 min 10-40 ºC	The maximum level of soluble casein was 40–50% of the total casein at all temperatures and pressures above 100 MPa.	Anema et al. (2005)
Sheep	100-500 MPa 10/30 min 25/50 ºC	Pressurization showed a tendency to increase milk fat globules in the range 1-2 μm. No differences regarding FFA content were observed.	Gervilla et al. (2001)
Cow	100-400 MPa 10-60 min RT	No changes reported in lactose or lactulose after HPP.	Lopez-Fandiño et al. (1996)
Cow	600 MPa 30 min RT	Degradation or hydrolysis of lactose did not occur under pressure.	Huppertz et al. (2004a)
Human	400/500/ 600 MPa 5 min 12 ºC	HPP allowed a better maintenance of the levels of vitamin C, fatty acids and tocopherols, similar to thermal pasteurization.	Moltó-Puigmartí et al. (2011)
Cow	450 MPa 30 min RT	No changes reported in vitamin B_1 and B_6.	Sierra et al. (2000)

Table 2.15 - Examples of the main effects of HPP on the immunoglobulins of milk.

Milk	HPP conditions	Effect on immunoglobulins	References
Human	400/600 MPa 3/6 min 10 ºC	Original immunoglobulin levels maintained under 400 MPa, while at 600 MPa losses were similar to thermal pasteurization.	Contador et al. (2013)
	200/400/600 MPa 2.5/15/30 min 8 ºC	Immunoglobulins were preserved at 200 and 400 MPa. 600 MPa for longer processing times resulted in higher losses, similar to thermal pasteurization.	Sousa et al. (2014)
	200-800 MPa 1 sec -15/0/10/30/50 ºC	Stable immunoglobulin levels under low pressures, while at 800 MPa, all combinations reduced the control levels.	Ramírez et al. (2021)

2.4.2.2 HPP effect on the nutritional properties of fresh cheese

HPP have been applied to fresh cheese (**Table 2.16**), as a possible solution for minimal processing of this highly nutritious and perishable dairy product while inactivating the spoilage microbiota, and potentially extend its shelf-life.

Fresh cheeses processed under 400 MPa (20 ºC) in Sandra, Stanford, and Goddik (2004) study, showed no changes in moisture, total solids, and fat content, while pH and protein content increased slightly. Under lower pressures (291 MPa), Okpala, Piggott, and Schaschke (2010) reported that moisture content did not vary from HPP cheeses using pressure up to 150 MPa, but dropped significantly when pressure increased, resulting in an increase in the fat and protein content, with also lower lipid oxidation values. Under 300-400 MPa, Evert-Arriagada et al. (2012) found no changes in fat and total protein contents, water activity or pH values, but significant increases in whey loss and total solids content occurred after HPP. Similar results were observed by the same authors (Evert-Arriagada et al., 2014), under higher pressure (500 MPa), with no changes in total solids, fat and protein content, pH and whey loss just after processing, with a significant increase in whey loss over storage (4 ºC), especially from the 14th day onforward, resulting in an increased total solids content. Under 600 MPa, Van Hekken, Tunick, Farkye, and Tomasula (2013) reported no changes on most of the physicochemical parameters studied, observing increases only in the moisture and protein content in HPP cheeses pre-warmed at 20 ºC, while warming HPP cheeses at 40 ºC prior processing, tended to reduce these changes in the moisture and protein content.

HPP of fresh cheese made with goat's milk in Capellas, Mor-Mur, Sendra, and Guamis (2001) study, resulted also in increased whey loss after processing, without significant changes in total solids, ash, fat and soluble nitrogen.

Overall, HPP resulted in fresh cheeses whey loss, which generally contributed to a reduction in moisture content, increase in total solids and protein content, resulted from the compression of cheese matrix caused by high pressure, forming a new more compact cheese structure.

Table 2.16 - Examples of the main effects of HPP on the nutritional properties of fresh cheese.

Fresh Cheese	HPP conditions	Effect on nutritional properties	References
Cow	400 MPa 20 min 20 °C	HPP cheese presented pH values and protein content slightly higher than the control cheese.	Sandra et al. (2004)
Cow	9-291 MPa 1-29 min 25 ºC	Reduction in moisture content, above 150 MPa, and an increment of fat content. pH and TBARS values (lipid oxidation) decreased with increasing pressure.	Okpala et al. (2010)
Cow	300/400 MPa 5 min 6 ºC	Total solids content and whey loss of HPP cheeses were higher than control cheeses, with no changes reported in the other studied parameters.	Evert-Arriagada et al. (2012)
Cow	500 MPa 5 min 16 ºC	No changes were observed immediately after HPP. While over storage, total solids content and whey loss, tended to increase.	Evert-Arriagada et al. (2014)
Cow	200/400/600 MPa 3-20 min 20/40 ºC	Small increase on the protein content. HPP resulted in moisture content decrease, as pressure increased.	Van Hekken et al. (2013)
Goat	500 MPa 5/15/30 min 10/25 ºC	No changes were reported for most of the physicochemical parameters. HPP cheeses expelled significantly more whey than control cheeses.	Capellas et al. (2001)

2.4.3 HPP effect on rheological and textural properties

2.4.3.1 HPP effect on the viscosity of milk

HPP of milk can result in some cases in the increase of milk viscosity, which seems to be dependent of pressure intensity and duration (Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003; Huppertz, Kelly, & Fox, 2002; Trujillo et al., 2007), as detailed in **Table 2.17**. For lower pressures, below 30 MPa, milks' viscosity remained stable during 8 weeks of storage at 4 ºC (Li, Joyner, Carter, & Drake, 2018), with a slight increase from 2.3 to 2.8

mPa·s under 300 MPa, reported by Adapa, Schmidt, and Toledo (1997). Although viscosity was not statistically affected by HPP treatment in Mussa and Ramaswamy (1997) study, a linear viscosity increase was obtained directly dependent on treatment intensity (up to 400 MPa) and duration, with Trujillo et al. (2007) also observing a visual increase in viscosity after HPP of 500 MPa. In Huppertz et al. (2003) work, increased values in viscosity in HPP above 200 MPa for periods longer than 5 min, were found, with a clear effect of HPP treatment and duration. Similarly Harte et al. (2003) reported a higher viscosity in raw milk processed under pressures equal and above 300 MPa. Viscosity increment could be related to disintegration of the casein micelles into smaller structures and denaturation of β-lactoglobulin, resulting in large protein aggregates which increases milk viscosity, but seem not take place under treatments bellow 200 MPa (Trujillo et al., 2007).

Table 2.17 - Examples of the effects of HPP on the viscosity of milk.

Milk	HPP conditions	Effect on viscosity properties	References
Cow	13.8/20.7/27.6 MPa 0.3 sec 85°C	After 8 weeks no changes in viscosity were observed in all processing conditions.	Li et al. (2018)
Cow	310 MPa 0.3 sec 10 ºC	Slight increase in viscosity from 2.3 to 2.8 mPa·s, after HPP.	Adapa et al. (1997)
Cow	200-400 MPa 5-120 min RT	Increases in viscosity were strongly affected by HPP intensity and duration.	Mussa & Ramaswamy (1997)
Goat (colostrum)	400/500 MPa 10 min 20 °C	Samples processed under 500 MPa, presented visually higher viscosity.	Trujillo et al. (2007)
Cow	100-600 MPa 0-60 min 20 ºC	Viscosity of skimmed milk increased as pressure intensity and treatment time increased.	Huppertz et al. (2003)
Cow	300-676 MPa 5 min 4ºC	HPP of milk resulted in higher viscosity.	Harte et al. (2003)

2.4.3.2 HPP effect on the texture of fresh cheese

Fresh cheeses processed by HP resulted in several textural changes, which were generally pressure intensity dependent (**Table 2.18**). The effect of HPP in fresh cheese promoted increases in firmness, gumminess and chewiness immediately after processing, and during refrigerated storage textural losses were more pronounced in HPP cheeses, comparatively to unprocessed ones (Sandra et al., 2004). Similarly, Okpala et al. (2010) observed increased hardness in HPP fresh cheese (291 MPa), and reduced adhesiveness. In Evert-Arriagada et al. (2012) study, the increases in firmness were attributed to a decrease

in water content of HPP cheeses (300/400 MPa) comparatively to unprocessed cheeses. Under higher pressures, 500 MPa, Evert-Arriagada et al. (2014) reported similar textural changes, with HPP cheeses becoming more resistant to deformation, less fracturable and deformable than unprocessed ones, with such differences also increasing during storage (4 ºC). Pressure can disrupt the Ca-casein associations, and once the system is again in equilibrium the associations between caseins are different from before. In Van Hekken et al. (2013) study, cheeses warmed to 20 ºC prior HPP, showed increased hardness, chewiness, cohesiveness, fracture stress, and fracture rigidity, while those preheated to 40 ºC had less variations among treatments and were closest to the unprocessed ones. It was hypothesized that fresh cheese pre-treated at 40 ºC lost most of its free whey before HPP, being compressed a warmer and more flexible protein matrix, while at 20 ºC fresh cheese protein matrix lost most of its whey during compression. In fresh cheese made with goat's milk in Capellas et al. (2001) work, generally HPP fresh cheeses presented significantly higher fracture stress values, while fracture strain tended to decrease, as a result of whey loss.

Similar results were obtained regarding textural changes in HPP fresh cheeses, as pressurization induces changes in the cheese protein network, as whey is released during compression, decreasing the moisture content, and leading to a more compact structure that affects the microstructure organization.

Table 2.18 - Examples of the main effects of HPP on the textural properties of fresh cheese.

Fresh Cheese	HPP conditions	Effect on texture properties	References
Cow	400 MPa 20 min 20 °C	HPP cheeses had higher firmness, gumminess, and chewiness than the control ones.	Sandra et al. (2004)
Cow	9-291 MPa 1-29 min 25 ºC	HPP increased the hardness of cheeses, but decreased adhesiveness.	Okpala et al. (2010)
Cow	300/400 MPa 5 min 6 ºC	In general, HPP cheeses were significantly firmer than the control cheeses.	Evert-Arriagada et al. (2012)
Cow	500 MPa 5 min 16 ºC	Pressurized cheeses were more resistance to deformation, less fracturable and deformable, than the control ones.	Evert-Arriagada et al. (2014)
Cow	200/400/600 MPa 3-20 min 20/40 ºC	HPP cheeses warmed at 20 ºC tended to present higher values for hardness, chewiness, cohesiveness, fracture stress, and fracture rigidity.	Van Hekken et al. (2013)
Goat	500 MPa 5/15/30 min 10/25 ºC	Fracture stress values were significantly higher when compared to control cheeses.	Capellas et al. (2001)

2.4.4 HPP effect on sensorial properties

2.4.4.1 HPP effect on the sensorial properties of milk

The studies of HPP effect in the sensorial properties of milk available in the literature address majorly the colour parameters and are described in **Table 2.19**. Gervilla et al. (2001) observed a decrease in *L**, while greenness (*a**) and yellowness (*b**) increase as pressure also increases, resulting in significant total colour change (*ΔE*) when pressurized at 25 ºC for HPP above 300 MPa, that would be sufficient for consumers to detect milk colour alterations. Comparable results were obtained by Adapa et al. (1997), with HPP producing decreases in *L**, *a** and *b** colour parameters of milk. Despite the slight decrease observed only in *L** parameter after HPP of ≥200 MPa, reported by Mussa and Ramaswamy (1997), the effect was directly dependent by HPP intensity and duration, resulting in a more translucid milk. Casein micelles play an important role in light scattering and when HP is applied, noncovalent forces (hydrogen bonds, ionic interactions, and hydrophobic forces) are disrupted, leading to casein micelles disintegration into small fragments that increase the translucence of the milk (Harte et al., 2003; Trujillo et al., 2007). After processing under 600 MPa, Needs et al. (2000) observed strong alterations in all colour parameters, with milk appearance being more translucent and greenish, which could be reversed, to some extent, after heating at 43 ºC. Despite panellists capacity to distinguish HPP milk processed at 25 °C, namely due to colour changes, when milk was pressurized at 50 ºC, it resulted in no visual changes, with panellists ending up preferring this HPP milk, mainly due to its smoother and creamier taste, resulting in improved organoleptically properties of milk (Garía-Risco et al., 2000). Studies regarding HPP of milk resulted mainly in changes in lightness value (*L**), contributing to losses in milks' colour after HPP.

Table 2.19 - Examples of the main effects of HPP on the sensorial properties of milk.

Milk	HPP conditions	Effect on sensorial properties	References
Cow	310 MPa 0.3 sec 10 ºC	HP treatment changed milks' colour, producing lower *L**, *a** and *b** values.	Adapa et al. (1997)
Cow	300- 676 MPa 5 min 4 ºC	After HPP milk colour became more yellow.	Harte et al. (2003)
Sheep	100-500 MPa 10/30 min 25/50 ºC	*ΔE* rates increased with pressure, with a maximum at 500 MPa, resulted from *L** value reduction.	Gervilla et al. (2001)
Cow	400 MPa 15 min 25-60 °C	Milk processed under 400 MPa at 25 ºC for 15 min, were more transparent and green-yellow. However, at higher temperatures, no changes were noted.	Garía-Risco et al. (2000)
Cow	200–400 MPa 5-120 min RT	Changes in colour observed only for *L** parameter, reporting a small decrease.	Mussa & Ramaswamy (1997)
Cow (Skim milk)	600 MPa 15 min RT	Significant changes in *L**, *b** and *a** values.	Needs et al. (2000)

2.4.4.2 HPP effect on the sensorial properties of fresh cheese

As previously mentioned, HPP promote changes in the cheese matrix, which can for instance affect cheese brightness (**Table 2.20**). In Sandra et al. (2004) study, panelists found no differences for most attributes in HPP cheeses when compared to unprocessed ones, however HPP cheeses were slightly less crumbly than the control, also denoting some colour changes. In another study, Okpala et al. (2010) reported no changes in *L**, while a strong association between *a** and processing duration, and between *b** and processing intensity were observed, but it did not influence significantly total colour change. Similarly in Evert-Arriagada et al. (2012) study, HPP cheeses had an instrumentally detectable higher *b** values (more yellow), which was also identified by the panellists, characterizing HPP cheeses as more yellow, firmer, and less watery, but without off-flavours or great differences in flavour and aroma. When a higher pressure was applied (500 MPa), reduction in *L** and increases in *b** could also be detected in HPP cheeses (Evert-Arriagada et al., 2014), with also an increase in firmness noticeable by the panellists, while flavour, aroma, elasticity and off-flavour parameters remained unchanged with HPP treatment, which despite this changes did not affect panellists' preference. In another study (Van Hekken et al., 2013), despite the panellist capacity to distinguish between unprocessed and HPP cheeses, both presented similar texture hedonic scores, without noticeable changes in flavour. Capellas et al. (2001) reported no

visual changes in colour in the inner part of goat's fresh cheeses, while on the surface HPP (500 MPa) at 10 ºC resulted in greater total colour changes, mainly by the increases in *L** and *b** parameters, while HPP at 25 ºC, minimize total colour alterations.

In general, HPP of fresh cheeses resulted in most cases in some colour changes, especially due alterations in *b** and *L** colour parameters, and although textural changes were denoted by the panellists, no off-flavours were detected with HPP cheeses scoring similar values in flavour and aroma when compared to unprocessed cheeses.

Table 2.20 - Examples of the main effects of HPP on the sensorial properties of fresh cheese.

Fresh Cheese	HPP conditions	Effect on sensorial properties	References
Cow	400 MPa 20 min 20 °C	HP cheeses were slightly less crumbly than the control ones.	Sandra et al. (2004)
Cow	9-291 MPa 1-29 min 25ºC	Increases in *b** were linearly dependent with pressure intensity, while *a** was strongly affected by processing duration.	Okpala et al. (2010)
Cow	300/400 MPa 5 min 6 ºC	Panellists were able to distinguish HPP cheeses, due to colour and textural changes, with no off-flavours development.	Evert-Arriagada et al. (2012)
Cow	500 MPa 5 min 16 ºC	Panelist equally preferred pressurized to unprocessed fresh cheeses, although HPP resulted in the increase of firmness.	Evert-Arriagada et al. (2014)
Cow	600 MPa 3-10 min 20ºC	Panelists were able to distinguish the control from HPP cheeses, scoring around 3.4 "moderately liked".	Van Hekken et al. (2013)
Sheep	500 MPa 5/15/30 min 10/25 ºC	Processing at 10 ºC resulted in increased *L** and *b**, promoting a significant colour alteration.	Capellas et al. (2001)

2.4.5 HPP effect on milk endogenous enzymes

When enzymes are affected by HPP, the effect can result in enhanced or reduced activity, or even complete inactivation. In terms of molecular structure, it can be explained by the alterations in the quaternary, tertiary and secondary structure of enzymes, which directly affects the enzymes active site configuration (Eisenmenger & Reyes-De-Corcuera, 2009). Also HPP can induce the exposure of hydrophobic amino acids, exposure of sulfhydryl groups (SH) due to unfolding of proteins, reduction in the total SH content due to new disulphide bonds formation, hydration of charged groups, disruption of bounded water, and stabilization of hydrogen bonds (Chakraborty, Kaushik, Rao, & Mishra, 2014). Changes in enzymatic activity of the endogenous enzymes in milk available in the literature are described in **Table 2.21**.

Proteolysis phenomena affect the quality of dairy fresh products, mainly by the action of endogenous plasmin, which can promote the development of off-flavours and can play an important role in age-gelation of dairy products. Plasmin is the major indigenous proteinase in milk and is part of a complex enzymatic system, plasminogen (non-active plasmin), plasminogen activators and inhibitors, which are resistant to conventional thermal treatment (Huppertz et al., 2004b). Plasmin had also shown a greater stability in HPP studies (**Table 2.21**), as for instance Garía-Risco et al. (2000) reported no changes in plasmin residual activity after HPP (400 MPa) at 25 ºC, similarly to Lopez-Fandiño et al. (1996) (400 MPa at 25 ºC) and García-Risco, Recio, Molina, and López-Fandiño (2003) (400 MPa at 23 ºC), reporting decreases in plasmin activity to around 70-80%. Scollard, Beresford, Needs, Murphy, and Kelly (2000) observed a greater stability of plasmin to HPP, decreasing around 30% under pressures above 500 MPa, also reporting a reduced proteolysis under these conditions probably related to HPP effect in plasmin proteolytic activity. At higher temperatures (60 ºC) plasmin activity is severely more affected, with Garía-Risco et al. (2000) reporting a maximum of 86.5% activity reduction under 400 MPa. In Huppertz et al. (2004b) study, plasmin showed an enhanced activity under 100 MPa, maintaining its activity at 200 MPa, decreasing with HPP intensity, to a minimal of 25% under 600 MPa during 30 min.

In homogenized milk and resulting dairy products, the lipid fraction is highly susceptible to lipolysis, but thermal treatment generally results in lipase inactivation. Under HPP, this enzyme seems be more resistant, with some enhanced activity under 400 MPa after short treatment, remaining stable even after 100 min (Pandey & Ramaswamy, 2004). Also Buffa, Guamis, Pavia, and Trujillo (2001) and Trujillo, Royo, Ferragut, and Guamis (1999) hypothesize that lipoprotein lipase could be resistant to HPP (500 MPa), as a similar lipolysis level was achieved in cheeses made with HPP milk, when compared to unprocessed and thermal treated ones.

Lactoperoxidase has a known defensive activity against a great variety of microorganisms with a wide bacteriostatic or bactericidal effect, used also as an index enzyme for pasteurization in milk, that seems to be quite resistant to pressure (Kussendrager & van Hooijdonk, 2000). Lopez-Fandiño et al. (1996), observed no variations in lactoperoxidase activity even after 60 min under 400 MPa at 25 ºC, similar to Mazri, Sánchez, Ramos, Calvo, and Pérez (2012) at 20 ºC under higher pressures, 450-700 MPa.

When higher temperatures were applied (73 ºC) under pressures up to 700 MPa, Ludikhuyze, Claeys, and Hendrickx (2002) observed an antagonistic effect under 700 MPa at 73 ºC comparatively to the fast linear inactivation at thermal treatment at atmospheric pressure, at lower temperatures (30-50 ºC) lactoperoxidase was more resistant to HPP.

Alkaline phosphatase is commonly used in dairy products to assess the efficiency of thermal pasteurization as it is more heat resistant than the vegetative pathogens present in milk. Mussa and Ramaswamy (1997) observed a steady low decrease in lactoperoxidase activity, showing a higher resistance to HPP (200-400 MPa) when compared to the studied microorganisms, that was almost the double at the same pressure level. Above 300 MPa, Ludikhuyze, Claeys, and Hendrickx (2000) reported a higher dependence in the inactivation of alkaline phosphatase to temperature than HPP, as initially inactivation increased with pressures up to 300 MPa and then decreased with further pressure increases, while raising of temperature resulted in a constant inactivation over time. Acid phosphatase is also present in milk, in lower concentration than alkaline phosphatase, being also one of the heat-stable indigenous milk enzyme (Balci, Ledward, & Wilbey, 2002). Under HPP, this enzyme was higher stable under pressures of ≤200 MPa in Balci et al. (2002) study, while under higher pressures (400 MPa) the activity was reduced to 40%.

In general, the information currently available in the literature suggests a greater resistance of the most representative enzymes present in milk to HPP, indicating also, that in order the achieve significant enzymatic inactivation, HPP should be combined with temperature.

Table 2.21 - Examples of the main effects of HPP on the endogenous enzymes of milk.

Milk	HPP conditions	Effect on endogenous enzymes	References
Cow	400 MPa 15 min 25 to 60 °C	Under 400 MPa at 25 °C only slightly changed plasmin activity, while higher temperatures considerably increased plasmin activity.	Garía-Risco et al. (2000)
Cow	100 to 400 MPa 15 min 23 ºC	A slight decrease in plasmin activity (20-30%) reported under 400 MPa.	García-Risco et al. (2003)
Cow	100-400 MPa 10-60 min 25 ºC	Plasmin retained its activity under the processing conditions used.	Lopez-Fandiño et al. (1996)
Cow	50-800 MPa 1/10/30 min 20 ºC	Plasmin was more susceptible to HPP above 500 MPa from processing times longer than 10 min.	Scollard et al. (2000)
Cow	100 to 600 MPa 10 or 30 min 20 ºC	HPP under 100 MPa for 10-30 min had little effect on plasmin activity, while HPP at 200–400 MPa progressively reduced its activity.	Huppertz et al. (2004b)
Cow	300-400 MPa 0-180 min 3 ºC	All pressure treatments resulted in an increased lipase activity.	Pandey & Ramaswamy (2004)
Goat	500 MPa 15 min 20 ºC	The level of lipolysis in cheese made from HPP milk was similar to the one made with unprocessed raw milk.	Buffa et al. (2001)
Goat	500 MPa 15 min 20 ºC	Higher lipolysis levels were reported between cheeses made with HPP milk, comparatively to the pasteurized one.	Trujillo et al. (1999)
Cow (Skim Milk)	450-700 MPa 0-42 min 20 ºC	No lactoperoxidase inactivation was observed under the studied conditions.	Mazri et al. (2012)
Cow	100-400 MPa 10-60 min 25 ºC	No activity alteration was reported for lactoperoxidase under the processing conditions used.	Lopez-Fandiño et al. (1996)
Cow	150-750 MPa/ 120 min 15-73 ºC	At 73ºC and pressure between 150 and 700 MPa completely inhibited lactoperoxidase activity, however at a slow rate when compared to atmospheric pressure.	Ludikhuyze et al. (2002)
Cow	200-400 MPa 5-120 min RT	Slight decrease in alkaline phosphatase activity, indicating some resistance under these conditions.	Mussa & Ramaswamy (1997)
Cow	100- 750 MPa 1- 1200 min 25 to 63 ºC	Alkaline phosphatase presented a significant pressure resistance, being an antagonistic effect observed for lower pressures at high temperature.	Ludikhuyze et al. (2000)
Cow	200-800 MPa Periods of 10 min 10 ºC	Acid phosphatase activity was not affected under 200 MPa, decreasing significantly at ≥400 MPa.	Balci A., et al. (2002)

2.4.6 General overview

HPP in now an industrial viable option for dairy products pasteurization, allowing the elimination of vegetative bacteria, available in batch or semi-continuous processing. In general, most of milk constituents seem to be stable during and after HPP, such as vitamins, lactose, lipids, immunoglobulins, and enzymatic profile, with changes being mainly reported regarding casein micelles organization and protein aggregation, more evident at higher pressures that to some extent may contribute the minor alterations of viscosity and colour. As for fresh cheese, HPP compression of cheeses induced mostly textural changes, resulting in moisture content and free whey losses, which contributed to some colour alteration, without additional changes in sensorial and nutritional properties being described.

During HS the intensity of high pressure is considerably lower when compared to the ones commonly applied during HPP, but food products are maintained during considerably longer periods under pressure. The information gathered in this thesis, can contribute to a better understanding of the possible effects in dairy products during prolonged storage under mild pressure levels.

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CHAPTER 3

Chapter 3

This chapter is based on the first manuscript submitted as a short communication

Hyperbaric storage at room temperature with several short intermittent interruption periods at atmospheric pressure results in similar microbial growth inhibition and inactivation as without interruption

Ricardo V. Duarte, Ana M. Gomes, Ivonne Delgadillo, Jorge A. Saraiva

3.1 Introduction

Moderate pressure is employed in hyperbaric storage (HS), usually in a range between 25-150 MPa and during prolonged storage periods. These long storage periods restrict very noticeably the number of experiments one can carry out and so on the number of samples to be studied, unless a large number of pressure vessels are available, what is not currently the case at all. To reduce the limitation of this situation and to facilitate these experiments, the same pressure vessel can be filled with several samples, with sampling taking place over time and requiring several compression and decompression (C/D) cycles, with each one taking usually about 5 minutes. During each sampling, samples are under atmospheric pressure (AP) and room temperature (RT) perishability, what should not be a problem due to the short time which samples are exposed to these conditions, even considering the possibility of several C/D cycles. A rather analogous situation occurs when foods are taken out from the refrigerator to AP/RT conditions and go back into the refrigerator, but with a striking difference. While temperature changes are mass/time dependent, what is particularly important for bulky foods, this meaning it takes some time for foods to cool/heat in consecutive cycles of in/out of the refrigerator, while pressure changes are mass/time independent. This peculiar feature of pressure is advantageous when pressuring a food for HS, but it could be disadvantageous when decompressing for sampling, due to the instantly and homogeneously loss of pressure. L,.oool

Although we have tested the possible effect of compression/decompression cycles in several HS/RT works in our research group, this was never reported expressly by us in the literature, with the exception of a PhD thesis (Lopes, 2018). In this work a brief study was reported to assess the effects of several C/D cycles during milk fermentation for yoghurt

production under pressure, with each C/D cycle taking about 5 min. For this, yoghurt production was carried out under pressure in two sets of experiments, where in the first one sampling was performed every two hours (with a total of 3 C/D cycles taking place), while in the second experiment, samples were only removed from the pressure vessel after 6 hours (at the end of the fermentation). The results showed that the C/D cycles performed to collect samples during the fermentation under pressure had no effect on the fermentative process, with similar values of pH, titratable acidity and fermentation rate being observed, when compared to fermentation under pressure without interruptions.

To better evaluate the effect of several C/D cycles during HS/RT of foods, concerning microbial behaviour, a systematic study was carried out in the present work, with a large number of C/D cycles. For this, raw cow's milk samples were kept under 75 MPa (the lowest pressure level that usually causes microbial inactivation, *Chapter 2.1*) at RT for a total of 31 days in three different vessels, with three specific C/D cycles during storage, with one being only C/D on each sampling day for microbial evaluation, while the other two were intentionally C/D one time or three times every day, respectively.

3.2 Material and Methods

Raw cow's milk was kindly supplied by a local dairy farm association being packed under aseptic conditions, inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) in previously UV-light sterilized, low permeability polyamide-polyethylene bags (90 micron, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal), 10 mL of raw milk per replica, and heat-sealed individually. The experiments were performed in a custom designed high pressure equipment SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), equipped with three pressure vessels of 30 mm inner diameter and 500 mm height, at variable uncontrolled room temperature (RT, 18-22 ºC) under 75 MPa.

The study was carried out for 31 days, and sampling took place at days 2, 7, 9, 14 and 31 of storage. The three different C/D conditions studied were in detail: condition 1 (Cond 1), where samples were only C/D when a sample was removed from the vessel for microbiological evaluation at the specific sampling period (at days 2, 7, 9, 14 and 31, thus resulting in 5 C/D cycles in total); condition 2 (Cond 2), where samples were intentionally

C/D once a day (hence resulting in a total of 31 C/D cycles); condition 3 (Cond 3), where samples were also intentionally C/D three times a day, to simulate a situation of a great number of C/D cycles (therefore resulting in a total of 93 C/D cycles). For the three conditions, in each C/D cycle profile, samples remained at AP/RT about 5 min per cycle, in a total time of 25/0.42, 155/2.58, and 465/7.75 min/h, respectively for condition 1, 2, and 3.

Quantification of total aerobic mesophiles (TAM) and Enterobacteriaceae (ENT) counts were evaluated after 2, 7, 9, 14 and 31 days of storage. At each sampling period, raw milk samples were serially diluted in Ringer's solution and plated on the appropriate media for microbiological evaluation. TAM were enumerated on plate count agar, incubated at 30 °C and 20 ºC for 3 and 5 days, respectively (ISO 4833:2013), while ENT counts were determined on violet red bile glucose agar and incubated at 37 °C for 1 day (ISO 21528:2017).

TAM and ENT inactivation along HS/RT was verified to follow a first order inactivation kinetics and D_p-values determination was carried, in cases where measurable values were obtained (values below the quantification and detection limits were not considered). A D_p-value is the time needed at a constant pressure, to reach a decimal reduction in the microbial load (expressed here in days) and was calculated based on the negative inverse of the log linear slope of **Equation 3.1**.

$$Log\ (N)\ = Log\ (N_0) - \frac{t}{D_p} \qquad \textbf{Equation 3.1}$$

where N is the microbial load (CFU/ml) under a certain pressure (MPa) for certain time (t) in days, and N_0 is the initial microbial load (CFU/mL).

All experiments were carried out in triplicate and all analyses were done in triplicate. The results for the different storage conditions were compared using Analysis of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance.

3.3 Results and Discussion

At each sampling period TAM and ENT counts were evaluated being the results shown in **Figure 3.1** and **3.2**. Raw milk presented initial TAM counts of 6.66 ± 0.09 log

CFU/mL, which were gradually reduced over time, reaching after 31 days of storage, no statistically ($p > 0.05$) different values of 4.85 ± 0.07, 4.76 ± 0.06, 4.90 ± 0.20 log CFU/mL for the three storage conditions studied, respectively in condition 1, 2 and 3, indicating that the number of C/D cycles does not cause changes in TAM behaviour during HS/RT, at least up to 31 days at 75 MPa/RT and for up to at least 93 C/D cycles.

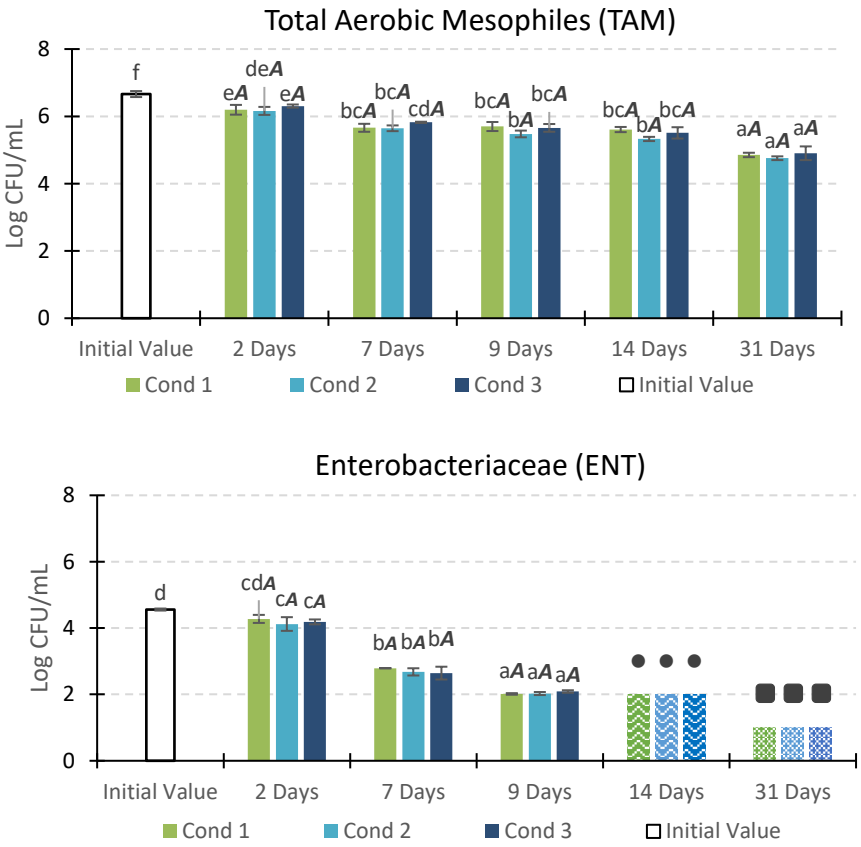

Figure 3.1 - TAM and ENT microbial evolution in raw milk stored by HS under 75 MPa at room temperature (RT), at the different compression/decompression (C/D) conditions: represented by Cond 1 (total of 5 C/D cycles), where samples were only C/D when a sample was removed for microbiological evaluation, Cond 2, and Cond 3, where samples were intentionally C/D once (total of 31 C/D cycles) or three times (total of 93 C/D cycles) a day, respectively. The symbols • and ■ represent microbial counts below the quantification (2 log CFU/mL) and detection limit (1 log CFU/mL), respectively. Different letters denote statistically significant differences ($p < 0.05$) between all the different storage conditions and storage times (a-f) and only within each storage period between the 3 storage conditions (A).

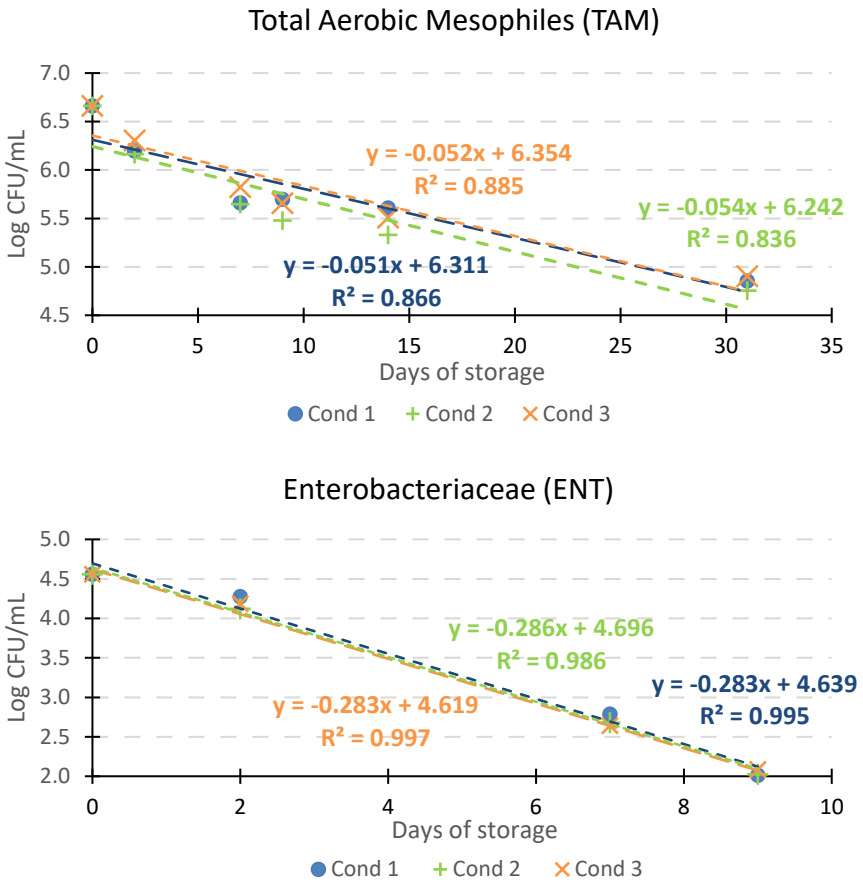

Figure 3.2 - Log linear decrease of TAM and ENT microbial counts present in raw milk (expressed in log CFU/mL), throughout HS storage under 75 MPa/RT at the different decompression/compression (C/D) conditions, represented by represented by Cond 1 (total of 5 C/D cycles), where samples were only C/D when a sample was removed for microbiological evaluation, Cond 2, and Cond 3, where samples were intentionally C/D once (total of 31 C/D cycles) or three times (total of 93 C/D cycles) a day, respectively.

Equivalent results were obtained regarding ENT, although the inactivation rate was higher compared to TAM. As it can be seen in **Figure 3.2**, inactivation of ENT occoured gradually over time, without a significant ($p > 0.05$) effect regarding the number of times each vessel was C/D, with similar counts being observed for the same sampling period at each diferent storage conditon (**Figure 3.1**). For instance, after 7 days at HS/RT, raw milk ENT counts were 2.79 ± 0.01, 2.68 ± 0.11 and 2.64 ± 0.19 log CFU/mL for conditions 1, 2 and 3, respectively ($p > 0.05$), with all the conditions reaching quantification and detection limit levels (2 and 1 log CFU/mL, respectively), at the 14th and 31st days of storage, respetively.

As for the D_p-values (**Table 3.1**), both for TAM and ENT similar values were obtained for the three different storage conditions (around 19.4 days for TAM and 3.5 days for ENT), confirming no effect on microbial inactivation rate regarding the number of C/D cycles performed.

Table 3.1 - D_p-values (days) determined for total aerobic mesophiles (TAM) and Enterobacteriaceae (ENT) loads in raw milk, stored by HS under 75 MPa/RT at the different compression/decompression (C/D) conditions, represented by Cond 1 (total of 5 C/D cycles), where samples were only C/D when a sample was removed for microbiological evaluation, Cond 2, and Cond 3, where samples were intentionally C/D once (total of 31 C/D cycles) or three times (total of 93 C/D cycles) a day, respectively.

Conditions	**D_p-values** *(days)*	
	TAM	**ENT**
Cond 1	19.81 ± 0.85^a	3.50 ± 0.11^a
Cond 2	18.81 ± 0.48^a	3.55 ± 0.10^a
Cond 3	19.63 ± 1.33^a	3.56 ± 0.13^a

As a preservation methodology, since food products are stored under HS inside a vessel/container, they could be subjected to several C/D cycles when the vessel would be opened for product removal, closed and recompressed to maintain HS conditions. Based on the results presented in this work, no significant changes regarding microbial behaviour were observed, even when comparing condition 1 with condition 3, being the first one subjected to a few number of cycles (total of 5 C/D) and the other one, subjected to a larger number of cycles (total of 93 C/D), resulting in a total time at AP/RT perishability conditions of 0.42 and 7.75 hours, respectively. In what concerns physicochemical and nutritional parameters, based on our results (*Chapter 4*) for raw milk stored under 75 and 100 MPa at RT for 60 days (total of 5 C/D cycles, resulting in 25/0.42 min/h at AP/RT perishability conditions), also no considerable changes were observed along storage, with values similar to those of the initial raw milk, prior to storage, regarding pH, titratable acidity, density, total solids content, density, colour, lipid oxidation, viscosity, fatty acids and volatile organic profile.

The results of this work indicate so that HS/RT can be used to preserve foods in a practical situation, where several C/D cycles can occur, what is very important for instance for industrial applications. Also, these results show also that experimental HS/RT work can

be accelerated by using a single vessel to study several samples, since C/D the vessel several times do not affect the results.

3.4 Conclusion

The results obtained in this focused evaluation, indicate that several compression/decompression (C/D) cycles of raw milk do not change the microbial behaviour observed during HS/RT, even when comparing 5 C/D cycles with 93 C/D after 31 days. This is very important, since for practical applications, several C/D cycles could have to be done, with foods remaining at atmospheric pressure and room temperature perishability conditions for some minutes at each C/D cycle. Moreover, these results show also that experimental HS/RT work can be accelerated using a single vessel to study several samples along time, since C/D the vessel several times do not affect the results.

3.5 References

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CHAPTER 4

Chapter 4.

This chapter is based on the second manuscript submitted for publication

A microbiological perspective of raw milk preserved at room temperature under hyperbaric storage comparatively to refrigeration

Ricardo V. Duarte, Carlos A. Pinto, Ana M. Gomes, Ivonne Delgadillo, Jorge A. Saraiva

4.1 Introduction

Milk is considered one of the most complete foods, rich in essential nutrients including protein, fat, carbohydrates, vitamins, and various mineral needed for a healthy growth and development (Sharabi, Okun, & Shpigelman, 2018), with milk and dairy products widely consumed all over the world, representing an important part of the human diet.

After collection, raw milk temperature is around 38 ºC, and thus it needs to be cooled rapidly and kept at refrigeration (RF) temperatures, as its rich nutritional profile, near neutral pH and high-water activity makes milk the perfect environment for the proliferation of several microorganisms (Lundén, Tolvanen, & Korkeala, 2004). Microbial composition and diversity in raw milk can be associated to diverse types and origins of contaminations, occurring during pre- or post-harvest. Microorganisms can already be present when milk is excreted (pre-harvest), for example, if the mammary gland is infected (mastitis), which is the most common disease associated with dairy cattle (Angulo, LeJeune, & Rajala-Schultz, 2009). This inflammation cannot always be visible and the source of infection ranges from bacteria, yeasts, mycoplasma, and algae, that can subsequentially be excreted into milk (Bradley, 2002). Also when the milk is being excreted it can come in contact with commensal microbiota that live in the teat skin, or on the epithelial lining of the teat canal or via the lactiferous duct (Isaac et al., 2017). Thereby, by the time the milk leaves the animal, microbial contamination may occur even in a healthy animal. Post-harvest contamination can derive from the dairy farm environment during production, collection, processing, distribution, and storage of milk. These contaminants may result from faecal, animal feed, mud, water, soil, human handling, farm utensils, distribution pipes, bulk, or transport tanks (Damm, Holm, Blaabjerg, Bro, & Schwarz, 2017). *Staphylococcus*, *Campylobacter*, *Listeria*, *Escherichia*, *Salmonella*, *Micrococcus*, *Clostridium*, *Yersinia*

enterocolitica and *Bacillus* (vegetative and spore cells) are commonly associated with milk contamination when improper or poor sanitary conditions are in place (Papademas & Bintsis, 2010). Maintenance of low temperature (4-10 ºC) once the milk is collected or transported for further processing remains one of the most crucial factors for the overall quality in raw milk, since it slows down microbial growth and chemical deterioration (Koutsoumanis, Pavlis, Nychas, & Xanthiakos, 2010). However, psychrophile microorganisms can proliferate under low temperatures, releasing lipases and proteases responsible for organoleptic changes, like rancidity and bitter off-flavours (McClements, Patterson, & Linton, 2001). Additionally, heat pasteurization is also ineffective in the inactivation of spores commonly found in the farm environment, like *Bacillus cereus* (Heyndrickx, 2011), and thus, spoilage of raw milk can easily occur when proper processing protocols are not followed correctly during pre- or post-harvest of milk (LeJeune & Rajala-Schultz, 2009).

Hyperbaric storage (HS) is a preservation methodology based on high pressure as a hurdle for microbial growth a like RF, that uses moderate pressures ranging from 25 to 100-220 MPa during lengthy periods of time, in fact during the whole storage period (Moreira et al., 2015; Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). This new methodology was accidently discovered when several perishable foods (sandwiches, soups, and apples) were recovered in good consumable conditions from a submersible that was sunken after 10 months at 1540 m depth (~15 MPa) at 3 ºC (Jannasch, Eimhjellen, Wirsen, & Farmanfarmalan, 1971). The combination of low temperature and pressure was assumed to be the main cause for the good preservation state observed for those recovered foods, and so, a few studies were subsequently carried out using those combined conditions in different foods (Charm, Longmaid, & Carver, 1977; Mitsuda, 1972). However, the feasibility to use HS at room temperature (RT) re-emerged in the recent decade, as a possibility to substitute RF, since no energy is required to control the temperature throughout the storage (Duarte et al., 2014; Queirós et al., 2014). This novel food preservation methodology is considered environmentally friendlier than conventional RF, as energy is only applied shortly in the compression and decompression phases of the pressure vessel, with considerably lower energy requirements (Bermejo-Prada, Colmant, Otero, & Guignon, 2017; Segovia-Bravo et al., 2012). One of the first studies concerning HS at RT was focused on strawberry juice (low pH) stored under 25, 100 and 220 MPa at 20 ºC for 20 days, successfully inhibiting microbial growth even under the lower tested pressure, 25 MPa (Segovia-Bravo et al., 2012).

In the following years, the possibility to store more perishable food products, watermelon, and melon juice (low acidity), was tested under a combination of different pressures (25-150 MPa) at and above RT (25-37 ºC), however only during short periods of time, from 8 to 60 hours (Fidalgo et al., 2014; Queirós et al., 2014; Santos et al., 2015). All authors were able to consistently observe that for these juices a minimal pressure of 50 MPa was required to inhibit microbial growth, and above 75 MPa microbial inactivation even during short periods of time at and above RT was achieve (Queirós et al., 2014). Later, HS at and above RT was reported to be able to extend the shelf-life of non-liquid highly perishable food products (minced pork meat, whey cheese and fresh salmon) stored for longer periods, 1 to 10 days, under pressures above 75 MPa (Duarte et al., 2017; Fernandes et al., 2018; Fidalgo, Lemos, Delgadillo, & Saraiva, 2018). All these results point to the possible increase of highly perishable foods shelf-life under HS at and above RT, potentially replacing and improving the common RF preservation effect.

To the best of the authors knowledge, the present study is the first work regarding HS of milk, and so, in this work the effects of HS on endogenous microflora (total aerobic mesophiles, total aerobic psychrophiles, lactic acid bacteria, Enterobacteriaceae, coliform bacteria, yeasts and moulds), and inoculated surrogate pathogens (*Listeria innocua*, *Escherichia coli*), pathogenic (*Salmonella senftenberg*) and bacterial spores (*Bacillus subtilis*) at 50, 62, 75 and 100 MPa in raw milk under naturally variable/uncontrolled RT was evaluated and compared with RF storage under atmospheric pressure (AP). In order to evaluate the possible effect of HS on microbial recovery, for the endogenous microflora, a post-HS study was also conducted under AP/RF.

4.2 Materials and Methods

4.2.1 Raw milk samples preparation and storage

Raw milk was kindly supplied by a local dairy farm association company and milk samples were packed under aseptic conditions, inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) in UV-light sterilized, low permeability polyamide-polyethylene bags (90 micron, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal), and heat-sealed individually, avoiding as much as possible leaving air inside.

HS experiments were performed in a high pressure equipment SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), equipped with a pressure vessel of 30 mm inner diameter and 500 mm height), at variable uncontrolled room temperature (RT). Determination of HS effect on endogenous microbial flora was determined in two different sets of experiments. In the first experiment, raw milk samples were stored at RT, refrigeration (RF, 4 ºC) at atmospheric pressure (AP, 0.1 MPa), and under 50/62/75 and 100 MPa at variable uncontrolled RT (18 - 22 ºC) during 7, 14, 28, 39 and 60 days. In the second experiment, raw milk samples presented higher initial microbial load than those of the first experiment, and were stored under 0.1, 50, 75 and 100 MPa for 1, 5, 15, 35, 60 and 130 days at RT and, a post-HS (PHS) experiment under refrigeration was conducted on those samples that had been previously stored for 15, 60 and 130 days under 75 and 100 MPa. For the PHS, samples were then stored under RF at AP for 5, 14, 30 and 60 days, to assess the possible effect promoted by prolonged high pressure exposure on microbial recovery under AP/RF conditions.

4.2.2 Inoculated pathogenic surrogate and pathogenic microorganisms

To study the effect of HS on pathogenic surrogate microorganisms, raw milk was inoculated with *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090, and pathogenic *Salmonella senftenberg* ATCC 43845. The three microorganisms were previously grown in Tryptic Soy Broth (TSB; Liofilchem, Italy) at 37 ºC for 24 h to ensure they reached the stationary phase. Late stationary phase is a well-known higher resistant pressure phase comparatively to the exponential-phase, where cells display a more rigid/thicker membrane and higher nucleoid condensation, which is believed to increase their viability under stress/high pressure (Mañas & Mackey, 2004). The grown microorganisms were inoculated into raw milk to achieve a final concentration around 4-5 log CFU/mL, and placed under different conditions, 50, 75 and 100 MPa at RT and AP/RF for comparison, during 3, 7, 10, 14, 21 and 31 days.

4.2.3 Bacillus subtilis endospores inoculation

Bacillus subtilis ATCC 6633 endospores preparation was performed as described by Pinto, Santos, Fidalgo, Delgadillo, and Saraiva (2018). Briefly, a liquid culture of *B. subtilis* was grown overnight in brain-heart infusion (BHI) broth at 30 ºC for 24 h, and afterwards

spread-plated into BHI-agar plates, which were incubated at 30 ºC for 15 days to allow sporulation to occur. Sporulation was confirmed by phase-contrast microscopy, then spores were harvested by 3-fold centrifugation in cold sterile distilled water (10 min at 5000 × g at 4 ºC) and stored at 4 ºC until use. Endospores were then inoculated into raw milk, in order to reach a final concentration around 5-6 log CFU/mL. *Bacillus subtillis* vegetative and endospores endogenous and inoculated loads were studied throughout the storage period, during 1, 4, 7, 21, 31 and 60 days under AP/RF, AP/RT and under 50, 75 and 100 MPa at RT. To clearly distinguish between endospore germination and inactivation, aliquots of milk were heat-treated at 80 ºC for 20 min, allowing to inactivate germinated spores and vegetative forms (Black et al., 2005).

4.2.4 Microbial analyses

After each experiment, samples were serially diluted in Ringer's solution, except for *B. subtilis*, which was serially diluted in physiological solution (0.9% NaCl) and plated on the appropriate media. Total aerobic mesophiles (TAM) and total aerobic psychrophiles (PSY) were enumerated on plate count agar (PCA), incubated at 30 ºC and 20 ºC for 3 and 5 days, respectively (ISO 4833:2013). Enterobacteriaceae (ENT) counts were determined on violet red bile glucose agar (VRBGA), incubated at 37 ºC for 1 day (ISO 21528:2017). Lactic acid bacteria (LAB) counts were determined on Man Rogosa Sharpe agar (MRS) and incubated at 30 ºC for 3 days (ISO 11133:2014). Coliform bacteria (COL) were enumerated on chromocult coliform agar (CCA), incubated at 37 ºC for 1 day (ISO 4832: 2007). Yeasts and moulds (YM) were enumerated using rose bengal chloramphenicol agar (RBCA) at 25 ºC for 5 days (ISO 21527:2008). *Listeria innocua* ATCC 33090 was determined in PALCAM Listeria agar base with the selective supplement PALCAM (FD061) and incubated at 37 ºC for 2 days (ISO 11290-1:2017). *Escherichia coli* ATCC 25922 was enumerated in CCA after incubation at 37 ºC for 1 day (ISO 9308-1:2014). *Salmonella senftenberg* ATCC 43845 was incubated on xylose lysine deoxycholate agar at 37 ºC for 1 day (ISO 6579-1:2017). *Bacillus subtillis* was enumerated in BHI-agar and incubated at 30 ºC for 1 day (ISO 7932:2004).

All the results were expressed as decimal logarithm of colony forming units per millilitre of raw milk (log CFU/mL).

4.2.5 D_p-value and z_p-value determination

Determination of the D_p- and z_p-values was carried out for the microbial groups analysed in this study for which inactivation was verified and measurable (values below the quantification and detection limits were not considered), and for all cases a first order inactivation kinetics was verified. D_p-value is the time needed at a constant pressure, to reach a decimal reduction in the microbial load (expressed here in days) and was calculated based on the negative inverse of the log linear slope (**Equation 4.1**), while z_p-value, the pressure resistance (here expressed in MPa), was calculated based on the D_p-values of the different HS conditions for a specific microorganism type, determined as the negative reciprocal of the slope as shown **Equation 4.2**:

$$Log\ (N)\ = Log\ (N_0) - \frac{t}{D_p} \qquad \textbf{Equation 4.1}$$

N is the microbial load (CFU/ml) under a certain pressure (MPa) for certain time (t) in days, and N_0 is the initial microbial load (CFU/mL). The slop was obtained from the log linear decreased throughout storage, under a certain pressure.

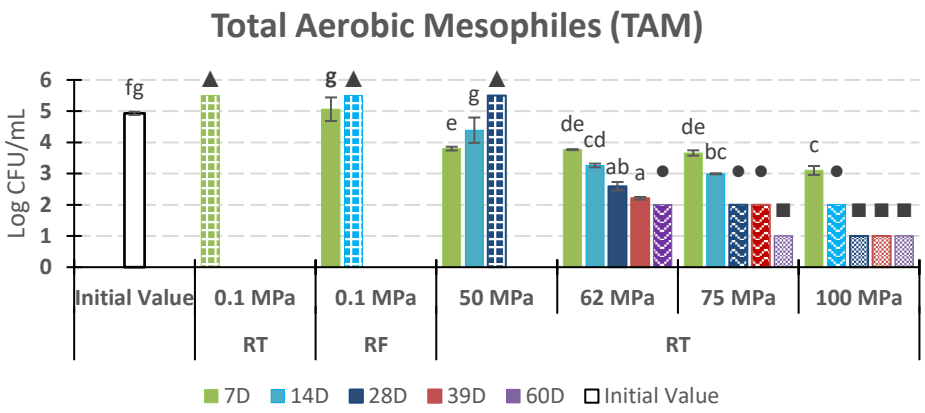
$$\text{Log D} = \text{Log D}_0 - \frac{P - P_0}{z_p} \qquad \textbf{Equation 4.2}$$

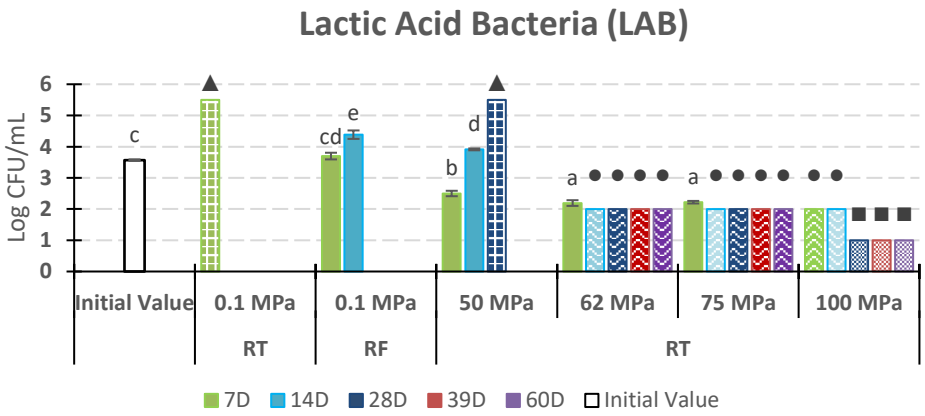
where D and D_0 (in days) are D_p-values at pressures P and P_0 (in MPa), respectively, being P_0 a reference pressure, here considered as zero MPa.

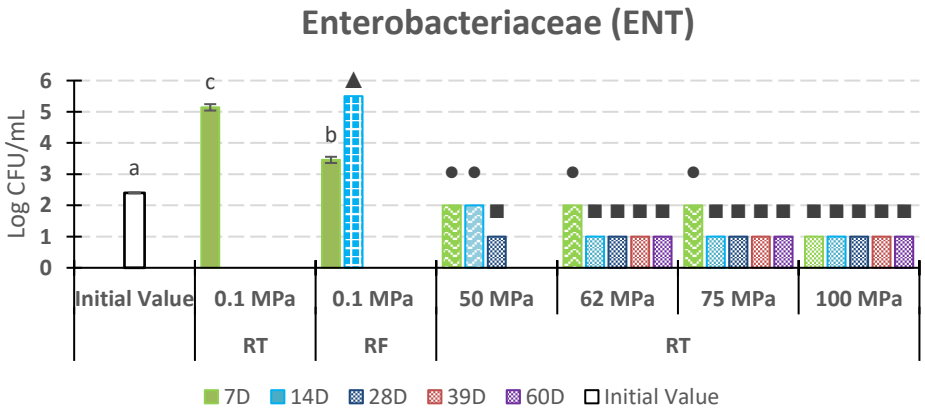
4.2.6 Statistical analysis

All experiments were carried out in triplicate and all analyses were done in triplicate. The different storage conditions were compared using Analysis of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance.

4.3 Results and Discussion







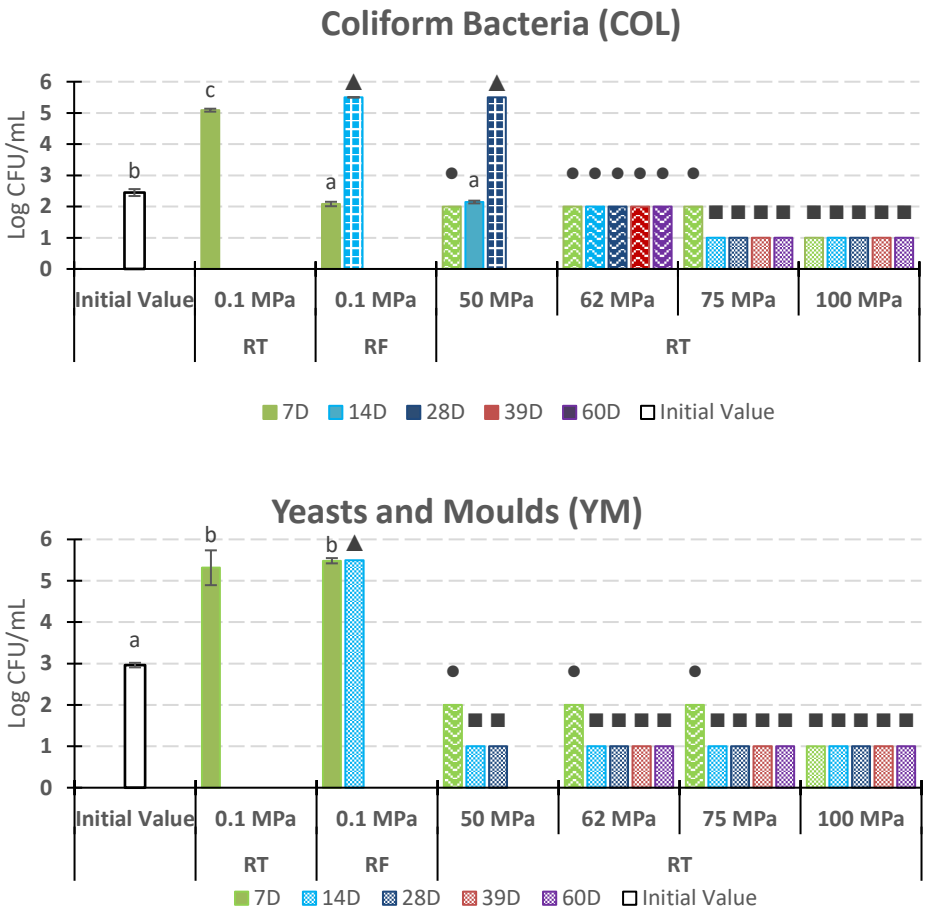


Figure 4.1 - TAM, LAB, ENT, COL and YM microbial evolution during HS at uncontrolled room temperature (RT) of raw milk used in the first set of experiments, and comparison with storage under refrigeration (RF) and RT at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where ▲, • and ■ represent counts above the acceptable (5.5 log CFU/mL), and below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively.

4.3.1 Microbial analyses

The acceptable microbial limits for cow's raw milk vary between countries legislation (Ledenbach & Marshall, 2009). In the EU, TAM counts below 5 log CFU/mL reflect good milk production hygiene, to be considered for further thermal processing (Nunes, 2009), while raw milk used to produce dairy products, immediately before transformation, should also contain TAM counts below 5.5 log CFU/mL (EC Regulation Nº 853/2004). This study was divided in two stages, with raw milk samples from the first experiment revealing a microbial load within the limits allowed for raw milk before pasteurization in the EU (TAM counts below 5 log CFU/mL (EC Regulation Nº 853/2004)), and those from the second

experiment containing a higher microbial load above this limit in order to simulate a worst-case scenario. Samples from the first study presented initial microbial counts around 4.93 ± 0.05, 3.57 ± 0.02, 2.96 ± 0.06, 2.45 ± 0.11 and 2.40 ± 0.02 log CFU/mL for TAM, LAB, YM, COL and ENT respectively (**Figure 4.1**). It is important to note that in this study, when samples from a storage condition achieved TAM counts above 5.5 log CFU/mL, the acceptable limit considered, the samples were withdrawn from the experiment and no further analyses were performed regarding such storage condition. As expected, samples stored at room temperature and atmospheric pressure (AP/RT) after 7 days of storage presented higher ($p < 0.05$) microbial counts well above the acceptable threshold (≥5.5 log CFU/mL for TAM and LAB), and of >5.00 log CFU/mL for COL, ENT and YM (**Figure 4.1**). Refrigerated storage (AP/RF) was able to slow down ($p > 0.05$) the microbial growth of TAM, LAB and COL bacteria up to the 7th day of storage, while ENT and YM presented higher counts ($p < 0.05$), comparatively to the initial ones, 3.46 ± 0.10 and 5.48 ± 0.06 log CFU/mL, respectively. After 14 days at AP/RF storage, raw milk was microbiologically unacceptable, with TAM, ENT, YM and COL counts reaching values above 5.5 log CFU/mL, while LAB counts presented an overall increase ($p < 0.05$) of approximately 1 log unit. Previous works reported that even under AP/RF, TAM and psychrophile bacteria (PSY) are capable of proliferate in milk and release extracellular hydrolytic enzymes (some thermoresistant) resulting in overall nutritional quality losses (Pinto, Martins, & Vanetti, 2006); YM metabolism may produce by-products that cause off-odours and unpleasant flavours later on when transformed into dairy foods (Giudici, Masini, & Caggia, 1996); and LAB, COL and ENT may produce gas, and several metabolites that favour the development of off-flavours (Bintsis, 2018; Frank, 2007).

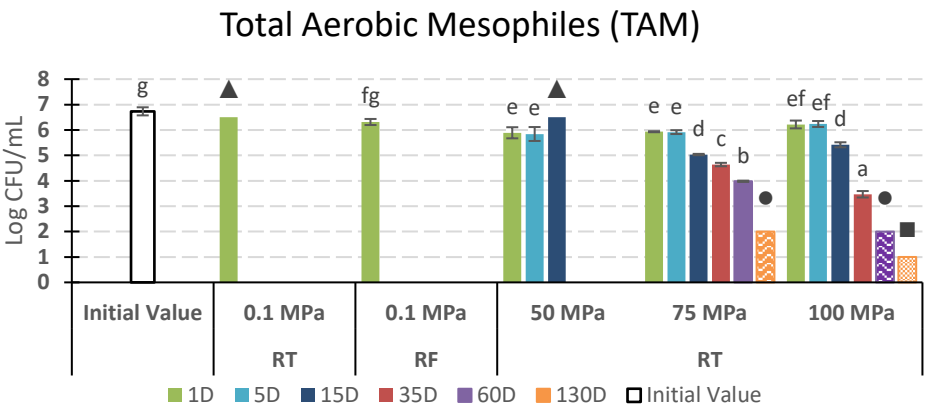
Regarding HS, at the 7th day of storage at the lower pressure (50 MPa), all studied microorganisms were affected, being reduced either to counts below the quantification limit of 2 log CFU/mL, in the case of COL, ENT and YM, or as observed for TAM and LAB counts, undergoing a decrease ($p < 0.05$) of approximately 1 log unit. At the 14th day of storage TAM, LAB and COL were able to grow to values similar to the initial ones (still within the acceptable limit), extending the microbial shelf-life of raw milk under 50/RT comparatively to AP/RF, and it was only at the 28th day of storage that microbial counts reached values above the acceptable limit. This behaviour of TAM and LAB was also observed when watermelon juice and fresh salmon were stored under 50 MPa at 15 ºC, after

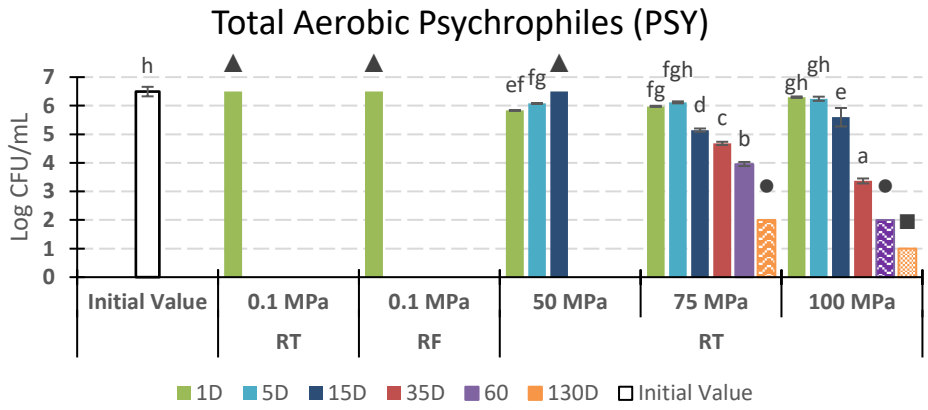
3 and 6 days of storage, respectively (Fidalgo et al., 2019; Lemos, Ribeiro, Fidalgo, Delgadillo, & Saraiva, 2017). TAM microbial group is very heterogeneous, since for example, several microorganisms in milk can grow on PCA medium, from gram-positive, like *Bacillus* spp., to gram-negative bacteria like *E. coli* and other coliforms. So, the initial decrease in TAM counts may be related to the decrease in gram-negative bacteria groups as observed for ENT and COL counts since gram-negative bacteria tend to be more sensitive to high pressure (Tomasula et al., 2014).

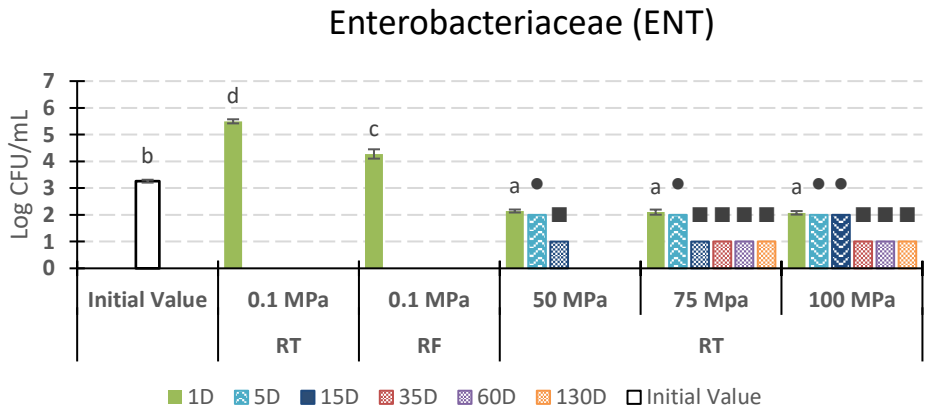
For HS at 62 and 75 MPa, a similar effect on the microbial load was observed for both storage conditions and after 7 days of storage, LAB, YM, COL and ENT counts were reduced to microbial counts below 2 log CFU/mL, with a significant ($p < 0.05$) reduction for TAM, which presented similar values between these two storage conditions (3.77 ± 0.02 and 3.66 ± 0.08 log CFU/mL at 62 and 75 MPa, respectively). TAM counts were gradually reduced ($p < 0.05$) throughout the storage period, reaching the quantification limit at the 60th day of storage at 62/RT. This inactivation effect was faster for samples stored at 75/RT, which reached counts lower than 2 log CFU/mL right after 28 days of storage, and below 1 log CFU/mL (detection limit) at the 60th day of storage, which is in agreement with previous observations reported by Santos, Castro, Delgadillo, and Saraiva (2020), who observed a greater inactivation effect at 75 MPa over 60 MPa, for TAM and LAB counts in raw bovine minced meat throughout storage. When raw milk was stored at 100 MPa just after 7 days, ENT, YM and COL bacteria were all inactivated below the detection limit, LAB were inactivated below 2 log CFU/mL, and TAM were significantly ($p < 0.05$) reduced to 3.10 ± 0.14 log CFU/mL (2 log units reduction). Overall, the microbial load of those samples remained low with LAB and TAM achieving counts below 1 log CFU/mL at the 28th day of storage, with no further changes until the end of the study.

As observed in **Figure 4.1**, for 50 MPa the results were comparable to RF (microbial growth slowdown) but to a greater extent, thus pointing to a possible longer microbial shelf-life extension. Additionally, higher pressures (62-100 MPa) resulted in progressively higher microbial inactivation and so better microbial proliferation control throughout storage, pointing to a minimal pressure of 62-75 MPa to maintain raw milk microbiologically stable, for at least 60 days of storage without temperature control. Noteworthy, at 100 MPa all studied microbiological groups were at least below the quantification limit after 14 days and below the detection limit onwards. Thus, all HS conditions resulted in better microbial

preservation than AP/RF and it is important to highlight, HS yielded these results at RT with no energetic costs throughout storage with considerable microbial inactivation.







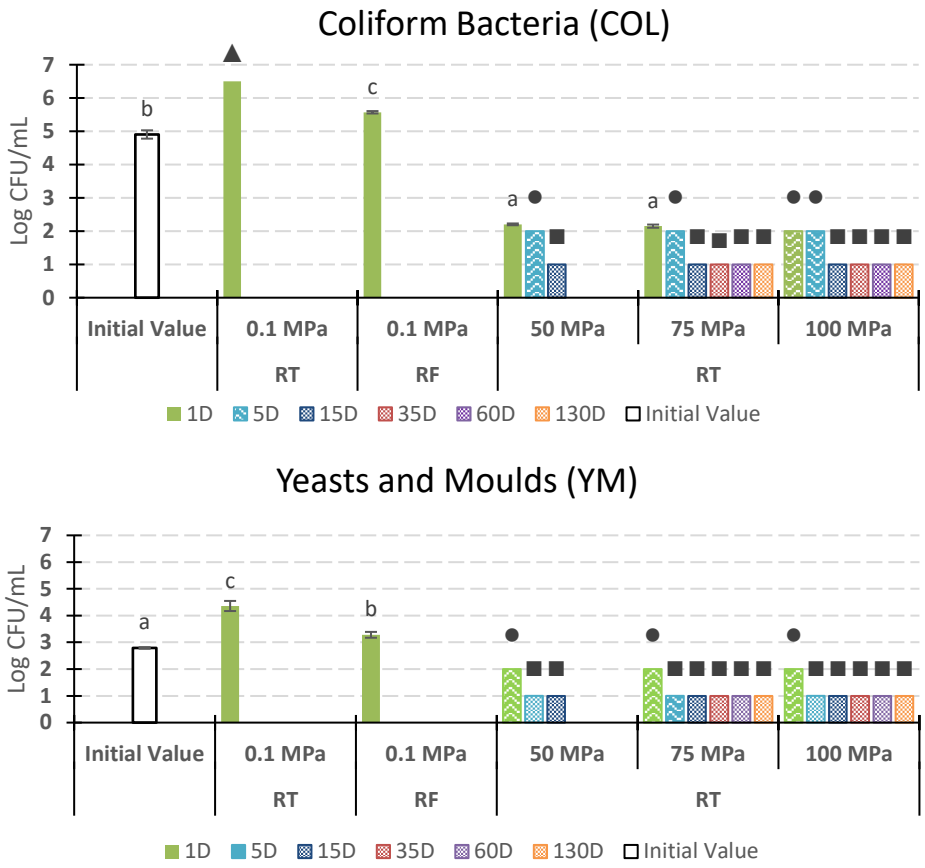


Figure 4.2 - TAM, PSY, ENT, COL and YM microbial evolution during HS at uncontrolled room temperature (RT) of raw milk used in the second set of experiments, and comparison with storage under refrigeration (RF) and RT at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively. While ▲, represent counts above the limit defined for this storage experiment interruption (6.5 log CFU/mL), due to considerable initial spoilage.

In the second part of the experiment, raw milk with a higher microbial load was used to simulate a worst-case scenario in order to study the effect of HS on samples with higher microbial spoilage levels and for longer storage periods (130 days) at 50, 75 and 100 MPa (since in the previous study, 62 and 75 MPa storage achieved comparable results, only HS at 75 MPa was further selected) at RT and compared to storage under AP at 4 ºC. The initial microbial load was 6.73 ± 0.16, 6.49 ± 0.17, 4.90 ± 0.12, 3.26 ± 0.05 and 2.79 ± 0.03 log CFU/mL for TAM, PSY, COL, ENT and YM respectively (**Figure 4.2**). As mentioned previously, TAM counts in raw bovine milk above 5.5 log CFU/mL are beyond the acceptable limit, so in this part of the study a higher microbial limit was considered (6.5 log

CFU/mL) for experiment interruption. Due to the higher spoilage levels of the milk used in the second set of experiments, initially shorter sampling periods were selected, 1 and 5 days, comparatively to 7 days studied in the first set. Just after 1 day, even at lower temperatures (AP/RF) a significant increase in COL, ENT and YM counts were observed ($p < 0.05$), which was significantly more pronounced under storage at AP/RT, above 1 log unit (**Figure 4.2**). Differently and interestingly, even at the lowest pressure, 50 MPa, COL and ENT were significantly affected ($p < 0.05$) just after 1 day of storage, with a reduction of 2.70 and 1.12 log units, respectively and with YM counts being reduced to below the quantification level. As observed in the first set of experiments, for this pressure level, the more baro-resistant microbial groups (TAM and PSY) also underwent significant reductions ($p < 0.05$) in the first days, with a reduction of approximately 1 and 0.7 log units in the first day of storage, respectively. TAM and PSY growth was slowed down up to the 5th day of storage ($p < 0.05$), presenting counts around 5.84 ± 0.27 and 6.08 ± 0.01 log CFU/mL respectively, however, at the 15th day both reached counts above 6.5 log CFU/mL, while ENT, COL and YM reached counts below the detection limit. At 75 MPa, again ENT, COL and YM were highly susceptible to pressure, presenting counts below the detection limit by the 15th day of storage. Concurrently, storage at 75/RT in the first day reduced TAM counts around 0.80 log units ($p < 0.05$), staying stable until the 5th day, followed by a gradual reduction ($p < 0.05$) throughout storage, reaching the quantification level at the 130th day. A similar inactivation effect on PSY counts was observed, which were gradually reduced over time ($p < 0.05$), noteworthy the remarkable reduction of ≥4.5 log units at the 130th day of storage, when compared to the initial load. It is relevant to note the importance in quality and proper management of raw milk, and its impact in the initial microbial load, as it took more than 4 times longer for samples used in the second experiment to reach the quantification limit, when compared to samples used in the first experiment (initial load of 4.93 ± 0.05 and 6.73 ± 0.16 log CFU/mL, regarding TAM counts, respectively). As for 100/RT the inactivation effect was more pronounced when compared to 75/RT (**Figure 4.2**), with TAM and PSY counts inactivated faster throughout the storage, reaching values below the detection limit after 130 days of storage.

Differences in the inactivation rates between the different HS conditions can also be assessed by the calculated D_p-values (*Annex A,* ***Figure A.1*** *and* ***Table A.1***). Storage under 75 and 100 MPa resulted in D_p-values of 25.6 and 12.8 days for TAM, respectively, similarly

to PSY, D_p-values of 25.6 and 13.0 days at 75 and 100 MPa, respectively. When comparing the D_p-values of TAM and PSY under 100/RT over 75/RT, the inactivation was 2 times faster under 100/RT, with both microbial groups reaching counts below the quantification level at day 60 and 130 of storage under 100 and 75 MPa, respectively.

4.3.2 Post-hyperbaric Storage

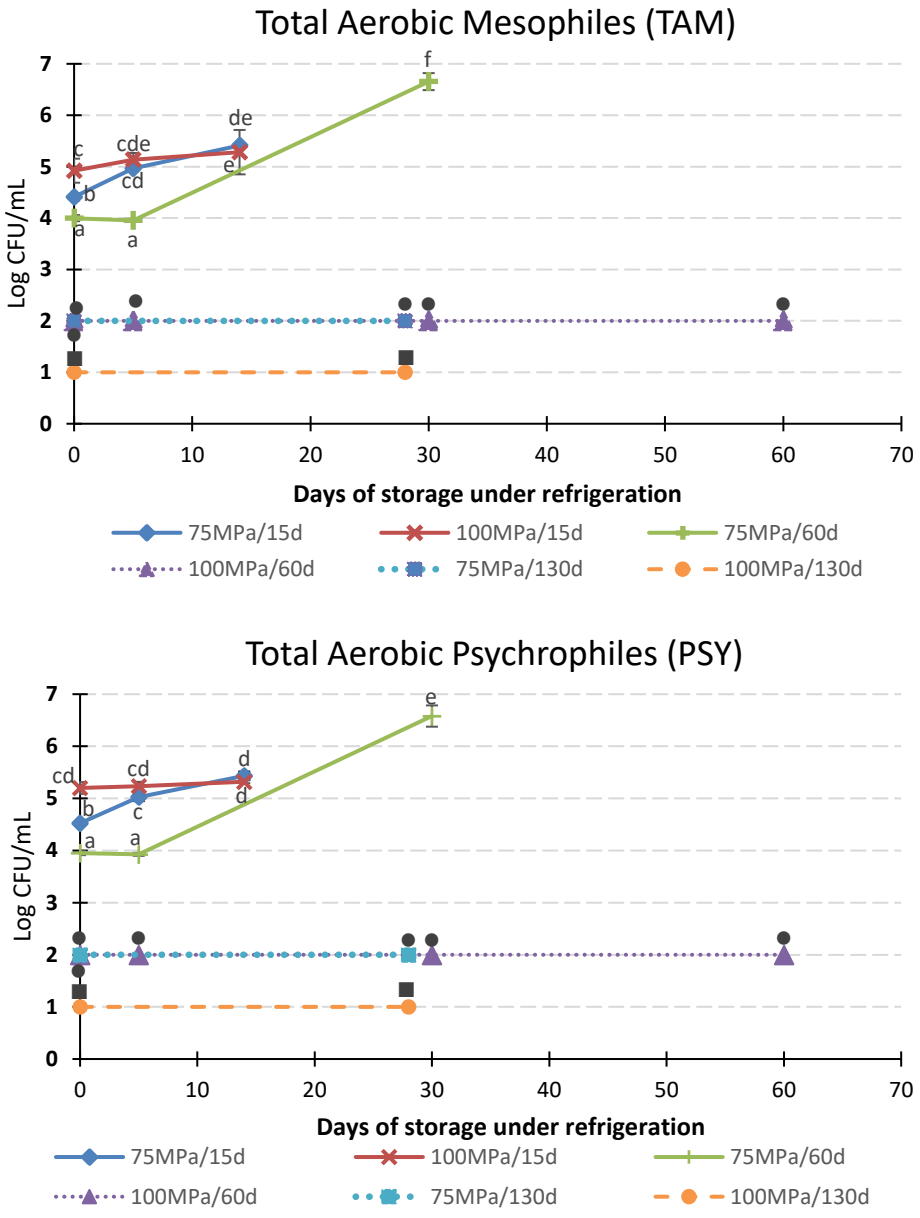


Figure 4.3 - TAM and PSY microbial evolution during PHS under refrigeration (4 ºC) of raw milk used in the second set of experiments, stored under HS of 75 and 100 MPa for 15, 60 and 130 days at room temperature (RT). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively.

A post-hyperbaric storage (PHS) was carried out after samples (used in the second set of experiments) had been put under HS and consisted in storing them at AP/RF, to evaluate possible impairment in microbial recovery. The samples selected for PHS were the ones stored first under 75 and 100 MPa for 15, 60 and 130 days at RT, presenting distinct levels of exposure and intensity to pressure. Samples stored under HS for 15 days at both 75 and 100 MPa (75MPa/15d and 100MPa/15d), showed reduced TAM microbial growth over time when stored at AP/RF (**Figure 4.3**), increasing around 1 and 0.6 log units after 15 days ($p < 0.05$), respectively, which is still lower than the acceptable limit selected in the PHS study (6.5 log CFU/mL).

Samples stored under 75MPa/60d presented no signs of microbial proliferation in the first 5 days under AP/RF ($p > 0.05$), however at the 30th day of storage, TAM and PSY reached counts of 6.65 ± 0.17 and 6.58 ± 0.20 log CFU/mL, respectively ($p < 0.05$). On the other hand, for the higher pressure, samples that initially presented counts below the quantification limit (100 MPa/60d), remained low even after 60 days under AP/RF, regarding both TAM and PSY counts. The same behaviour was observed for samples stored at 75MPa/130d and 100MPa/130d, to which TAM and PSY counts remained below the quantification and detection limit, respectively, after 28 days under AP/RF. Other microbial groups (ENT, COL and YM) that were already below the detection limit for samples initially stored under 75 and 100 MPa for 15, 60 and 130 days at RT, remained undetectable (≤1 log CFU/mL) during the PHS period (*results not shown*). Low HP (20-200 MPa) has proven to interfere with several mechanisms associated to cellular viability, affecting, for instance, membrane stability, ribosomes association, nutrient uptake, gene expression such as replication and transcription (Abe, 2007). The magnitude to which these effects may result in cellular death, are not only related to the intensity of HP, but also on other factors such as HP duration, pH, and medium composition (Bull, Hayman, Stewart, Szabo, & Knabel, 2005). After prolonged exposure to pressure during HS, the remaining viable microbial cells would supposedly require more time and resources for full cellular recovery, which would allow microbial growth after the imposed sub-lethal damage. As reported before, temperature plays a crucial role in cell recovery after HPP, in a study conducted with *E. coli* (Koseki & Yamamoto, 2006) and another with *L. monocytogenes* (Bull et al., 2005), both microorganisms presented a better recovery rate when incubated at 25 and 15 ºC,

respectively, when compared to post incubation under AP/RF. In the present work, the results obtained during the PHS period may indicate that the degree of intracellular injury could be related to the duration and intensity of HS. For instance, samples kept at 75MPa/15d and 100MPa/15d, when stored under RF, presented different growth rates. Regarding PSY counts, 75MPa/15d showed significant growth ($p < 0.05$) both at day 5 and 15, while on the other hand, PSY counts of 100MPa/15d condition, remained stable during the 15 days ($p > 0.05$) at AP/RF. This may indicate, since no information in available regarding the effect on microbial recovery after such longer exposure times to HP, that longer periods under HS increase the intensity of sub-lethal damages, which will decrease the ability to recover afterwards. Samples kept at 100MPa/60d, when placed at AP/RF, presented a stable microbial load evolution, even after 60 days at AP/RF. This may indicate a greater microbial stability of raw milk when stored at AP/RF after HS, which can also contribute to an extended shelf-life under PHS.

4.3.3 Inoculated microorganisms

Campylobacter, *Listeria monocytogenes*, pathogenic *E. coli* and *Salmonella* are among the most commonly and important epidemiological pathogens found in milk, which contamination may derive mainly from improper raw milk handling or processing (LeJeune & Rajala-Schultz, 2009; Quigley et al., 2013). Raw milk was inoculated with two pathogenic-surrogate microorganisms, *E. coli* ATCC 25922 and *L. innocua* ATCC 33090, with pathogenic *S. senftenberg* ATCC 43845 (to a final concentration around 5 log CFU/mL) and then stored under AP/RF, and also under 50, 75 and 100 MPa at RT. Prior to raw milk inoculation, evaluation of endogenous *E. coli*, *Salmonella* and *Listeria* was conducted, with the last two being undetected (below the detection limit), while *E. coli* was below the quantification level (2 log CFU/mL).

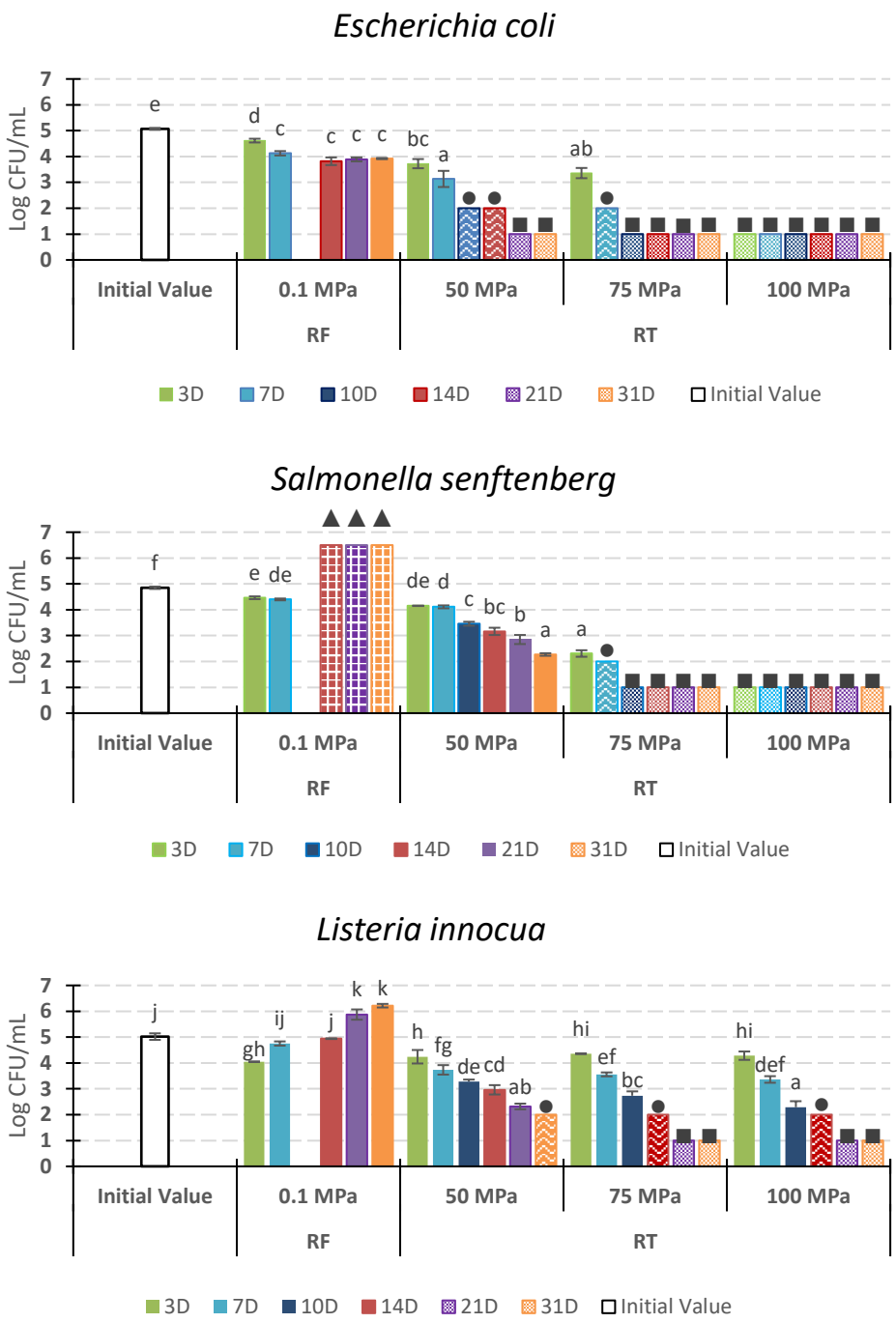


Figure 4.4 - Evolution of viable cell numbers of inoculated *Escherichia coli*, *Salmonella senftenberg* and *Listeria innocua* during HS at room temperature (RT) of raw milk, and comparison with storage under refrigeration (RF) at atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/mL) and detection limits (1 log CFU/mL), respectively. While ▲ represent counts above the limit defined for this storage experiment interruption (6.5 log CFU/mL).

After inoculation, the initial load for *E. coli*, *S. senftenberg* and *L. innocua* was 5.07 ± 0.04, 4.85 ± 0.04 and 5.02 ± 0.13 log CFU/mL, respectively (**Figure 4.4**). When stored under RF, all microorganisms were initially affected ($p < 0.05$), with a reduction around 0.4, 1.0 and 0.4 log units on day 3, for *E. coli*, *S. senftenberg* and *L. innocua*, respectively. This could result from difficulties in adaptation for the inoculated microorganisms to the new environment (raw milk), and since raw milk was not heat treated, this initial decrease could be related to the competition between the endogenous microbiota, like lactic acid bacteria with the inoculated microorganisms (Arias, Monge-Rojas, Chaves, & Antillón, 2001). *Escherichia coli* was able to retain its counts at constant levels during refrigerated storage without significant growth after the 7th day ($p > 0.05$), similarly to that observed by Zapico, Gaya, Nuñez, and Medina (1995) and Guraya, Frank, and Hassan (1998), wherein *E. coli* stored at AP/RF maintained similar counts from the beginning until the last days of storage, 7th and 35th days, respectively. Indeed, this microorganism is able to survive and maintain high viable cell numbers, even after longer storage periods at refrigerated temperatures (Guraya et al., 1998). When placed under HS, at the 3rd day, *E. coli* counts were gradually reduced ($p < 0.05$) at the lowest pressure (50 MPa), reaching counts bellow the quantification and detection limits at day 10 and 21 of storage, respectively, corresponding to a D_p-value of 3.7 days (*Annex A, **Figure A.2** and **Table A.1***). The inactivation effect was stronger for 75 MPa, reaching values below the detection limit at the 10th day, remaining constant throughout the storage. Under 100 MPa, *E. coli* counts were already absent at day 3 (and even after 31 days of storage), highlighting the fast inactivation effect of this storage condition, when compared to the lowest one studied. *Escherichia coli* O157:H7 is the most prominent pathogenic strain of *E. coli*, which can cause food poisoning illness even in low numbers (that could be as low as 10 cells), and thus, if *E. coli* O157:H7 survives pasteurization, it is important to keep this microorganism absent (Bolton, Crozier, & Williamson, 1996; Phillips, 1999).

Salmonella senftenberg, the other gram-negative microorganism studied, presented a similar behaviour under HS, being inactivated below the detection limit after 10 and 3 days when stored under 75 and 100 MPa, respectively. At 50/RT, *S. senftenberg* counts were gradually reduced ($p < 0.05$) along storage, reaching a minimum of 2.27 ± 0.05 log CFU/mL at the end of storage experiments (**Figure 4.4**), resulting in a calculated D_p-value of 12.7 days (*Annex A, **Figure A.2** and **Table A.1***). While some species of *Salmonella* do not grow

at temperatures below 6 ºC, others are able to grow although at a slower rate (Muir, 1996) and, despite the initial decrease in *S. senftenberg* counts when placed under RF, it ended up reaching higher counts (≥6.5 log CFU/mL) at the 14th day, outlining the need to implement suitable preservation methods capable to inhibit the growth or even inactivate several critical pathogenic microorganisms that can grow under AP/RF before/after pasteurization, and thus preventing food safety issues.

Listeria monocytogenes is a well-known gram-positive psychrophilic microorganism, capable to grow under refrigerated temperatures as low as 0.4 ºC and up to 42 ºC (Muir, 1996; Sergelidis et al., 1997). In the present study, after the 3rd day of storage under AP/RF, *L. innocua* was able to increase its counts slowly, surpassing the initial load on day 21, reaching around 6.22 ± 0.07 log CFU/mL at the 31 day of storage ($p < 0.05$), presenting in this case a rate increase of 0.076 log CFU/mL per day (*Annex A*, ***Figure A.2***). Initially, inoculated samples stored under HS presented similar values at day 3 when compared to AP/RT, however, *L. innocua* counts decreased continuously in the following days under all three HS conditions ($p < 0.05$). *Listeria innocua* counts were gradually reduced under 50/RT throughout the storage ($p < 0.05$), reaching values below 2 log CFU/mL at day 31. This trend is quite interesting considering that, in previous studies performed by Pinto et al. (2017) it was demonstrated that *L. innocua* was able to proliferate in watermelon juice stored under HS at 50 MPa (for 10 days), reaching values above 6 log CFU/mL. The difference between this study and the aforementioned one, may be due to the common presence of lactic acid bacteria in milk, which has shown to contribute to the inhibition of spoilage and pathogenic microorganisms, present in the composition of dairy products (Grattepanche, Miescher-Schwenninger, Meile, & Lacroix, 2008). Storage under 75 and 100/RT caused similar reductions between these two storage conditions on *L. innocua* counts ($p > 0.05$) relatively to similar storage periods, with the exception being on day 10, where 100/RT samples presented a significant reduction around 0.5 log units ($p < 0.05$), comparatively to samples stored under 75/RT. As mentioned, all HS conditions were able to inactivate *L. innocua*, however at different rates, with D_p-values of 8.6, 4.5 and 3.7 days for 50/RT, 75/RT, and 100/RT respectively (*Annex A*, ***Figure A.2*** *and* ***Table A.1***), with a z_p of 138.9 MPa. *L. monocytogenes* is stated in the literature to have a minimal dose that may cause food poisoning of around 10 to 100 cells (Golnazarian, Donnelly, Pintauro, & Howard, 1989; Schlech, 1988) with milk and other dairy products considered one of the main vehicles types

for human infection, with a lethality around 30% caused from listeriosis (Barancelli, Silva-Cruz, Porto, & Oliveira, 2011; Rocourt, BenEmbarek, Toyofuku, & Schlundt, 2003). Even after processing, *L. monocytogenes* can recover at lower temperatures during storage, as described by Ritz, Pilet, Jugiau, Rama, and Federighi (2006), where *L. monocytogenes* was able to recover and grow when placed under AP/RF after HPP of 400 MPa/10min. *Listeria* is a persistent problem in the food industry, mainly due to its ability to produce biofilms, a three-dimensional matrix of extracellular polymeric substances, that acts as a reservoir for *Listeria* colonies, offering protection against antimicrobial agents (Djordjevic, Wiedmann, & McLandsborough, 2002). These protected reservoirs can also allow the growth of spoilage bacteria, being located in places where water is abundant and where cleaning in not performed adequately (Borucki, Peppin, White, Loge, & Call, 2003). Considering the aforementioned and the results obtained regarding *L. innocua*, this may be a good indication for the implementation of HS in the future.

Under HS (75 and 100 MPa), *L. innocua* appears to be more pressure resistant than the other ones studied, with *L. innocua* counts reaching values below the detection limit under 75/RT and 100/RT at day 21 for both conditions, comparatively to raw milk inoculated with *E. coli* and *S. senftenberg* that reached the same level of inactivation on day 3 and 7 for 100/RT and 75/RT, respectively. As mentioned before, one of the main targets of HP for pasteurization is the microbial membrane (Georget et al., 2015; Morimatsu, Inaoka, Nakaura, & Yamamoto, 2019), affecting its fluidity, stability, and integrity of membrane-bound protein, compromising the normal membrane functions that can result in no osmotic response and in intercellular material leakage (Abe, 2007; Huang, Lung, Yang, & Wang, 2014). Gram-positive microorganisms are characterized by a thicker peptidoglycan layer when compared to gram-negative microorganisms, which reflects a greater pressure resistance (Alpas et al., 1999; Patterson, Quinn, Simpson, & Gilmour, 1995).

4.3.4 Bacillus subtilis vegetative and endospores load

Bacillus spp. are widely present in the natural microbiota of raw milk and can be introduced from soil, bedding materials, silage, faeces, water, and feed (Magnusson, Christiansson, & Svensson, 2007; Slaghuis, Te Giffel, Beumer, & André, 1997). *Bacillus cereus* is of high interest in the dairy industry since this pathogen can form heat-resistant endospores and produce toxins (Gopal et al., 2015). *B. subtilis* is also commonly found in

dairy environments and has been used as surrogate endospores form of *B. cereus* in several food inactivation models (Jagannath & Tsuchido, 2003). Non inoculated raw milk was microbiologically evaluated, as a control, for *B. subtilis* total endogenous vegetative and total endogenous endospores loads, the ones that survived the heat treatment (80 ºC for 20 min), in all tested storage conditions. After inoculation, raw milk contained both endogenous and inoculated endospores. Initially it was observed that the endogenous vegetative load of *Bacillus* was naturally high in raw milk samples, ranging from 6.06 ± 0.04 to 6.22 ± 0.02 log CFU/mL, with an endogenous endospores load of 3.26 ± 0.07 log CFU/mL, which is within the values reported in the literature (Magnusson et al., 2007), increasing to 5.59 ± 0.10 log CFU/mL after inoculation (**Figure 4.5**). Endogenous endospores load in control samples, presented a similar behaviour in all storage conditions, comparatively to the inoculated endospores ones, and thus are not represented in **Figure 4.5**.

Under AP/RT storage, regarding inoculated samples, both vegetative and endospores load increased significantly after 1 day ($p < 0.05$), around 2 and 0.2 log units respectively, reaching a total of 8.54 ± 0.02 and 6.40 ± 0.36 log CFU/mL, respectively, on the 4th day of storage ($p < 0.05$). This increase in overall *B. subtilis* load was also observed in non-inoculated samples, possibly due to the increase of microbial population in raw milk samples leading to nutrient depletion and pH decrease which often initiate endospores complex development (Coorevits et al., 2011).

Under AP/RF, inoculated samples of *B. subtilis* vegetative load increased around 1.6 log units after 7 days ($p < 0.05$), remaining constant ($p > 0.05$) until the end of the storage period (8.07 ± 0.09 log CFU/mL), with the endospores load remaining relatively constant throughout storage ($p > 0.05$).

Interestingly, storage under HS/RT presented different results, regarding the lower (50 MPa) and the higher pressures (75 and 100 MPa). As for the vegetative load of inoculated samples, 50/RT was insufficient to inhibit the growth of *B. subtilis* (Gram-positive), allowing a significant growth ($p < 0.05$) throughout the studied period, reaching counts of 7.14 ± 0.02 log CFU/mL on the 31st day. Storage at 75/RT and 100/RT, were able to successfully inactivate *B. subtilis* vegetative load along the storage time ($p < 0.05$), being the inactivation superior for 100/RT, with both storage conditions allowing a gradual decrease in microbial counts to 3.08 ± 0.05 and 2.40 ± 0.17 log CFU/mL after two months of storage, under 75/RT and 100/RT, respectively. These two storage conditions presented a D_p-value of 21.7 and

16.0 days, regarding *B. subtilis* vegetative load inactivation, under 75 and 100 MPa, respectively (*Annex A,* ***Figure A.3*** *and* ***Table A.1***). Regarding the endospores load, storage at 50/RT slightly reduced their counts until the 21st day ($p < 0.05$), to 4.73 ± 0.10 log CFU/mL, which increased at the 31st day, to 5.10 ± 0.08 log CFU/mL ($p > 0.05$), while a significant reduction in the endospores load ($p < 0.05$) was observed at 75/RT, about 1 log unit just after one day and inactivation to counts below the quantification level (2.30 log CFU/mL) on the 21st day, reaching counts below the detection level (1.30 log CFU/mL) on the 60th day. A faster inactivation effect was observed for 100/RT ($p < 0.05$), reducing endospores counts below the quantification level on the 7th day, and reaching undetectable counts on the 31st day, remaining thereafter constantly low, until the end of the storage period. In fact, 100/RT was more than two-fold faster at inactivating endospores, with a D_p-value of 2.4 days, comparatively to 75/RT, D_p-value of 6.7 days, while a higher D_p-value was achieved under 50/RT of 27.0 days, resulting in a z_p of 47.6 MPa (*Annex A,* ***Figure A.3*** *and* ***Table A.1***). Endospores are highly resistant to extreme conditions such as pressure, extreme heat or cold, drought, biocides, and UV irradiation (Gopal et al., 2015), although low pressure (40-100 MPa) has been proved to induce germination in combination with the available nutrients, through activation of the nutrient-like receptors gerA gerB and gerD, by inducing conformational changes in their active sites (Wuytack, Soons, Poschet, & Michiels, 2000). Therefore, HS may trigger endospores germination, followed by outgrowth inhibition due to pressure, and thus resulting in endospore inactivation under pressures equal to above of 75 MPa, as observed in the present work for raw milk. The level of pressure required to promote endospores inactivation seems to be related to the products pH value and overall nutritional composition, since as Pinto et al. (2019) observed for *Alicyclobacillus acidoterrestris* spores in apple juice (pH 3.50), a minimum of 25 MPa at RT was sufficient for both endospores and vegetative load inactivation, while on a more optimal growth matrix (like BHI-broth, pH 6), higher pressures (≥50 MPa) were required in order to achieve the same microbiological effect (Pinto et al., 2018). Noteworthy, storage under 100 MPa successfully reduced the high levels of *B. subtilis* endospores, at a rate of 1 log unit per 2.4 days, to constant undetectable levels from the 31st day, until the end of the storage period. Interestingly, D_p-values for *B. subtilis* spores were found to be lower than for its vegetative form, which might be hypothesised above, HS may trigger endospores germination, thus stimulating them to germinate, followed by outgrowth inhibition due to pressure. As far as

the authors are aware, this was the first study that allowed the determination of D_p and Z_p-values in some of the endogenous microflora, inoculated pathogenic surrogate vegetative bacteria and in *B. subtilis* endospores, studied under HS conditions.

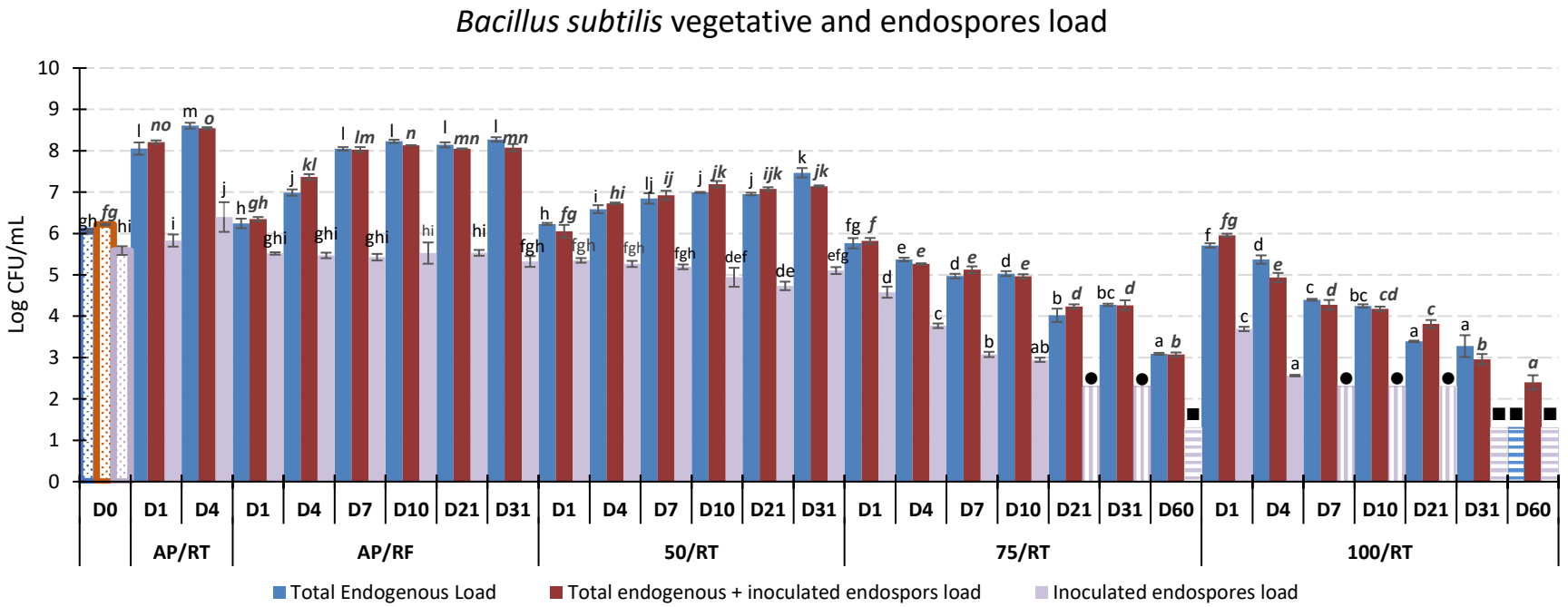


Figure 4.5 - Total vegetative endogenous load, total vegetative endogenous plus inoculated endospores load and inoculated endospores load (non-germinated) of *Bacillus subtilis* evolution during HS (50, 75 and 100 MPa) at room temperature (RT) in raw milk, and comparison with storage under refrigeration (RF) and RT under atmospheric pressure (AP). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below 2.30 and 1.30 log CFU/mL, respectively.

4.4 Conclusions

In this study, despite the raw milk level of spoilage, HS at uncontrolled RT performed much better than RF, requiring pressures between 62-75 MPa, to not only inhibit the microbial growth of TAM, PSY, LAB, ENT, COL and YM, but also to promote microbial inactivation to undetectable levels at least for two months. Post-hyperbaric storage of samples under 75 and 100 MPa, points to HS capacity to slow down microbial recovery from sub-lethal damage, when stored further under AP/RF, leading to a more microbial stable product after HS. Also, HS was able to restrain the growth of the surrogate pathogenic microorganisms studied, contributing to a microbiological safer product, even under 50 MPa. Furthermore, it is noteworthy the capacity of HS (≥75 MPa) to inactivate *B. subtilis* endospores, a highly resistant bacterial form to thermal treatment and very relevant endospore in the food industry.

Despite the need for further scientific and technological research, HS could have a significant impact when applied to raw milk contributing significantly to its increased microbial safety and considerable enhanced shelf-life, compared to refrigeration (up to at least two months, the longest storage period studied in this work). In addition, being *quasi*-energetically costless, comparatively to refrigeration and so it deserves further studies, namely in what concerns the nutritional and sensorial quality.

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CHAPTER 5

Chapter 5.

This chapter is based on the third manuscript submitted for publication

Physicochemical, nutritional, and endogenous enzymes assessment of raw milk preserved under hyperbaric storage at variable room temperature

Ricardo V. Duarte, Susana Casal, José A. Lopes-da-Silva, Ana M. Gomes, Ivonne Delgadillo, Jorge A. Saraiva

5.1 Introduction

Hyperbaric storage (HS) is a novel preservation methodology that employs milder pressure values (20-150 MPa) than the ones (400-660 MPa) commonly used in high pressure processing (HPP). One of the reasons why HS is rising a substantial research interest for food preservation is a fact that it can be applied at variable room temperature (RT), since energy is only required to pressurize and depressurize the vessel, where food would be stored and additionally, since no energy is spent in maintaining constant low temperatures, as in refrigeration (RF), a significant energy reduction is foreseen for food preservation by HS. In fact, Bermejo-Prada, Colmant, Otero, and Guignon (2017) estimated that comparatively to conventional RF, storage of 800 kg of strawberry juice during 15 days at HS/RT, would allow an energy cost 26-fold lower than RF, but would require so far higher investment in HS equipment. However, the equipment forecasted in the study cited above, is based on the ones currently available for HPP, that are highly more complex and demanding, as they need to achieve fast and elevated pressures (up to 600 MPa), so requiring a more robust vessel that can endure higher pressures, than the ones for HS (Fidalgo et al., 2014; Queirós et al., 2014). Also, Bermejo-Prada et al. (2017) reported an estimated reduction in almost 25.8-fold for HS in carbon-footprint (per kg strawberry juice), resulting in a more sustainable preservation methodology that in return would account for negligible emission tax when compared to RF.

Another main reason for HS research interest is the potential considerable increment in food shelf-life, with very interesting microbial results being reported for several types of food products. Initially HS was studied for juices preservation as case a study, while recently other foods, including solid foods matrices are being evaluated. The HS studies in juices,

were carried out for strawberry juice (an acidic juice), having been found that most of the physicochemical parameters remained stable even under low pressures as 25 MPa at 20 ºC during 15 days, as the low pH acts synergistically with HS to restrain microbial growth (Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). Later, non-acid juices (melon and watermelon juice) at and above RT but during shorter storage periods, up to 60 hours, have shown great stability in most of the parameters studied, requiring pressures up to 50 MPa to achieve a similar preservation as RF, while pressures equal to above 75 MPa, allowed a greater microbial stability, even at 25-37 ºC, causing microbial inactivation (Fidalgo, Pinto, Delgadillo, & Saraiva, 2021; Santos, Castro, Delgadillo, & Saraiva, 2020). Other highly perishable food matrices have been analysed, like whey cheese, ham, and carrot soup, all characterized by almost neutral pH and high water activity, all demonstrating a better preservation compared to RF by HS at and above RT (25-37 ºC), for periods up to 8 hours, retaining colour, pH, titratable acidity, and restraining lipid oxidation under 100 MPa (Duarte et al., 2014; Fernandes et al., 2015; Moreira et al., 2015). Further, longer storage periods (10 days) were assessed in watermelon juice (50-100 MPa) and whey cheese (100 MPa) at RT, reporting an initial microbial growth inhibition under 50 MPa, while 75-100 MPa allowed microbial inactivation in both food products, resulting in an increased shelf-life comparatively to RF (Duarte et al., 2017; Pinto et al., 2017). More recently the feasibility to store fish/meat products under HS was studied for longer storage periods (up to 60 days), with promising results. Both food products revealed great microbial stability by HS above 50 MPa, with microbial reductions being verified in both endogenous and inoculated microbial loads (Fidalgo et al., 2021; Santos, Delgadillo, & Saraiva, 2020). Thus, although HS at RT has proven its capability to control microbial growth, it is important to perform more insightful analysis in other important foods, like milk not only regarding the microbial quality but also physicochemical and biochemical quality parameters, to gain further knowledge about the potential of HS to possibly substitute RF with prolonged shelf-life.

Raw milk is a highly perishable food product with short shelf-life, due to its high nutritional profile, near neutral pH and high-water activity, resulting in a good environment for the development of several microorganisms, that jeopardize the overall quality and safety of milk as well as the other dairy products produced from it, thus requiring refrigerated storage to slow down microbial growth, prior processing (LeJeune & Rajala-Schultz, 2009). So far there are no results for the effect of HS for milk preservation, with the exception of a

study carried out recently by our group and submitted for publication (*Chapter 4*), with raw milk stored under HS conditions, 62-100 MPa at RT showing increased microbial stability at RT during 60 days, comparatively to RF, with microbial inactivation observed to undetectable counts for endogenous microbial load (up to more than 5 log units reduction) and inoculated vegetative surrogate pathogenic microorganisms, *Escherichia coli* and *Listeria innocua*, and pathogenic *Salmonella senftenberg*, as well as bacterial spores (*Bacillus subtilis* endospores) throughout storage. These results clear indicate the great potential of HS for raw milk preservation, compared to RF, with potential increased shelf-life and microbial safety. So, in present study, several quality/nutritional parameters and activity of endogenous enzymes of raw milk were studied for the cases where good microbial preservation was also observed in the above indicated study compared to preservation by RF. Therefore, raw milk samples stored under HS (50-100 MPa) at variable RT (18 - 22 ºC) and stored at atmospheric pressure (AP) at RF and RT during 60 days were studied. An overall assessment in milk pH, titratable acidity, total solids content, density, colour, viscosity, volatile organic and fatty acids profile, lipid oxidation, total protein, soluble protein, free amino acids and alkaline phosphatase and lactoperoxidase activities, were performed and compared to the milk prior to storage and milk stored in the different storage conditions.

5.2 Material and Methods

5.2.1 Samples preparation and storage conditions

Cow's raw milk was collected from a local dairy farm association company, kept under refrigeration during transportation, then packaged under aseptic conditions, inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain). Samples were double packed in UV-light sterilized, low permeability polyamide-polyethylene commercial food packaging bags (90 micron, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal), and heat-sealed individually, avoiding as much as possible leaving air inside.

HS samples were stored under 50, 75 and 100 MPa at room temperature (RT, 18 - 22 ºC) using a SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK) system, equipped with a pressure vessel of 30 mm inner diameter and 500 mm height. For comparison, raw

milk samples were also stored at RT and RF (4 ºC) at AP (0.1 MPa) during 7, 14, 28, 39 and 60 days. Once raw milk stored under the different storage conditions was considered microbiologically unsuitable (*Chapter 4*), that storage condition was stopped.

5.2.2 Physicochemical parameters

pH was measured directly in the sample at constant RT with a proper calibrated pH meter (Testo 205, Testo, Inc., New Jersey, USA). The total solids content and density were determined using a portable density and ºbrix meter (Handheld Refractometer Atago, ATC-1E, Tokyo, Japan) at 20 and 15 ºC, respectively. Titratable acidity (TA) of raw milk samples was determined by titrating 5 mL of diluted raw milk (2 mL of milk in 3 mL of distilled water) to pH 8.4 with a previously standardized sodium hydroxide 0.01M solution, using an automatic titrator (Titromatic 1S, Crison Instruments, S.A., Barcelona, Spain). The results were expressed as grams of lactic acid per litter of milk, based on **Equation 5.1**:

$$TA = \frac{N\ NaOH\ x\ mL\ NaOH\ x\ 90.08}{mL\ of\ sample} \qquad \textbf{Equation 5.1}$$

5.2.3 Colour

Colour was measured using a Minolta Konica CM 2300d equipment (Konica MinoltaCM 2300d, Osaka, Japan), calibrated before each sample measurement. 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: red/green colour (a^*), yellow/blue colour (b^*) and luminosity (L^*) parameters. The colour parameters L^*, a^*, and b^* were measured and the total colour change (ΔE^*) was calculated by **Equation 5.2**:

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \qquad \textbf{Equation 5.2}$$

where ΔE^* represents total colour difference between a respective sample and the initial one prior to storage, with L^*_0, a^*_0, and b^*_0 representing the respective parameter at day zero.

5.2.4 Alkaline phosphatase and lactoperoxidase activity

Alkaline phosphatase (ALP) activity was assayed with *p*-nitrophenylphosphate (*p*-NPP) as a substrate, as described by Negrão et al. (2003) with some modifications. Initially

raw milk was mixed with 4 mM *p*-NPP in buffer solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and incubated during 30 min at 37 ºC. The reaction was stopped by the addition of 2M Na0H and the *p*-nitrophenol released was measured at 405 nm using a micro-plate spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific, Waltham, Massachusetts, USA). *p*-NPP is hydrolysed rapidly in the presence of alkaline phosphatase to *p*-nitrophenol, resulting in an intense yellow colour measured at Abs at 405 nm, in AU (absorbance units). Activity was expressed in D $Abs_{405\ nm}$/min and the results presented in relative percentual values in relation to the values of the initial raw milk, prior to storage.

Lactoperoxidase (LPO) activity assay was performed based on the method described by Marín, Sánchez, Pérez, Puyol, and Calvo (2003). Briefly, raw milk was mixed with a solution of 0.325 mM ABTS (in 0.1 M sodium phosphate buffer, pH 6.0) and left for 30 min at 20 ºC, then 0.1 mM hydrogen peroxide was added and mixed quickly to start the reaction, with the absorbance ($Abs_{412\ nm}$) measured during 1 min. The enzymatic activity was calculated as the slope of the curve relating Abs increment versus time and expressed as $DAbs_{412\ nm}$ AU/min.

All enzymatic assays were performed in triple replicates for each storage condition, with the residual activity calculated by **Equation 5.3**:

$$Residual\ acitivity\ (\%) = \frac{A}{A_0}\ x\ 100 \qquad \textbf{Equation 5.3}$$

where A is the enzymatic activity in raw milk samples after storage and A_0 is the enzymatic activity of the sample at day zero.

5.2.5 Viscosity

Milk viscosity was assessed through a controlled-stress rheometer (AR-1000, TA Instruments, New Castle, USA) equipped with a cone-and-plate geometry (acrylic cone, 6 cm diameter and 2° angle). Prior to the analysis, samples were allowed to achieve a constant temperature (20 ± 0.5 °C) on the rheometer lower fixed flat plate for 300 sec. A circulatory thermostatic bath (Circulating Bath 1156D, VWR International, Carnaxide, Portugal) was connected to this plate, ensuring that the target temperature was achieved and maintained. Before placing the samples on the rheometer plate, each sample was mixed gently and

carefully transferred onto the rheometer measuring system, avoiding the trapping of air bubbles between the cone and the plate. Flow curves were obtained by applying a continuous stress ramp, from 0 to 2 Pa during 3 min. Rheological results were monitored using a TA Instruments software package. The apparent viscosity measured at a shear rate of 300 s^{-1}, within the Newtonian region, was used to compare among samples.

5.2.6 Volatile organic Compounds

Volatile organic compounds (VOC) profile determination was based on the method described by Yue et al. (2015). It was performed by headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS). Raw milk (2 mL) was pipetted into 35 mL vials, followed by the internal standard (50 µL of cyclohexanone aqueous solution at 25 µg/mL), being immediately sealed with a metallic cap with silicon septum. After equilibration at 50 ºC during 30 min, with agitation (500 rpm), the SPME fiber (DVB/CAR/PDMS; 50/30 µm; Supelco Inc.) was exposed to the sample's headspace during 30 min still at 50 °C, for volatiles adsorption. The fiber was inserted in the injection port of the GC equipment, Agilent GC-7890 gas chromatographer equipped with a mass spectrometer Agilent 5977B, and a DB-5 MS Capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent, USA). The injector port was heated to 260 °C and injections were performed in splitless mode with helium at a linear velocity 1 mL/min. The oven temperature was programmed at 35 ºC during 5 min, increasing to 100 ºC at a rate of 4 ºC/min, followed by an increase of 10 ºC/min until 225 ºC and held for 0.25 min (total of 33.5 min). The ion source and interface temperatures were maintained at 230 and 280 °C, respectively, and the electron impact ionization mass spectra recorded with an ionization energy 70 eV. Mass spectra were scanned from 20 to 350 m/z in full scan mode. Identification of the volatile compounds was based on computer matching with the reference mass spectra of the MS library of the National Institute of Standards and Technology 2011 (NIST 11), retention times, retention index and with individual standards when available. Using cyclohexanone as internal standard equivalents basis, volatiles' profile semi-quantitative determinations was calculated from the full scan areas and the results were expressed in µg of internal standard equivalents per mL of milk.

5.2.7 Fatty Acids Profile

For the fatty acids profile determination, a similar method to what is described by Sobral, Casal, Faria, Cunha, and Ferreira (2020) was performed. Briefly, 100 μL of internal standard solution (10 mg/mL of undecanoin, C11:0 triglyceride, in heptane) was evaporated to dryness under a gentle stream of nitrogen (Stuart®, Staffordshire, USA) and 1 mL of milk was added, followed by the addition of isopropanol (2 mL) for protein precipitation, cyclohexane (2 mL) and NaCl aqueous solution (1%) (1.5 mL). After Agitation and centrifugation (5000 rpm, 5 min) the supernatant was collected, evaporated under a nitrogen stream at room temperature and redissolved with heptane (2 mL). For the preparation of fatty acid methyl esters (FAME), 2M potassium hydroxide (200 μL) was added and the samples carefully vortexed for 1 min. Finally, 50 μL of injection standard (20 mg/mL of methyl tridecanoate (C13:0 methyl ester)) was added. The hexane layer containing the FAME was transferred into 1 mL GC vials.

FAMEs profile was analysed using a GC (Chrompack CP-9001 model, Netherlands) with flame ionization detection (FID). 1 μL was injected, with the injector and detector temperatures set to 250 ºC and 270 ºC, respectively. Separation of the fatty acids was achieved on a Select FAME (50 m × 0.25 mm x 0.25 μm) column (Agilent, USA) using helium as carrier gas (pressure of 140 kPa), heated from 120 ºC (3 min hold) to 220 °C (5 min hold) at a 3 °C/min rate. Fatty acids identification and FID calibration was accomplished with a certified reference standard mixture (TraceCert – Supelco 37 component FAME mix, USA) and the results were expressed in relative percentages of their FAMEs.

5.2.8 Secondary Lipid oxidation by-products

Lipid oxidation was determined by malondialdehyde (MDA) quantification, using the 2-thiobarbituric acid reactive substances (TBARS) method with adaptations (King, 1962). Briefly, 1 mL of raw milk was mixed with 2 mL 7.5% trichloroacetic acid, then vortexed for approximately 60 sec, followed by centrifugation at 4000 × g at 4 ºC for 20 min (Universal 320-R, Hettich Group, Tuttlingen, Germany). After filtration (Whatman nº1), 1 mL of the resulting extract was added to 1 mL of 46 mM 2-thiobarbituric acid, vortexed and immersed in boiling water for 40 min, and then cooled down in cold water. Triplicates were measured using a micro-plate spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a Brand

plate of 96 wells, at 532 nm. Standard solutions of MDA in 7.5% trichloroacetic acid were prepared from 1,1,3,3-tetramethoxypropane and a calibration curve was prepared at a concentration ranging from 0.2 to 10 μg/L. TBARS results were expressed as μg of malondialdehyde per mL of milk.

5.2.9 Protein profile

Overall protein profile was assessed by determination of total nitrogen through the Kjeldahl method, soluble protein (SP) by Bradford method and free amino acids (FAA) using the EZ:Faast Amino Acid Analysis Kit available for GC-FID. Micro-Kjeldahl procedure was performed with a Kjeltec system 1002 Distilling unit (Tecator, Sweden) and the crude protein content determined by multiplying the total nitrogen content by 6.38 (AOAC Official Method 2001.14, 2002). The total soluble protein was determined based on the Bradford method (Bradford, 1976) with few modifications. Initially milk was diluted in distilled water (1:100 v/v), followed by centrifugation at 4000 × g at 4 ºC for 15 min (Universal 320-R, Hettich Group, Tuttlingen, Germany). Then 50 μL of the supernatant was added to 250 μL of dye Coomassie Blue G25 in a microplate, shaken for 30 sec and incubated 20 min at room temperature. The absorbance was measured at 595 nm (Microplate Spectrophotometer Multiskan GO, Thermo Scientific, Waltham, MA, USA) and soluble protein was expressed in mg per 100 mL of milk. A calibration curve was prepared using BSA as standard at concentrations ranging from 0 to 0.5 mg/mL. For FAA determination and quantification, milk was centrifuged (17000 × g at 4 ºC for 5 min), the supernatant was collected and centrifuged again. 100 μL of the second supernatant were used for the analysis of FAA using the EZ:Faast Amino Acid Analysis Kit (GC-FID) (Badawy, Morgan, & Turner, 2008) and the results were expressed in nmol per mL of milk.

5.2.10 Statistical analyses

All experiments were carried out in triplicate and all analyses were done in triplicate. Analysis of Variance (ANOVA) was performed within all the different storage conditions, followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance. Additionally, principal component analysis (PCA) was performed in order to identify statistical patterns in VOC data.

5.3 Results and Discussion

Table 5.1 - pH, titratable acidity (g lactic acid/L), total solids (%), density (g/mL), colour, viscosity (mPa·s) and lipid oxidation (µg MDA/mL) parameters of raw milk prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–j) indicate significant differences ($p < 0.05$) between the different conditions for each parameter.

Condition	**Initial**	**AP/RT**	**AP/RF**		**50MPa/RT**			**75MPa/RT**					**100MPa/RT**				
Days	0	7	7	14	7	14	28	7	14	28	39	60	7	14	28	39	60
pH	6.68j	4.14a	6.64hij	6.53c	6.66ij	6.59efgh	6.43b	6.63ghi	6.60efgh	6.57ef	6.56ef	6.51cd	6.61fghi	6.58efg	6.57ef	6.55de	6.51cd
Titratable acidity (g lactic acid/L)	1.73a	11.66f	1.77ab	2.22de	1.79ab	2.01bcd	2.41e	1.87abc	2.08cd	2.09cd	2.13d	2.24de	1.76ab	2.02bcd	2.13d	2.22de	2.26de
Total Solids (%)	11.83d	9.17a	10.42b	10.67bc	11.83d	11.67cd	11.58cd	11.17bcd	12.17d	11.97d	11.33bcd	11.25c	11.25bcd	11.92d	12.00d	11.48bcd	11.58cd
Density (g/mL)	1.037b	1.029a	1.036b	1.033ab	1.038b	1.038b	1.038b	1.036b	1.038b	1.038b	1.033ab	1.033ab	1.036b	1.037b	1.038b	1.034ab	1.036b
Colour																	
L*	54.11ab	55.79b	54.21ab	53.40a	53.81ab	53.59ab	52.77a	53.54ab	53.12a	53.73ab	53.09a	52.81a	53.39a	53.96ab	54.14ab	52.92a	52.68a
a*	-0.77bc	-0.89ab	-0.78abc	-0.82abc	-0.86abc	-0.83abc	-0.87ab	-0.82abc	-0.83abc	-0.89a	-0.86abc	-0.89a	-0.75c	-0.79abc	-0.85abc	-0.82abc	-0.84abc
b*	2.74a	2.75a	2.78a	2.65a	2.52a	2.51a	2.52a	2.62a	2.62a	2.81a	2.59a	2.78a	2.96a	2.93a	2.79a	2.86a	3.11a
ΔE	*NP*	1.70ef	0.56a	0.71abc	0.41a	0.59ab	1.37cdef	1.07bcde	1.00abcd	0.73ab	1.03abcde	1.89f	0.79abc	0.48a	0.77abc	1.19bcde	1.48def
Viscosity (mPa·s)	2.87a	*NP*	31.12c	*NP*	4.99ab	10.63d	*NP*	2.84a	2.80a	2.76a	2.74a	3.00a	2.96a	2.91a	2.83a	2.80a	2.96a
Lipid oxidation (µg MDA/mL)	0.83ab	1.18c	0.72a	0.78ab	0.89abc	0.93ab	0.91ab	0.82ab	0.74a	0.80ab	0.88ab	1.03bc	0.75a	0.73a	0.78ab	0.76a	0.84ab

(*NP* – parameters not performed under these condition)

5.3.1 Physicochemical parameters

pH, titratable acidity, total solids, and density were assessed in all samples under the different storage conditions (**Table 5.1**). Initial raw milk presented a pH value of 6.68 ± 0.01, which is within the values reported in the literature (Zareba, Ziarno, & Obiedzinski, 2012). As expected, storage conditions that allowed fast and considerable microbial growth (*Chapter 4*), resulted in an increasing acidity ($p < 0.05$), just after 7 days, in the case of AP/RT with a pH of 4.14 ± 0.02 (**Table 5.1**). A less pronounced but significant ($p < 0.05$) decrease was also observed for storage under AP/RF after 14 days to 6.53 ± 0.01, and at 50/RT after 28 days to 6.43 ± 0.01 ($p < 0.05$). Raw milk stored under 75 and 100 MPa also presented a slight decrease in pH throughout storage, despite being statistically significant ($p < 0.05$) the decrements were much smaller, with the pH decreasing to around 6.51 ± 0.2 for both storage conditions after 60 days (compared to the initial value, 6.68).

Regarding titratable acidity (TA), the initial value observed was similar to values reported in the literature, 1.73 ± 0.02 g/L (Júnior, Beloti, da Silva, & Tamanini, 2013). A substantial increase was observed for samples stored under AP/RT, with TA increasing to 11.66 ± 0.18 g/L after 7 days (**Table 5.1**). As mentioned, due to the highly perishable nature of milk, high water activity and neutral pH, it provides a good environment for microbial growth, that results in increasing organic acids concentration that are responsible for higher acidity (Júnior et al., 2013), as observed. Initially on day 7, the acidity of all the other storage conditions increased slightly ($p > 0.05$) comparatively to samples prior to storage. Milk acidity continued to increase for all storage conditions, however at different rates, with samples stored under AP/RF and 50/RT reaching similar values, just after 14 and 28 days, 2.22 ± 0.16 and 2.41 ± 0.05 g/L, respectively, while when stored under 75 and 100/RT, milk reached a maximum of 2.24 ± 0.13 and 2.26 ± 0.03 g/L after 60 days, respectively.

For total solids, samples prior storage had a value of 11.83 ± 0.63% comparable to the values reported in the literature, 9.7 to 12.5% (Boci, Bardhi, & Cakraj, 2013). Under AP/RT and AP/RF after 7 and 14 days respectively, a significant decrease ($p < 0.05$) was observed, reaching a minimum of 9.17 ± 0.26% and 10.67 ± 0.38%, respectively. Storage under pressure maintained the TS value similar throughout the storage period with slight variations observed ($p > 0.05$), with values of 11.25 ± 0.42 and 11.58 ± 0.14 at day 60 at 75 and 100/RT, respectively.

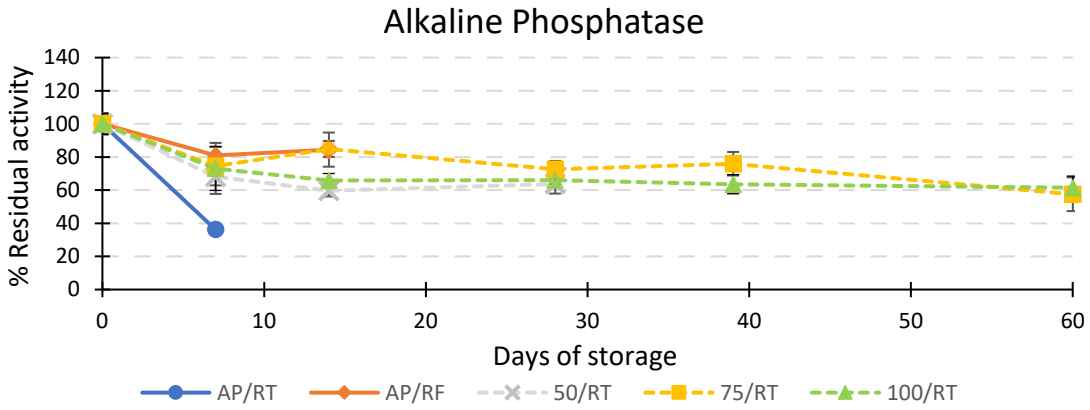
As for density, the values obtained were within the ones observed by Júnior et al. (2013) for bovine raw milk stored under AP/RF, which should be between 1.023 and 1.040 g/mL, as values outside this range may indicate adulteration, like water addition (Júnior et al., 2013). The only variation ($p < 0.05$) observed within all the storage conditions, was referred to AP/RT that presented a significant decrease from 1.036 ± 0.003 to 1.029 ± 0.001 g/mL after 7 days, with storage under RF and HS resulting in no modifications in milk density ($p > 0.05$).

5.3.2 Colour

The colour parameters L^*, a^* and b^* were monitored in milk stored under the different conditions, and total colour change (ΔE^*) was calculated, followed by comparison to the milk initial values prior to storage (**Table 5.1**). Raw milk presented L^*, a^* and b^* values of 54.11 ± 0.81, -0.77 ± 0.04 and 2.74 ± 0.24, respectively. L^* (lightness values) ranged from 55.79 ± 0.03 (AP/RT at day 7) to 52.68 ± 0.14 (100/RT at day 60), with no significant changes ($p > 0.05$) observed comparatively to the initial value. Overall, HS presented a decreasing tendency ($p > 0.05$) in raw milk L^* parameter, without substantial changes ($p > 0.05$). Regarding a^* (greenness), the only significant variation was observed under 75/RT at day 28 and 60 ($p < 0.05$), to values of -0.89 ± 0.02, for both periods. As for b^* (yellowness), no changes were observed under the different storage conditions ($p > 0.05$), ranging from 2.51 ± 0.04 to 3.09 ± 0.04. In milk HPP studies, L^* parameter is usually the most affected ($p < 0.05$), decreasing after processing (Gervilla, Ferragut, & Guamis, 2001), which could be due to casein micelles disintegration into smaller fragments that increase the translucence of milk, thus affecting this colour parameter (Adapa, Schmidt, & Toledo, 1997). However, HPP studies apply significant higher pressures comparatively to HS, that in the present work presented overall a slight decrease ($p > 0.05$) in this parameter throughout the storage. Comparatively to the overall colour changes, ΔE^*, at the 7th day of storage, only samples stored at AP/RT showed a significant increase ($p < 0.05$) to values of 1.70 ± 0.03, comparatively to storage under AP/RF, 0.56 ± 0.16. On the 14th day a slight increase ($p > 0.05$) was observed for most storage conditions, comparatively to the respective storage at day 7, that tended to increase as time went by. At the 28th day samples at 50/RT presented a significant increased on ΔE^* value of 1.37 ± 0.30, while storage at 75 and 100/RT maintained ΔE^* value similar to day 7. However, at the end of the storage, at day 60, values increased

to 1.89 ± 0.17 and 1.48 ± 0.15 under 75 and 100/RT respectively, possible related to the observed decrease in L^* parameter in these samples. Accordingly to Drlange (1994), all of the ΔE^* values for samples in this study are considered to have a "small difference" ($0.5<\Delta E^*<1.5$) perceptible by the consumer's eyes comparatively to the initial raw milk colour, with samples under AP/RT at day 7 and 75/RT at day 60 having "distinct differences" ($1.5<\Delta E^*<3$).

5.3.3 Alkaline phosphatase and lactoperoxidase activity



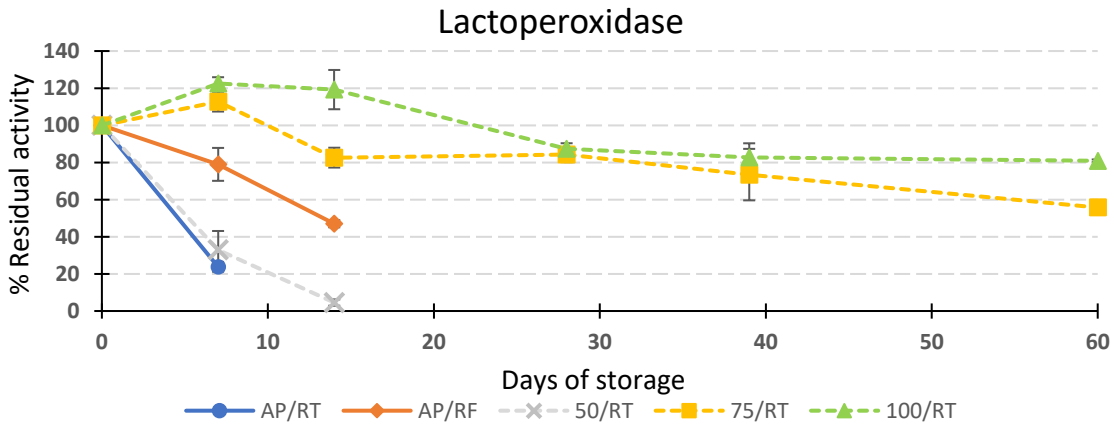
Alkaline Phosphatase	Days					
Condition	0	7	14	28	39	60
AP/RF	e	bcde	de	#	#	#
AP/RT	e	a	#	#	#	#
50/RT	e	bcd	ab	bcd	#	#
75 /RT	e	bcd	cde	bcd	bcde	ab
100/RT	e	bcd	bcd	bcd	bcd	bc

- residual activity not determined under this storage condition

Figure 5.1 - Alkaline phosphatase residual activity throughout storage under the different storage conditions (AP/RT, AP/RT, 50/RT, 75/RT, and 100/RT). Different letters (a–e) indicate significant differences ($p < 0.05$) between the different conditions.

Alkaline phosphatase (ALP) is an enzyme naturally present in milk, mainly bounded to the fat globule membrane, that can catalyse the hydrolysis of phosphate monoesters, yielding phosphate and the corresponding alcohol (Machado, Santos, Júnior, Costa, & Paiva, 2009). ALP is also commonly used as a standard assay for rapid validation of the milk pasteurization process as it is slightly more resistant to thermal treatment than the non-

sporogenic pathogenic microorganisms present in milk (Rankin, Christiansen, Lee, Banavara, & Lopez-Hernandez, 2010). **Figure 5.1** shows the residual activity of ALP during all storage conditions, throughout storage. After 7 days, ALP activity was reduced for all the storage conditions, reaching a significant decrease ($p < 0.05$) of 35%, at AP/RT with the rest of the storage conditions presenting residual activities like AP/RF (around 81%). ALP activity tended to decrease over time ($p > 0.05$), but for each storage condition the activity was similar when compared to the same storage condition at day 7. And so, even though an initial reduction in ALP residual activity to around 70% ($p < 0.05$) under HS was verified and reached further residual activities in the following weeks to around 58% and 61% under 75 and 100/RT at day 60, respectively, that were not significant ($p > 0.05$). Similarly Fidalgo, Delgadillo, and Saraiva (2020) also observed a decrease in acid phosphatase in Atlantic salmon under 75 MPa at 25 ºC, after 25 days to 23% opposingly to storage under 60 MPa at 10 ºC after 30 days, which maintained acid phosphatase activity stable (111%).



Lactoperoxidase	Days					
Condition	0	7	14	28	39	60
AP/RF	fg	e	bc	#	#	#
AP/RT	fg	b	#	#	#	#
50/RT	fg	b	a	#	#	#
75/RT	fg	gh	ef	ef	de	cd
100/RT	fg	h	h	ef	ef	e

- residual activity not determined under this storage condition

Figure 5.2 - Lactoperoxidase residual activity throughout storage under the different storage conditions (AP/RT, AP/RT, 50/RT, 75/RT, and 100/RT). Different letters (a–h) indicate significant differences ($p < 0.05$) between the different conditions.

Lactoperoxidase (LPO) is also a commonly enzyme present in milk, being one of its indigenous antimicrobial agents (Marín et al., 2003). This enzyme catalyses oxidation reactions in the presence of hydrogen peroxide and helps the production of products with a wide antimicrobial activity, such as pseudohalogens, thiocyanates, or halogens (Kussendrager & van Hooijdonk, 2000). At AP/RT the LPO activity was significantly reduced ($p < 0.05$) to 24% of its initial value after 7 days (**Figure 5.2**). Under AP/RF, LPO the activity decrease was slower, but still significant ($p < 0.05$) when compared to its initial activity to around 79% to 47%, after 7 and 14 days, respectively. Storage under 50/RT presented a behaviour alike AP/RT, with a more pronounce reduction ($p < 0.05$) in LPO activity up to 33% and 5% of the initial value, after 7 and 14 days, respectively. This enzyme might be more susceptible to the changes observed under these storage conditions, AP/RT, AP/RF and 50/RT, namely high microbial activity, decrease in pH and increasing acidity, that all together can promote LPO denaturation or reduce its activity (Kussendrager & van Hooijdonk, 2000; Moussa, Mankai, Fekih, & Hassouna, 2013). Storage under 75 and 100/RT presented overall a much better maintenance in LPO residual activity throughout storage, comparatively to the other storage conditions performed. At day 7, both storage conditions presented increased LPO activity, comparatively to the initial one, to values around 113% ($p < 0.05$) and 122% ($p < 0.05$), for 75 and 100 MPa, respectively. After 14 and 28 days, despite the decrease in LPO activity observed for both storage conditions, no significant variations were observed comparatively to the initial LPO activity ($p < 0.05$). Overtime, LPO activity was slightly more affected during storage under 75/RT, ending at day 60 with an activity of 56% of the initial value, while storage under 100/RT maintained LPO residual activity similar to the one observed at day 28 and 39, around 80% of the initial value, at the end of the storage period ($p < 0.05$). Knowledge about HPP effect, especially for low and mild pressures, in enzymes activity is scarce and HPP effect is not always linear, being dependent in several variables, such as HPP pressure level, duration, temperature, pH and the matrix environment, and can be specific for a determined enzyme (Naik, Sharma, Rajput, & Manju, 2013). In HPP studies, LPO is described as highly resistant without significant activity inactivation even after 60 min at 400 MPa or 15 min at 700 MPa (Lopez-Fandiño, Carrascosa, & Olano, 1996; Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012), however, despite that the prolonged HS effect in the enzymatic activity is not extensively discussed in the literature, two works have evaluated peroxidase (POD) activity during HS/RT in

watermelon and strawberry juice, for 10 and 15 days, respectively (Bermejo-Prada & Otero, 2016; Pinto et al., 2017). While Pinto et al. (2017) observed a significant reduction overtime in POD activity, to 54.6 and 16.8% after 10 days under 75 and 100 MPa, respectively, Bermejo-Prada and Otero (2016) reported a constant POD activity throughout storage, decreasing only to 85% under 200 MPa at 20 ºC. In the present study, milk stored at 100/RT, resulted in the decrease of LPO activity to 80% on the 28th day of storage, remaining stable until the end of the storage period, possibly retaining LPO antimicrobial activity.

5.3.4 Viscosity

Apparent viscosity was determined for all studied storage conditions (**Table 5.1**), with the exception for samples stored for 7 days at AP/RT and for 14 days at AP/RF, since these samples presented visible signs of spoilage (clots, swelling, and increased viscosity). The initial viscosity value for raw milk was similar to values reported in the literature, 2.87 ± 0.20 mPa·s (Li, Joyner, Carter, & Drake, 2018). Storage at AP/RF presented an almost 10-fold increase ($p < 0.05$) in milk viscosity to 31.12 ± 5.98 mPa·s after 7 days, while under HS conditions (50, 75 and 100/RT) it remained unchanged ($p > 0.05$) for this storage time. After 14 days, samples under 50RT presented a viscosity of 10.63 ± 1.39 mPa·s ($p < 0.05$), while samples under 75 and 100/RT showed no changes in viscosity ($p > 0.05$) throughout the entire storage period, with values of 3.00 ± 0.02 and 2.96 ± 0.07 mPa·s, respectively, at the end of the storage (60 days). The considerable microbial growth observed in samples at AP/RF and 50/RT (*Chapter 4*), may induce changes in milk composition, such as a decrease in pH and an increase in extracellular proteases and polymeric substances released by lactic acid bacteria, that have shown to increase the viscosity of milk (Vaningelgem, Zamfir, Adriany, & De Vuyst, 2004). Several studies have reported that HPP causes an increase in milk viscosity, directly dependent on treatment intensity from pressures above 200 MPa during 30 min, with a slight increase in milk viscosity observed also in treatments bellow that pressure (Thom Huppertz, Fox, & Kelly, 2003). These changes are mostly related to HPP effect in casein micelles, promoting changes in caseins shape, from spherical to non-spherical, micelles disruption or even reduction in particle size (Thom Huppertz et al., 2003; Needs, Stenning, Gill, Ferragut, & Rich, 2000). Apparently under HS at 75 and 100 MPa, such changes seem to do not occur, or at least not at a level enough to significantly change viscosity.

5.3.5 Volatile organic compounds

A total of 19 volatile organic compounds (VOC) were identified in almost all samples, mostly free fatty acids (FFA) and their ethyl esters, alcohols, and aldehydes (**Table 5.2**). FFA were the most abundant VOC in the initial raw milk (n=5), namely acetic, butanoic, hexanoic, octanoic and decanoic acids, followed by 3-hydroxybutan-2-one, similarly to what is reported for milk in literature (Tunick, Iandola, & Van Hekken, 2013; Yue et al., 2015). In lower concentrations some ethyl esters (n=3), alcohols (n=2) and aldehydes (n=2), with 3-methylbutanal being detected only in the initial raw milk, prior to storage.

Storage under AP/RT at day 7, resulted in significant changes ($p < 0.05$) in the major VOC classes, except for aldehydes, despite the slight increase in hexanal concentration. Overall, a significant increment ($p < 0.05$) in all FFA was observed, to concentrations almost up to 10-fold for acetic, butanoic, hexanoic and decanoic acids, what can result mainly from microbial action and lipase activity on fatty acids, and in a smaller degree, degradation of lactose and amino acids, that all together can be responsible for perceptible rancid flavour in milk (Vagenas & Roussis, 2012; Zareba et al., 2012). Esters were also more abundant ($p < 0.05$) in samples stored under AP/RT at day 7, when compared to the initial milk, which was particularly more pronounced for ethyl acetate, butanoate, and hexanoate. Additionally, ethyl octanoate and decanoate that were absent in the initial milk, were now detected in abundancy. The content in alcohols also increased considerably ($p < 0.05$) comparatively with the sampler prior storage, especially for 3-methylbutan-1-ol (64-fold higher**)**, with 2-methyl-1-butanol being now present. Both alcohols and esters can influence the flavour of dairy products when present in high concentrations, with alcohols being mainly derived from amino acid metabolism or fermentation of lactose and esters from esterification of short-chain alcohols and free fatty acids, both potentially indicating high microbial and enzymatic activities (Nursten, 1997; Toso, Procida, & Stefanon, 2002), which is coherent to what was reported for this storage condition (microbial levels above the acceptable level (≥5.5 log CFU/mL) for AP/RT samples at day 7, *Chapter 4*). In what concerns aldehydes, for these samples only hexanal was found, showing a significant 5-fold increase under AP/RT ($p < 0.05$) that may derive from unsaturated fatty acids oxidation (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001).

At the 7th day of storage under AP/RF, the evolution of milk VOC profile was similar to AP/RT samples, however the increments of the main VOC occurred at a slower rate,

namely for most FFA and esters, as under low temperature, microbial growth and enzymatic activity are slowed down, as it was observed in a previous study regarding the microbial evolution between these two storage conditions, AP/RF and RT (around 5 log units for AP/RF after 7 days, *Chapter 4*). After 14 days, the differences were more pronounced, with all FFA and 3-methylbutan-1-ol presenting significantly higher concentrations ($p < 0.05$), when compared to the sample prior storage, with ethyl octanoate and decanoate now present (microbial counts reaching values above the acceptable limit at the 14th day, *Chapter 4*).

Under HS at the lowest pressure (50 MPa), VOC concentration was comparable ($p > 0.05$) with AP/RF samples for the same storage period, with the exception for total FFA, that were statistically found at a lower concentration ($p < 0.05$), while aldehydes were statistically higher ($p < 0.05$) when compared to the corresponded AP/RF ones. Despite these samples showing a slower degradation rate pattern overall when compared to AP/RF, they presented significant increases ($p < 0.05$) in all identified VOC comparatively to the initial milk, being detected the presence of ethyl octanoate and decanoate at the 28th day of storage, possibly resulting from the increased microbial load observed for this storage condition at the end of the storage period (above the acceptable limit, *Chapter 4*). Toluene is a common compound reported in milk and dairy products, resulting from *β*-carotene degradation, and was detected in HS samples (Condurso, Verzera, Romeo, Ziino, & Conte, 2008). As for the upper pressures (75 and 100 MPa), a better overall preservation of the initial VOC profile was achieved, even after 60 days, for all FFA, esters, alcohols and aldehydes, with the exception for 3-hydroxybutan-2-one, which concentration decreased considerably, especially ($p < 0.05$) under 50 and 100/RT. Comparing storage under 75 and 100/RT, the later one presented a VOC profile more similar to that of the initial milk, with lower changes in all FFA, with no nonanoic acid formation being detected (similar to milk prior storage) and also relatively for esters (only ethyl acetate was present, in low concentration), without the formation of fatty acids ethyl esters, thus possibly indicating a better preservation of raw milk under these conditions. Overall alcohols content remained low and constant under 100/RT ($p < 0.05$), despite the formation of 2-methyl-1-butanol and 2-ethylhexan-1-ol in low concentrations. As far as the authors are aware, the information available regarding the effect of low pressures during extended periods on VOC of foods is very scarce and absent at variable RT. Anyway, for the sake of comparison, the results observed in the present work, are in accordance with what was reported by Fidalgo et al. (2019), which observed a similar fresh-

like salmon VOC profile for samples stored under 60 MPa, at 10 ºC up to 30 days. Regarding raw milk under variable RT, a slower matrix degradation evolution for both 75 and 100/RT for 60 days was observed comparatively to the sample prior storage and a much better preservation of the VOC profile than those under AP/RF, which may also indicate a better control in microbial and enzymatic parameters.

The complete set of VOC data from samples stored under the different storage conditions were subjected to multivariate statistical analyses, and the results from PCA are shown in **Figure 5.3**, which presents the scores and loadings (**Table 5.3**), that explain 71.94% of the total variance, with 56.82% of the total variance for PC 1 and 15.12% for PC 2. **Table 5.3** shows the compounds more associated with the initial milk prior storage, like 3-hydroxybutan-2-one and 1-hexanol-2-ethyl scored on the positive PC 1, while the negative PC 1, is associated with FFA, esters and some alcohol development. As it can be observed in **Figure 5.3**, samples stored at AP/RF and 50/RT at day 7, and all samples under 75 and 100/RT are closer to the sample prior to storage (positive PC 1), while samples under AP/RF at day 14, 50/RT at day 14 and 28 are apart from the initial one, with samples under AP/RT being the more distant ones (negative PC 1). If the same exercise is carried out with only the data set for the three major classes of identified VOC (total FFA, esters, and alcohols), a similar pattern is observed, but with a better differentiation (99.17% of total variance), with 91.95% and 7.22% of the total variance being explained by PC 1 and PC 2, respectively (**Figure 5.4**).

Table 5.2 - Volatile organic compounds of raw milk prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) expressed in μg/mL. Different letters (a–f) indicate significant differences ($p < 0.05$) between the different conditions.

Condition	**Initial**	**AP/RT**	**AP/RF**		**50MPa/RT**			**75MPa/RT**					**100MPa/RT**				
Days	**0**	**7**	**7**	**14**	**7**	**14**	**28**	**7**	**14**	**28**	**39**	**60**	**7**	**14**	**28**	**39**	**60**
Free Fatty Acids	0.63 ab	6.42 g	1.76 d	5.05 f	1.74 cd	3.05 e	3.56 e	0.60 ab	0.78 ab	0.99 abc	1.37 bcd	1.05 abcd	0.64 ab	0.62 ab	0.55 a	0.81 ab	0.72 ab
acetic acid	0.08a	2.80c	0.74a	2.21bc	0.55a	1.51b	1.99b	0.15a	0.17a	0.21a	0.48a	0.22a	0.15a	0.10a	0.05a	*nd*	*nd*
butanoic acid	0.18ab	0.96f	0.41bcd	0.82ef	0.45cd	0.61de	0.50d	0.13a	0.20ab	0.24abc	0.24abc	0.23abc	0.20ab	0.17a	0.19ab	0.26abc	0.23abc
hexanoic acid	0.26ab	2.06	0.41abc	1.53e	0.51bcd	0.68cd	0.81d	0.10a	0.24ab	0.35ab	0.32ab	0.29ab	0.18a	0.19ab	0.18a	0.27ab	0.31ab
octanoic acid	0.10ab	0.63d	0.14ab	0.37c	0.15ab	0.18abc	0.17ab	0.04a	0.10ab	0.14ab	0.27bc	0.26bc	0.08ab	0.12ab	0.10ab	0.20abc	0.14ab
nonanoic acid	*nd*	0.04a	0.04a	0.04a	0.03a	0.03a	*nd*	0.14b	0.03a	0.02a	0.02a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
decanoic acid	0.02a	0.15e	0.04abc	0.14de	0.04abc	0.04abc	0.10cde	0.04abc	0.05ab	0.04ab	0.08abcd	0.05abc	0.02ab	0.03ab	0.02ab	0.08bc	0.05abc
Esters	0.21a	6.87d	0.64ab	1.11bc	0.47ab	0.45ab	1.64c	0.20a	0.20a	0.14a	0.37a	0.15a	0.19a	0.17a	0.11a	0.07a	0.07a
ethyl acetate	0.14 abcd	1.43 f	0.29 cde	0.32 de	0.14 abc	0.21 abcd	0.45 e	0.14 abc	0.17 abcd	0.05 a	0.26 bcd	0.07 ab	0.19 abcd	0.17 abcd	0.11 abc	0.07 a	0.07 a
ethyl butanoate	0.05a	1.80c	0.29ab	0.49ab	0.30ab	0.17ab	0.66b	0.06a	0.03a	0.07a	0.06a	0.05a	*nd*	*nd*	*nd*	*nd*	*nd*
ethyl hexanoate	0.02a	3.49b	0.06a	0.19a	0.04a	0.07a	0.39a	*nd*	*nd*	0.02a	0.04a	0.06a	*nd*	*nd*	*nd*	*nd*	*nd*
ethyl octanoate	*nd*	0.70b	*nd*	0.10a	*nd*	*nd*	0.12a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
ethyl decanoate	*nd*	0.18b	*nd*	0.02a	*nd*	*nd*	0.02a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Alcohols	0.08 a	2.91 g	0.57 cdef	0.65 def	0.32 abcdef	0.68 ef	0.71 f	0.11 ab	0.31 abcdef	0.54 bcde	0.49 abcdef	0.35 abcdef	0.17 abcd	0.23 abcd	0.17abc	0.27abcde	0.13ab
3-methylbutan-1-ol	0.04a	2.68d	0.57bc	0.61c	0.25ab	0.23a	0.35abc	0.11a	0.21a	0.35abc	0.32abc	0.17a	0.13a	0.14a	0.10a	0.13a	0.07a
2-methyl-1-butanol	*nd*	0.31b	*nd*	*nd*	*nd*	0.08a	*nd*	*nd*	0.10a	0.13a	0.13a	0.07a	0.06a	0.09a	0.06a	0.06a	0.04a
butane-2,3-diol	0.04a	*nd*	*nd*	0.04a	0.07a	0.37b	0.37b	*nd*	*nd*	0.02a	0.03a	0.05a	*nd*	*nd*	*nd*	*nd*	*nd*
2-ethylhexan-1-ol	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.08a	*nd*	*nd*	*nd*	0.08a	0.03a
Aldehydes	0.04ab	0.06ab	*nd*	0.03ab	0.06ab	0.24c	0.14bc	0.02a	0.03ab	0.03a	0.04ab	0.05ab	0.02a	0.03a	0.03a	0.05ab	0.04ab
3-methylbutanal	0.03	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
hexanal	0.01a	0.06ab	*nd*	0.03a	0.06ab	0.24c	0.14bc	0.02a	0.03a	0.03a	0.04ab	0.05ab	0.02a	0.03a	0.03a	0.05ab	0.04ab
Ketones 3-hydroxybutan-2-one	0.48cd	*nd*	0.12ab	0.09a	0.55d	0.06a	0.04a	0.28abc	0.43cd	0.32bc	0.61d	0.23abc	0.08ab	0.06a	0.12ab	0.09ab	0.15ab
Others Toluene	*nd*	*nd*	*nd*	*nd*	*nd*	0.26b	*nd*	*nd*	0.22b	0.02a	0.04a	0.03a	0.02a	0.02a	0.02a	0.04a	0.03a

(*nd* – stands for not detected)

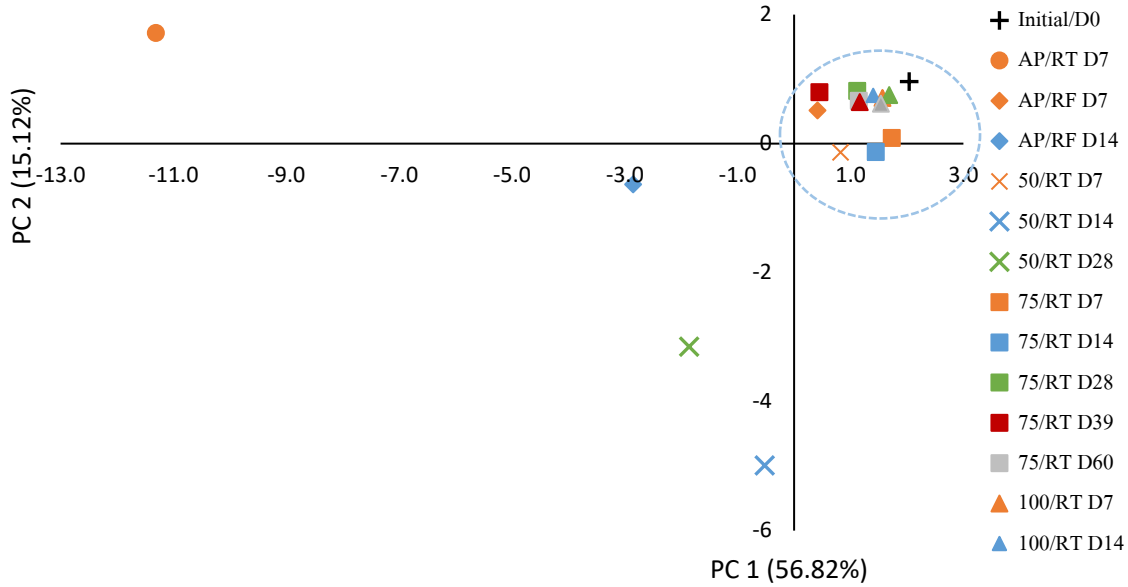


Figure 5.3 - Principal component analysis (PCA) score plot of the volatile organic compounds of raw milk prior storage (Initial) and stored under the different conditions (AP/RT, AP/RT and 50, 75 and 100/RT). Same storage periods have the same colour, while same storage conditions have the same symbol.

Table 5.3 - Loadings of the variables in the first two principal component analysis of the volatile organic compounds in raw milk.

Compounds	Principal Components	
	PC 1	PC 2
ethyl acetate	-0.964	0.091
acetic acid	-0.878	-0.389
3-hydroxybutan-2-one	0.394	0.232
3-methylbutan-1-ol	-0.961	0.205
2-methyl-1-butanol	-0.670	0.297
toluene	0.114	-0.573
butane-2,3-diol	-0.127	-0.936
hexanal	-0.219	-0.901
ethyl butanoate	-0.977	0.016
butanoic acid	-0.878	-0.296
ethyl hexanoate	-0.945	0.201
hexanoic acid	-0.945	-0.111
2-ethylhexan-1-ol	0.168	0.174
octanoic acid	-0.894	0.166
ethyl octanoate	-0.967	0.165
nonanoic acid	-0.173	-0.305
decanoic acid	-0.841	-0.062
ethyl decanoate	-0.955	0.196

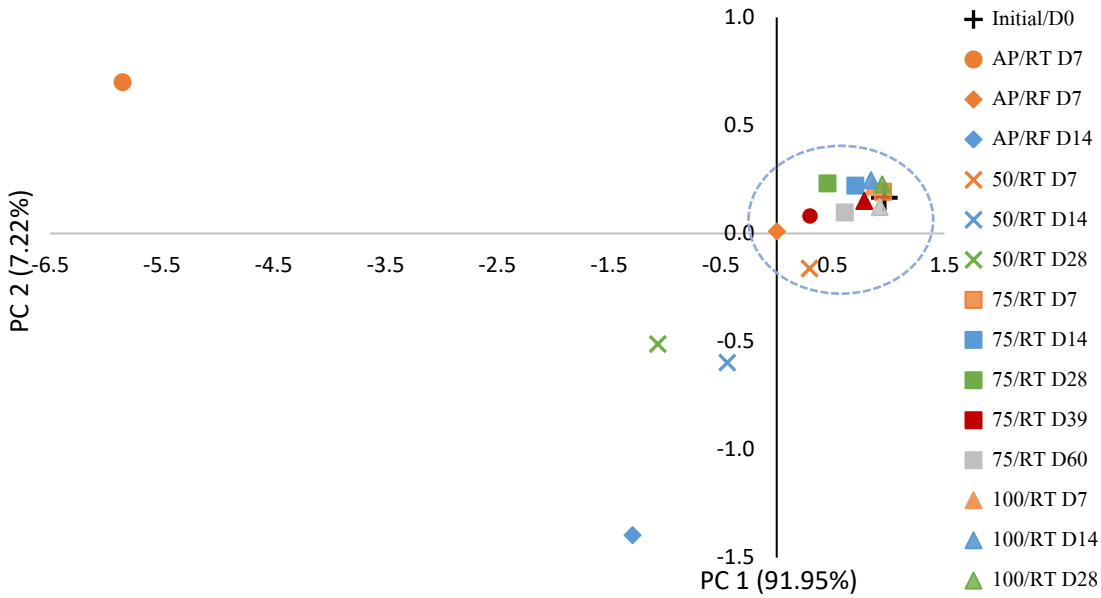


Figure 5.4 - Principal component analysis (PCA) score plot of the volatile organic compounds major classes (FAA, Esters and Alcohols) of raw milk prior storage (Initial) and stored under the different conditions (AP/RT, AP/RT and 50, 75 and 100/RT). Same storage periods have the same colour, while same storage conditions have the same symbol.

Table 5.4 - Loadings of the free fatty acids, esters, and alcohols in the first two principal component analysis of the volatile organic compounds in raw milk.

Compounds	Principal Components	
	PC 1	PC 2
Alcohols	-0.979	0.167
Esters	-0.974	0.198
Free fatty acids	-0.922	-0.387

5.3.6 Fatty Acids Profile

Milk fatty acid profile was characterized by a greater abundance in saturated fatty acids (SFA, 63.15-63.30%), followed by monounsaturated (MUFA, 30.28-30.92%) and polyunsaturated fatty acids (PUFA, 4.65-4.66%) (**Table 5.5**). Regarding individual fatty acids, the most abundant in the initial milk (% of total fatty acids) were palmitic (C16:0, 30.41 ± 0.36%), oleic (C18:1c9, 22.33 ± 0.34%), myristic (C14:0, 11.26 ± 0.07%), and stearic acid (C18:0, 10.84 ± 0.06), similar to what is reported for bovine raw milk in the literature (Dreiucker & Vetter, 2011; Lindmark Månsson, 2008), with some variations attributable to animal nutrition, seasonal feed changes, type of animal farming or stage of

lactation, among other factors (Arnould & Soyeurt, 2009). Overall, the different milk samples fatty acid profile did not present great changes, despite exhibiting a tendency to increase SFA content accompanied by a decrease in both MUFA and PUFA over time, particularly after 60 days under 75 and 100/RT ($p < 0.05$), comparatively to the initial milk prior to storage. The major variations regarding 75 and 100/RT were related to the increase ($p < 0.05$) of lauric, myristic and palmitic acids, and reductions in oleic acid (± 0.5%). However, when compared to storage at AP/RF after 7 days, the only significant reduction was on MUFA content after 60 days under 75/RT, presenting a significant decrease around 0.34%, which can be related to the increasing lipid oxidation values observed for that storage period (**Table 5.1**) (Ayeleso, Matumba, Ntambi, & Mukwevho, 2020).

Table 5.5 - Fatty acids profile of raw milk prior storage (Initial) and stored under the different storage conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) expressed in % of total fatty acids.

Condition	**Initial**	**AP/RT**	**AP/RF**		**50MPa/RT**			**75MPa/RT**					**100MPa/RT**				
Days	**0**	**7**	**7**	**14**	**7**	**14**	**28**	**7**	**14**	**28**	**39**	**60**	**7**	**14**	**28**	**39**	**60**
C8:0	1.54	1.52	1.50	1.52	1.49	1.52	1.53	1.51	1.53	1.49	1.52	1.55	1.48	1.54	1.49	1.48	1.56
C9:0	0.05	0.06	0.05	0.05	0.05	0.05	0.04	0.06	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.03	0.05
C10:0	2.99	3.01	2.99	2.96	2.98	3.01	3.03	2.97	3.02	2.99	2.99	3.09	2.95	3.02	2.98	2.95	3.10
C12:0	3.67a	3.72abc	3.71ab	3.67a	3.70ab	3.72abc	3.73abc	3.67aabc	3.72abc	3.72abc	3.69a	3.80bc	3.65a	3.72abc	3.71ab	3.69a	3.82c
C14:0	11.26a	11.39abc	11.36abc	11.34abc	11.32ab	11.40abcd	11.46bcd	11.39abc	11.44abcd	11.39abc	11.42abcd	11.58d	11.36abc	11.49bcd	11.39abc	11.42abcd	11.51cd
C15:0	0.97	0.97	0.97	0.96	0.97	0.97	0.97	0.96	0.97	0.97	0.96	0.97	0.96	0.97	0.97	0.97	0.97
i-C16:0	0.02	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.02
C16:0	30.41a	30.71ab	30.76ab	30.56ab	30.74ab	30.71ab	30.75ab	30.75ab	30.79ab	30.77ab	30.83ab	30.95b	30.77ab	30.80ab	30.72ab	30.86b	30.78ab
ai-C17:0	0.58	0.60	0.59	0.60	0.59	0.59	0.59	0.59	0.60	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59
C17:0	0.46	0.44	0.44	0.44	0.44	0.44	0.44	0.45	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
C18:0	10.84	10.89	10.94	10.79	10.92	10.87	10.89	10.90	10.88	10.93	10.95	10.83	10.93	10.91	10.91	10.94	10.83
C20:0	0.14	0.13	0.13	0.15	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
C21:0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
C22:0	0.05	0.04	0.04	0.04	0.04	0.04	0.07	0.04	0.05	0.05	0.06	0.05	0.05	0.04	0.04	0.05	0.05
C23:0	0.03b	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a	0.02a
C24:0	0.03b	0.02ab	0.02ab	0.02ab	0.02ab	0.02ab	0.02a	0.02ab	0.02ab	0.02ab	0.02ab	0.02a	0.02ab	0.02ab	0.02ab	0.02ab	0.02ab
Total SFA	63.21ab	63.56abc	63.58abcd	63.20a	63.45abc	62.89abc	63.77abcd	63.42abc	63.75bcd	63.61abcd	63.73bcd	64.12d	63.49abc	63.74cd	63.51abc	63.65abcd	63.92cd
C10:01	0.31	0.31	0.31	0.33	0.31	0.32	0.32	0.31	0.32	0.31	0.31	0.32	0.31	0.32	0.31	0.31	0.32
C12:1	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
C14:1*t*	0.21a	0.21bc	0.22bc	0.21bc	0.22bc	0.22bc	0.22bc	0.21bc	0.22bc	0.22bc	0.21bc	0.22c	0.21b	0.22bc	0.22bc	0.21bc	0.22bc
C14:1*c*	1.05a	1.07ab	1.07ab	1.06ab	1.07ab	1.07ab	1.07ab	1.06ab	1.07ab	1.07ab	1.06ab	1.08b	1.05a	1.07ab	1.07ab	1.07ab	1.08b
ai-C15:1	0.48a	0.49ab	0.49ab	0.49b	0.49ab	0.49ab	0.49ab	0.49ab	0.49ab	0.49ab	0.49ab	0.49ab	0.48ab	0.49ab	0.49ab	0.49ab	0.49b
C15:1	0.26a	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab	0.28b	0.26ab	0.26ab	0.26ab	0.26ab	0.26ab
C16:1*t*	0.05	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05
C16:1*c*	1.82	1.83	1.83	1.82	1.83	1.82	1.82	1.82	1.84	1.82	1.81	1.81	1.82	1.81	1.82	1.82	1.81
C17:1	0.21	0.21	0.22	0.22	0.22	0.21	0.21	0.22	0.21	0.22	0.22	0.21	0.21	0.22	0.21	0.21	0.21
C18:1*t*	0.54	0.55	0.54	0.56	0.56	0.56	0.55	0.54	0.54	0.55	0.56	0.53	0.55	0.54	0.55	0.56	0.56
C18:1*t*9	2.30	2.33	2.34	2.32	2.36	2.33	2.31	2.31	2.31	2.35	2.32	2.29	2.33	2.30	2.31	2.32	2.31
C18:1*ci*9	22.23b	22.08ab	22.08ab	22.15b	22.07ab	21.99ab	22.01ab	22.19b	21.96ab	22.02ab	22.05ab	21.78a	22.20b	22.02ab	21.97ab	22.03ab	21.77a
C18:1*c*11	0.91	0.91	0.91	0.93	0.92	0.92	0.91	0.92	0.91	0.92	0.92	0.90	0.93	0.91	0.91	0.92	0.91
C20:1*c*9	0.12	0.12	0.12	0.13	0.12	0.13	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
C24:1	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.04	0.04

Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

Table 4.5 (cont.) - Fatty acids profile of raw milk prior storage (Initial) and stored under the different storage conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) expressed in % of total fatty acids.

Condition	**Initial**	**AP/RT**	**AP/RF**		**50MPa/RT**			**75MPa/RT**					**100MPa/RT**				
Days	**0**	**7**	**7**	**14**	**7**	**14**	**28**	**7**	**14**	**28**	**39**	**60**	**7**	**14**	**28**	**39**	**60**
Total MUFA	30.66c	30.60abc	30.60bc	30.72c	30.64c	30.53abc	30.51abc	30.68c	30.47abc	30.57abc	30.54abc	30.26a	30.26c	30.69abc	30.47abc	30.55abc	30.29ab
C18:2*t*	1.00	1.02	0.96	1.04	1.05	1.03	1.01	0.98	0.99	1.00	1.02	0.97	1.01	0.99	0.99	1.03	1.02
CLA*c*9,*t*11	0.47	0.47	0.47	0.51	0.47	0.47	0.46	0.47	0.47	0.47	0.46	0.46	0.47	0.47	0.47	0.47	0.47
CLA*t*10,*c*12	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
C18:2*c*9,*t*12	0.22	0.23	0.22	0.23	0.23	0.23	0.23	0.22	0.22	0.23	0.23	0.22	0.22	0.22	0.23	0.23	0.23
C18:2*c*9,*c*12	2.16e	2.17e	2.14cde	2.16e	2.15cde	2.12abcd	2.12abc	2.14bcde	2.11abc	2.12abcd	2.11abc	2.08a	2.16de	2.11abc	2.10ab	2.11abc	2.10ab
C18:3*n*-6	0.05	0.06	0.05	0.06	0.05	0.05	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
C18:3*n*-3	0.22	0.21	0.21	0.23	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
C20:2	0.05	0.05	0.05	0.03	0.05	0.04	0.04	0.05	0.04	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.05
C20:3*n*-6	0.13c	0.13bc	0.13bc	0.12abc	0.12abc	0.12abc	0.12ab	0.12abc	0.12abc	0.11a	0.12ab	0.12abc	0.12abc	0.12ab	0.12ab	0.11a	0.12abc
C20:4*n*-6	0.23d	0.21cd	0.21cd	0.20bcd	0.20bcd	0.19abc	0.16a	0.19abc	0.18abc	0.20bcd	0.17ab	0.18abc	0.18abc	0.19abc	0.19abc	0.18abc	0.18abc
C20:5*n*-3	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.02	0.01
C22:5	0.05	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Total PUFA	4.65bc	4.66bc	4.57abc	4.71c	4.66bc	4.59abc	4.53ab	4.56abc	4.52ab	4.53ab	4.51ab	4.44a	4.59abc	4.51ab	4.51ab	4.55abc	4.54ab

Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

5.3.7 Secondary Lipid oxidation by-products

Lipid oxidation is responsible for the production of numerous undesirable compounds that impact negatively the sensory and nutritional qualities of dairy products, what can be enhanced by the presence of oxygen, light, endogenous and exogenous metals, and enzymes (Ajmal, Nadeem, Imran, & Junaid, 2018). MDA content was monitored as an indicator of secondary lipid oxidation development in all milk samples, being the results presented in **Table 5.1**. The initial value (0.83 ± 0.08 µg MDA/mL) observed is similar to the ones reported by Johnson et al. (2015) and the only storage condition that presented a significant change (increase) was AP/RT, reaching values of 1.18 ± 0.07 µg MDA/mL ($p < 0.05$) after 7 days. All the other storage conditions showed no significant ($p > 0.05$) variations in lipid oxidation values, despite showing a tendency to increase in HS samples at the end of the storage period. This resulted in a good maintenance of MDA values for all HS samples up to 60 days of storage (particularly at 75 and 100 MPa), compared to the values of the initial milk. Noteworthy is the fact that after 60 days at RT, samples stored under 75 and 100 MPa presented TBARS values (1.03 ± 0.11 and 0.84 ± 0.05 µg MDA/mL, respectively) below the 1.3 µg MDA/mL, which was associated with perceptible sensory changes in milk reported in Johnson et al. (2015) work.

Table 5.6 - Total protein (g/100mL), soluble protein (mg/100mL), free amino acids (nmol/mL) of raw milk prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–i) indicate significant differences ($p < 0.05$) between the different storage conditions for each parameter.

Condition	**Initial**	**AP/RT**	**AP/RF**		**50MPa/RT**			**75MPa/RT**					**100MPa/RT**				
Days	**0**	**7**	**7**	**14**	**7**	**14**	**28**	**7**	**14**	**28**	**39**	**60**	**7**	**14**	**28**	**39**	**60**
Total protein (g/100mL)	3.42	3.57	3.52	3.56	*NP*	3.36	3.37	*NP*	3.52	3.43	*NP*	3.36	*NP*	3.37	3.39	*NP*	3.38
Soluble protein (mg/100mL)	1.89bcd	1.06a	1.20a	1.24a	1.07a	1.48ab	1.52ab	1.78bc	2.24cde	2.38de	3.19fg	7.92h	1.90bcd	2.74ef	3.04f	3.69g	11.10i
FAA (nmol/mL)																	
Alanine	146.0a	321.8cd	295.9bcd	355.7d	*NP*	546.4e	675.6f	*NP*	271.7bc	303.6bcd	*NP*	546.0e	*NP*	251.5b	316.2cd	*NP*	651.7f
Glycine	103.7b	74.4b	20.0a	28.0a	*NP*	35.6a	38.4a	*NP*	144.3c	143.1c	*NP*	268.6e	*NP*	150.4c	173.2c	*NP*	372.3e
Valine*	64.1a	70.2a	30.8a	43.9a	*NP*	279.6b	339.2bcd	*NP*	352.8bcd	415.0cd	*NP*	990.9e	*NP*	290.9cd	424.7d	*NP*	1115.3e
Leucine*	38.0a	86.5ab	114.7ab	120.9ab	*NP*	188.8bc	425.0d	*NP*	265.1c	485.4de	*NP*	1325.3f	*NP*	293.3c	580.2e	*NP*	1502.0g
Isoleucine*	24.7ab	9.6a	12.6a	10.1a	*NP*	103.3bc	126.6cd	*NP*	129.7cd	169.7cd	*NP*	582.3e	*NP*	129.0cd	202.7d	*NP*	666.5i
Threonine	16.4a	12.6a	12.0a	13.3a	*NP*	41.0b	45.1b	*NP*	50.0b	73.6c	*NP*	250.3e	*NP*	87.9c	137.3d	*NP*	393.5f
Serine	16.7a	94.4def	32.3ab	34.3ab	*NP*	62.4bc	71.0cde	*NP*	63.0bcd	98.7ef	*NP*	429.7h	*NP*	112.1f	166.5g	*NP*	547.7i
Proline	63.0ab	152.9c	61.8a	77.5ab	*NP*	142.5c	124.8bc	*NP*	119.2bc	138.5c	*NP*	423.2e	*NP*	134.1c	232.5d	*NP*	778.7f
Asparagine	8.6ab	12.9b	10.0ab	6.2a	*NP*	10.2ab	8.7ab	*NP*	8.1ab	7.8ab	*NP*	21.20c	*NP*	6.8ab	9.8a	*NP*	24.2c
Aspartic acid	62.0a	155.2a	95.6a	133.0a	*NP*	86.5a	114.4a	*NP*	69.1a	92.6a	*NP*	324.9b	*NP*	71.1a	136.2a	*NP*	498.8c
Methionine*	6.2a	3.3a	1.4a	3.0a	*NP*	5.5a	6.1a	*NP*	37.0b	57.8c	*NP*	203.6e	*NP*	52.4c	95.2d	*NP*	295.5f
Hydroxyproline	12.1ab	13.7ab	10.5ab	13.2ab	*NP*	10.2ab	6.0a	*NP*	15.0b	13.3ab	*NP*	16.9bc	*NP*	12.1ab	13.4ab	*NP*	24.3c
Glutamic acid	585.8a	679.2ab	767.6ab	874.4ab	*NP*	609.3a	645.8a	*NP*	673.7ab	903.4ab	*NP*	2058.7c	*NP*	801.5ab	1031.6b	*NP*	1777.1c
Phenylalanine*	19.5a	10.7a	1.4a	0.6a	*NP*	28.0a	34.6a	*NP*	87.6b	149.1c	*NP*	426.6d	*NP*	101.0b	176.7c	*NP*	503.8e
Ornithine	18.0abcd	41.0e	15.4abc	11.6a	*NP*	18.5abcd	33.1de	*NP*	10.4a	12.7ab	*NP*	18.5abcd	*NP*	15.4abc	28.0bcde	*NP*	28.4cde
Lysine*	22.6ab	13.0a	34.6ab	30.7ab	*NP*	36.9ab	50.6bc	*NP*	25.9ab	46.9bc	*NP*	81.0d	*NP*	30.0ab	32.2ab	*NP*	68.8cd
Histidine*	72.0a	22.4a	14.8a	16.9a	*NP*	73.7a	82.6a	*NP*	310.7b	395.0b	*NP*	891.9d	*NP*	423.2b	575.6c	*NP*	1119.2e
Tyrosine*	2.6a	*ND*	2.8a	3.5a	*NP*	1.3a	1.1a	*NP*	5.8a	22.0a	*NP*	93.4c	*NP*	12.9a	54.6b	*NP*	160.7d
Tryptophan*	18.4a	19.3a	3.5a	3.6a	*NP*	30.5a	54.5ab	*NP*	96.0b	209.1c	*NP*	538.1d	*NP*	107.3b	250.2c	*NP*	665.8e
Cystine	11.7a	19.8a	9.9a	10.7a	*NP*	8.8a	14.4a	*NP*	14.1a	56.5b	*NP*	198.9c	*NP*	20.7a	79.3b	*NP*	208.6c
Total FAA (µmol/mL)	1.3a	1.8ab	1.6ab	1.8ab	*NP*	2.3bc	3.0cd	*NP*	2.8c	3.9de	*NP*	10.1f	*NP*	3.1cd	4.7e	*NP*	11.4g

NP – parameters not performed under these condition *ND* – not detected * - essential amino acids

5.3.8 Protein profile

Total protein was quantified prior and after storage at the different conditions (**Table 5.6**), with an initial value of 3.42 ± 0.13 g/100mL, which is in accordance with the literature for bovine milk (Silva et al., 2010) and no variations ($p > 0.05$) were observed between all the different storage condition, even after 60 days at 75 and 100/RT.

Regarding soluble protein (SP), an overall increase ($p < 0.05$) was observed, especially for longer storage periods (**Table 5.6**). SP content for initial milk was 1.89 ± 0.10 mg/100mL, decreasing after 7 days ($p < 0.05$) at AP/RF, 50/RT, and AP/RT to a minimum of 1.06 ± 0.10 mg/100mL, possibly related to nitrogen uptake for microbial metabolism (Hoskisson, Sharples, & Hobbs, 2003). Storage under 75 and 100/RT maintained SP concentration after 7 days ($p > 0.05$), which tended to increase as the storage period increased, with a significant variation being observed from day 14 to 39 ($p < 0.05$) of storage, under 75 and 100/RT, respectively. Specially, from day 39 until the end of the study, SP concentration had a pronounce increase of almost 4- to 6-fold under 75 and 100/RT, respectively, which can be attributed to the enzymatic activity of proteases like plasmin and microbial proteases (Moussa et al., 2013). SP increment in milk can occur in prolonged storage at RT, with Moussa et al. (2013) reporting an increase of 140% from day 0 to 60 days at 30 ºC in SP content of UHT milk, possible as a result of thermoresistant microbial proteases (mainly from *Pseudomonas* spp.), since plasmin tends to lose most of its activity at UHT conditions (Enright, Patricia Bland, Needs, & Kelly, 1999). HPP of raw milk (Huppertz, Fox, & Kelly, 2004), seemed to affect only plasmin activity for pressures higher than 250 MPa (10-30 min), which decreased throughout the storage at AP/RF and at 37 ºC, but in the end, resulted in a significant increase in SP content, even in treatments with reduced plasmin activity, thus pointing to the involvement of other proteases in this process.

Free amino acids (FAA) showed a similar behaviour during the study as to what was observed to SP, under the different storage conditions (**Table 5.6**), with a linear correlation being observed between SP and FAA (SP = FAA × 0.0008 – 0.3024, $r^2 = 0.97$). Initially raw milk had a total FAA of 1.3 ± 0.1 µmol/mL, being detected 20 aa, with all essential ones being present and characterized mainly by a high abundance of glutamic acid, alanine, glycine, histidine, and proline, which is in accordance with the literature (Ferchaud Roucher et al., 2013). Storage under AP/RT and AP/RF resulted in similar total FAA ($p > 0.05$), comparatively to the initial values, despite the tendency to increase at the end of storage for

these two conditions. While, on the other hand HS presented an overall significant increase throughout the storage period on the majority of FAA, increasing over time and were more pronounced as pressure also increased ($p < 0.05$). Milk stored under 75 and 100/RT had a similar FAA profile, but comparatively to the initial FAA, a greater variance ($p < 0.05$) was observed specially for tyrosine > methionine > leucine > tryptophan > serine > isoleucine > phenylalanine > threonine > cystine > valine > and histidine, with these FAA being associated with plasmin and microbial proteases enzymatic activity on caseins (Moussa et al., 2013). Since in this study, raw milk without any kind of processing was employed, a variety of active microbial proteases can be present initially, alongside with endogenous plasmin, which can promote casein proteolysis into small peptides or amino acids (Enright et al., 1999). The information regarding the effect of HPP in milk plasmin and other proteases, usually employed higher pressures (200-400 MPa) than the ones used in this study, and for shorter periods of time, and thus, it is difficult to make a straightforward comparation of the results obtained in these different conditions (Huppertz et al., 2004). However, when Atlantic salmon was stored under HS conditions (50-75 MPa at 10-25 ºC), several proteases (cathepsin B, D and calpains) have shown to maintain partial activity even after periods up to 50 days (Fidalgo et al., 2020), with these proteases activities being more affected by storage temperature than by the HS pressure level during storage, with storage at the lower temperature (10 ºC) causing changes in the myofibrillar fragmentation index to a lesser extent (Fidalgo et al., 2020).

5.4 Conclusion

The quality and nutritional parameters of raw milk evaluated in this study, point to a better preservation by HS, comparatively to conventional RF, particularly for pressures of 75 and 100 MPa. For instance, the only parameter found to be considerable affected by HS was FAA content, indicating a higher proteolytic activity dependent on pressure intensity that was more pronounced after 60 days under HS. And thus, further research regarding the HS effect on the proteolytic agents of raw milk, should be investigated in order to fully understand it, and its impacts in the sensorial properties of milk and its technofunctional properties to produce dairy products. On the other hand, HS of 75-100/RT presented an overall profile similar to raw milk prior to storage, for all the other parameters monitored,

clearly resulting in a better preservation methodology comparatively to RF for longer storage periods (with similar observations were also found for milk microbial quality in *Chapter 4*).

In conclusion, HS at 75 and 100 MPa at RT is a clear promising food preservation methodology for raw milk, leading possibly to considerable shelf-life extension with overall a similar quality to raw milk and to refrigerated milk (but in this case for a much shorter storage period) and should be further studied, given the high importance of milk in the human diet.

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CHAPTER 6

Chapter 6.

This chapter is based on the fourth manuscript submitted for publication

Extended shelf-life of fresh cheese by hyperbaric storage as a quasi-energetically costless, environmentally friendlier, and more sustainable alternative to refrigeration

Ricardo V. Duarte, Ana M. Gomes, Ivonne Delgadillo, Francisco J. Barba, Jorge A. Saraiva

6.1 Introduction

Storage under moderate pressure, hyperbaric storage (HS), was initially discovered by accident when a submersible that was sunken for 10 months at 1540 m depth (~15 MPa) at 3 ºC was rescued, and several perishable foods (sandwiches, soups and apples) were recovered in good consumable conditions, as lower temperatures and moderate pressure (~15 MPa) were able to synergistically slowdown microbial growth (Jannasch, Eimhjellen, Wirsen, & Farmanfarmalan, 1971). In the next years, HS studies emphasized the effect of low temperatures and pressure for food preservation, with minimal changes observed for rice, wheat and soy beans stored during one year (3.5 MPa at 1 ºC) by Mitsuda (1972), while Charm, Longmaid, and Carver (1977) preserved cod fish, beef and chicken during 36 days (-3 to 0 ºC under ≈20 MPa), reporting reduced microbial and enzymatic activity.

Over the last decade HS regained scientific interest as several studies analysed its feasibility at room temperature (RT) in several highly perishable foods, with results pointing to the potential replacement of refrigeration (RF), as HS resulted generally in enhanced microbial stability and safety, leading to increased shelf-life. In one of the first case-studies, low pH strawberry juice was stored at 20 ºC under 25, 100 and 220 MPa for 20 days, resulting in microbial growth inhibition even under 25 MPa, contrarily to the microbial proliferation observed at RF under atmospheric pressure (AP) (Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). Similar results were reported for more perishable low acidity juices (watermelon and melon juice), studied at and above RT (25-37 ºC) under 25-150 MPa from 8 to 60 hours, having been reported in general a more stable pH, titratable

acidity, total soluble solids, browning degree and cloudiness in HS/RT samples, although some colour losses were observed (Fidalgo et al., 2014; Queirós et al., 2014; Santos et al., 2015). In these studies, different sensibility to pressure by the studied microbiological groups were consistent, as generally Enterobacteriaceae, yeast and moulds were inactivated during storage under 50 MPa, while total aerobic mesophiles required pressures above 75 MPa, without significant changes in most of the physicochemical parameters, indicating that HS could be at least equivalent to RF, even when performed at RT. And thus, since during HS at RT energy is only applied in the compression/decompression phases, while no energy is required to control the temperature throughout the storage, this new preservation methodology has a considerable lower energy requirement being considered environmentally friendlier than conventional RF, with an estimated lower carbon foot print of 26-fold (Bermejo-Prada, Colmant, Otero, & Guignon, 2017).

More recently, solid highly perishable food products (minced pork meat, whey cheese and fresh salmon) have been studied for 24 h (in an industrial scale high pressure processing equipment) and further up to 25 days (Duarte et al., 2017; Fernandes et al., 2018; Fidalgo, Lemos, Delgadillo, & Saraiva, 2018). Whey cheese stored at HS/RT also maintained the pH, water activity, colour and fatty acids content after 10 days, while although no changes were reported for colour, water activity and pH of stored fresh salmon, an increase on the primary and secondary lipid oxidation products were observed. In another study Fidalgo, Pinto, Delgadillo, and Saraiva (2021) pointed to increased microbial shelf-life of fresh salmon for at least 30 days under HS, due to inactivation of the endogenous microbial population to counts below the detection limit of 1 log CFU/g under 75 MPa, while also reducing the inoculated pathogenic surrogate bacteria load (*Escherichia coli* and *Listeria innocua*). Similar HS preservation outcomes were reported by Santos, Castro, Delgadillo, and Saraiva (2020) in meats at RT during 60 days, as storage under 75/25 ºC reduced the endogenous microbial population throughout storage, avoiding meat spoilage, opposingly to samples kept under AP/RF, that were microbiologically unacceptable (above 7.00 log CFU/g) after 15 days. Storage under HS also inactivated to bellow the detection limit inoculated *E. coli* after 14 days, while *L. innocua* was slowly inactivated throughout storage. Meat samples stored under 75/25 ºC exhibited increased levels of lipid oxidation and drip losses through prolonged storage, maintaining mostly of the other physicochemical parameters stable for 60 days. Therefore, HS may contribute to increased shelf-life extension and enhanced

microbial safety of highly perishable foods as a more suitable alterative preservation methodology than RF.

Dairy products have a rich nutrient profile, supplying several vitamins and minerals, representing an important part of the human diet while contributing to a healthy growth and development (Sharabi, Okun, & Shpigelman, 2018). Fresh dairy products are widely consumed all over the world and are estimated to increase 1% worldwide, playing an important role in the sustainability of many countries' economy (OECD/FAO, 2020). Latin-style fresh cheese (non-ripen cheese) is a popular dairy product in some European and Latin American countries, made from pasteurized milk by acidic and enzymatic clotting, with a characteristic soft texture, mild flavour and low salt content (Bleoancă et al., 2016). The normal high moisture content and neutral pH level, provides the ideal postprocessing conditions for the growth of contaminant bacteria and other microbiota, limiting its shelf-life even under RF (Hnosko, San-Martin Gonzalez, & Clark, 2012). In this study two types of fresh cheese (FC) both made with pasteurized milk, one from cow's and the other from goat's milk, were stored under HS (50, 75 and 100 MPa) at variable RT and compared to AP/RF and AP/RT. Cheeses were stored during 60 days and the effect of HS on endogenous microbiota (total aerobic mesophiles, lactic acid bacteria, Enterobacteriaceae, coliform bacteria, yeasts and moulds, and total endospores load) and in the physicochemical parameters (pH, whey loss, moisture content, colour and lipid oxidation) were evaluated.

6.2 Materials and Methods

6.2.1 Fresh cheese samples preparation and storage

Two commercial fresh cheeses, both made form pasteurized milk, one from cow's and the other from goat's milk, were acquired from a local supermarket. Cheeses were kept under RF during transportation and until being used, being then packed under low temperature, into low permeability polyamide-polyethylene bags (90 micron, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal) previously sterilized with UV-light, under aseptic conditions inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) and heat-sealed individually.

The cheese samples were stored at room temperature (RT), refrigeration (RF, ≈4 ºC) at atmospheric pressure (AP, 0.1 MPa), and under 50, 75 and 100 MPa at RT (15 – 22 ºC),

with sampling occurring at 3, 7, 14, 28, 42 and 60 days. HS experiments were carried out in a multivessel high pressure equipment SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), using a mixture of propylene glycol and water (40:60) as the pressurizing fluid. Samples stored under AP/RF and AP/RT were kept immersed in the same pressurizing fluid and in the dark, to mimic as much as possible the same environment of HS samples inside the HP vessel, except for pressure.

6.2.2 Microbial analyses

After each experiment, cheeses were serially diluted in Ringer solution, except for the determination of total endospores load, which was serially diluted in physiological solution (0.9% NaCl) and plated on the appropriate media. Total aerobic mesophiles (TAM) and total endospores load (TEL) were enumerated on plate count agar (PCA), incubated at 30 ºC for 3 days (ISO 4833:2013). To assess TEL, aliquots of fresh cheese diluted in physiological solution were heat-treated at 80 ºC for 20 min to eliminate vegetative microorganisms, and then plated on the appropriate media. Enterobacteriaceae (ENT) counts were determined on violet red bile glucose agar, incubated at 37 ºC for 1 day (ISO 21528:2017). Lactic acid bacteria (LAB) counts were determined on man rogosa sharpe agar and incubated at 30 ºC for 3 days (ISO 11133:2014). Coliform bacteria (COL) were enumerated on chromocult coliform agar (CCA), incubated at 37 ºC for 1 day (ISO 4832: 2007). Yeasts and moulds (YM) were enumerated using rose bengal chloramphenicol agar at 25 ºC for 5 days (ISO 21527:2008). All the results were expressed as decimal logarithm of colony forming units per gram of fresh cheese (log CFU/g).

6.2.3 D_p-value determination

Determination of the D_p-values was carried out for the microbiological groups analysed in this study for which inactivation was verified and measurable (values below the quantification and detection limits were not considered), being verified for all cases a first order inactivation kinetics. A D_p-value is the time needed at a constant pressure, to reach a decimal reduction in the microbial load (expressed in days in this work) and was calculated based on the negative inverse of the log linear model slope (**Equation 6.1**).

$$Log\ (N)\ = Log\ (N_0) - \frac{t}{D_p}$$ **Equation 6.1**

N is the microbial concentration (CFU/g) under a certain pressure (MPa) for a certain time (t) in days, and N_0 is the initial load (CFU/g).

6.2.4 Physicochemical parameters

For each cheese sample, six random points were selected and at constant room temperature with a proper calibrated pH/temperature penetration meter (Testo 205, Testo, Inc., New Jersey, USA), the pH of the cheeses and the free whey were measured.

Cheese whey loss during storage was calculated by **Equation 6.2**, by weighing cheeses before and after each storage period, as performed by Evert-Arriagada, Hernández-Herrero, Guamis, and Trujillo (2014).

$$\mathrm{WL} = \frac{(P_1 - P_2)}{P_1} \times 100\%$$ **Equation 6.2**

where WL stands for whey loss in percentage, P_1 is the weight of the fresh cheese before storage and P_2 is the weight of the fresh cheese after storage.

The moisture content was determined by oven drying 1 g of weighed fresh cheese, in triplicate per sample, at 105 ºC for 72h, until constant weight was obtained, and expressed as percentage (**Equation 6.3**) (García et al., 2012).

$$\mathrm{MC} = \frac{(m_1 - m_2)}{m_1} \times 100\%$$ **Equation 6.3**

where MC stands for moisture content in percentage, m_1 is the weight of the fresh cheese before drying and m_2 is the weight of the fresh cheese after drying.

6.2.5 Colour

Minolta Konica CM 2300d equipment (Konica MinoltaCM 2300d, Osaka, Japan) was used in colour analyses, being calibrated before each sample measurement. CIELab parameters were determined through the original SpectraMagic NX software (Konica Minolta, Osaka, Japan), according to the International Commission on Illumination

regulations: red/green colour (a^*), yellow/blue colour (b^*) and luminosity (L^*) parameters. The colour parameters L^*, a^*, and b^* were measured in six random points of each cheese and the total colour change (ΔE^*) was calculated by **Equation 6.4**:

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad \textbf{Equation 6.4}$$

where ΔE^* represents total colour difference between a respective sample and the initial one prior to storage, with L^*_0, a^*_0, and b^*_0 representing the respective parameter at day zero.

6.2.6 Secondary lipid oxidation by-products

Lipid oxidation state was assessed by malondialdehyde (MDA) quantification, using 2-thiobarbituric acid reactive substances (TBARS) method adapted from King (1962). Initially 1 g of fresh cheese was crumbled into smaller pieces and homogenised with 3 mL 7.5% trichloroacetic acid, until being completely dissolved, followed by centrifugation at 4000 × g at 4 ºC for 20 min (Universal 320-R, Hettich Group, Tuttlingen, Germany). The resulted extract was filtered (Whatman nº1) and the same volume of 46 mM 2-thiobarbituric acid was added, vortexed and immersed in boiling water for 40 min, and then cooled down in cold water. Triplicates were measured using a micro-plate spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a Brand plate of 96 wells, at 532 nm. Standard solutions of MDA in 7.5% trichloroacetic acid were prepared from 1,1,3,3-tetramethoxypropane and a calibration curve was prepared at a concentration ranging from 0.2 to 10 μg/L. TBARS results were expressed as μg of malondialdehyde per g of cheese.

6.2.7 Statistical analysis

All experiments were carried out in triplicate and all analyses were done in triplicate. The different storage conditions were compared using Analysis of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance.

6.3 Results and Discussion

6.3.1 Microbial analyses

Both cow's and goat's FC were microbiologically analysed throughout storage under the different storage conditions, being the results shown in **Figure 6.1** and **6.2**, respectively. Initial microbial load for cow's FC regarding TAM, LAB, ENT, COL, YM and TEL were 6.84 ± 0.45, 5.03 ± 0.15, 5.41 ± 0.06, 5.10 ± 0.37, 4.90 ± 0.11 and ≤2.30 log CFU/g (quantification level for TEL), respectively, and goat's FC had initial counts of 7.19 ± 0.05, 6.04 ± 0.03, 5.83 ± 0.16, 5.61 ± 0.13, 5.20 ± 0.16 and 2.34 ± 0.06 log CFU/g, respectively. Both fresh cheeses presented an elevated initial microbial load, denoting its highly perishable nature (neutral pH and high-water activity) that jeopardize and limit its shelf-life even under refrigerated storage.

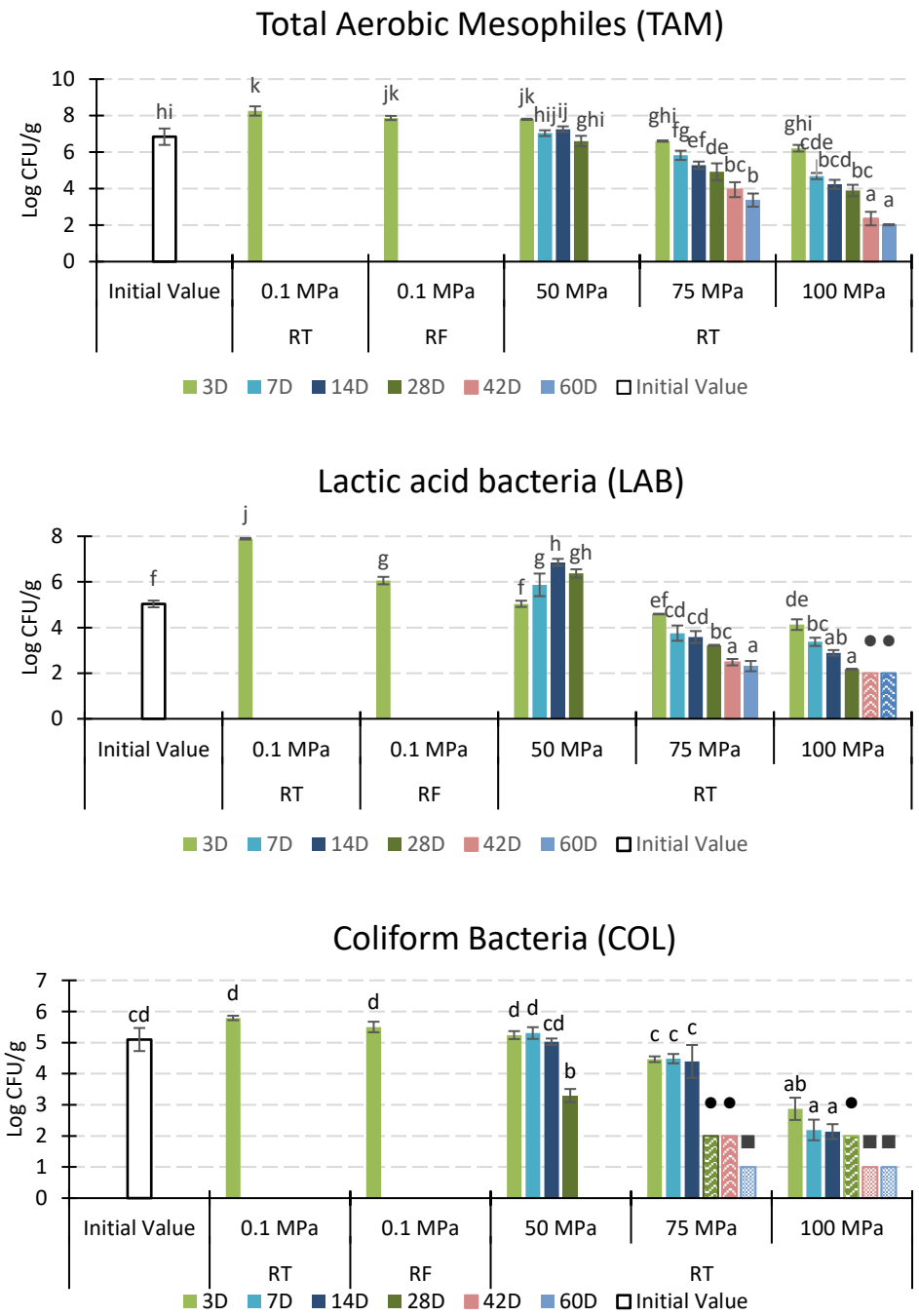
As expected, when cheeses were placed at AP/RT for 3 days, an increase in overall microbial counts was observed comparatively to the initial load, for both cheeses. Regarding cow's FC, significant increases of 1.41 and 2.86 log units were observed for TAM and LAB counts ($p < 0.05$), while the other microbiological groups presented slight counts increase ($p > 0.05$) after 3 days at AP/RT. A higher growth rate was observed in goat's FC for all microbiological groups ($p < 0.05$), of 1.68, 2.94, 0.96 and 1.50 log units for TAM, LAB, ENT and COL, respectively, while YM and TEL maintained constant counts after 3 days of storage.

Under AP/RF, the lower temperature was able to slow down microbial growth, comparatively to AP/RT, but still, in some microbiological groups a considerable growth ($p < 0.05$) was observed, comparatively to the initial load, after 3 days. For instance, TAM and LAB counts increased ($p < 0.05$) around 1 log unit in cow's FC, while regarding goat's FC, TAM, LAB, ENT and COL showed slightly increased counts ($p < 0.05$) of less than 1 log unit, although lower than the values observed for storage at AP/RT, as expected. Since after 3 days the microbial loads of cow's FC stored at AP/RF and AP/RT were high, the study was stopped here for these samples. While goat's FC storage was studied up to 7 days, with no growth observed for TAM and TEL ($p > 0.05$), while a slight increase over time ($p < 0.05$) was observed for the other microbiological groups.

As for storage under pressure (**Figure 6.1** and **6.2**), different impacts in microbial growth were observed, directly dependent on pressure intensity and microbiological

composition. Storage under 50/RT initially resulted in microbial growth inhibition of LAB, COL and ENT, while TAM increased around 1 log unit ($p < 0.05$), and a reduction in the microbial counts around 0.4 log units and to counts below the quantification limit (≤2.30 log CFU/g) were observed for YM and TEL, respectively. As the storage time went by, TAM counts stabilised around 7 log CFU/g, with COL, ENT and YM being more susceptible to storage under 50/RT, decreasing around 2 log units for COL and ENT, and to undetectable counts for YM (≤1 log CFU/g), after 28 days, with corresponding D_p-values of 14.7, 11.3 and 4.7 days (*Annex B*, ***Figure B.1*** *and* ***Table B.1***). On the other hand, LAB was more resistant under this storage condition, being able to gradually grow and reach counts of 6.37 ± 0.18 log CFU/g after 28 days. Also endospores formation was observed ($p < 0.05$), increasing to counts of 3.02 ± 0.15 log CFU/g, which could be a result of increments of microbial population in parallel with pH decrease and nutrient depletion, triggering endospores formation (Coorevits et al., 2011). A slightly different situation was verified for storage under 75/RT that inactivated overall and progressively the microbial population over time, reducing gradually all the analysed microbiological groups. For instance, a continued counts reduction was observed, being more pronounced for TAM and LAB, of around 3.5 and 2.7 log units, respectively, after 60 days, while COL and ENT maintained counts around 4.4 log CFU/g after 14 days, being inactivated to counts bellow the quantification and detection limit, after 42 and 60 days (more than 4 log units reduction), respectively. YM were highly sensitive to storage under 75/RT, presenting counts below 2 log CFU/g at the 7th day of storage, and below 1 log CFU/g from the 28th day onforward. Also, endospores load decreased, being reduced to undetectable levels on the 42nd day of storage. Increasing the storage pressure to 100 MPa resulted in an even faster microbial inactivation, as reflected by the D_p-values, comparatively to storage under 75/RT (*Annex B*, ***Figure B.1*** *and* ***Table B.1***). In fact, storage under 75 and 100 MPa resulted in D_p-values of, 17.8 and 13.4 days for TAM, 23.9 and 10.9 days for LAB, respectively, and of 9.6 days for ENT under 75 MPa. Alike storage at 75/RT, COL, ENT and YM were highly susceptible to 100/RT, being inactivated faster to undetectable counts after 42, 14 and 7 days, respectively, while for TAM and LAB, a reduction of around 5 and 3 log units were achieved after 60 days ($p < 0.05$), respectively. As for TEL, just after 3 days no viable counts were detected, like what was observed in a previous study conducted by the same authors (*Chapter 4*) in raw milk stored

under HS conditions, reporting the endospores inactivation capacity of HS (≥75 MPa) in *Bacillus subtilis* endospores.



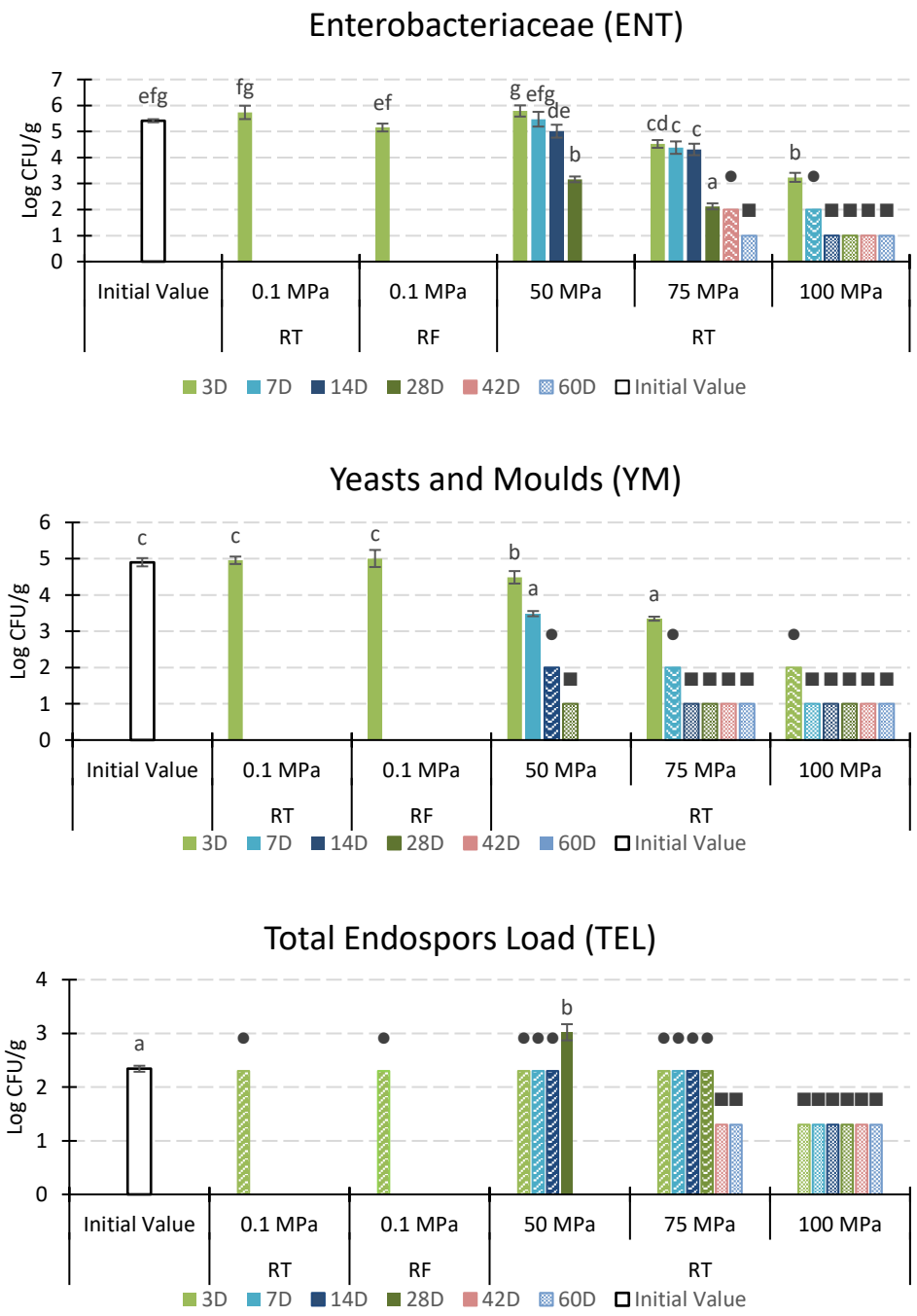
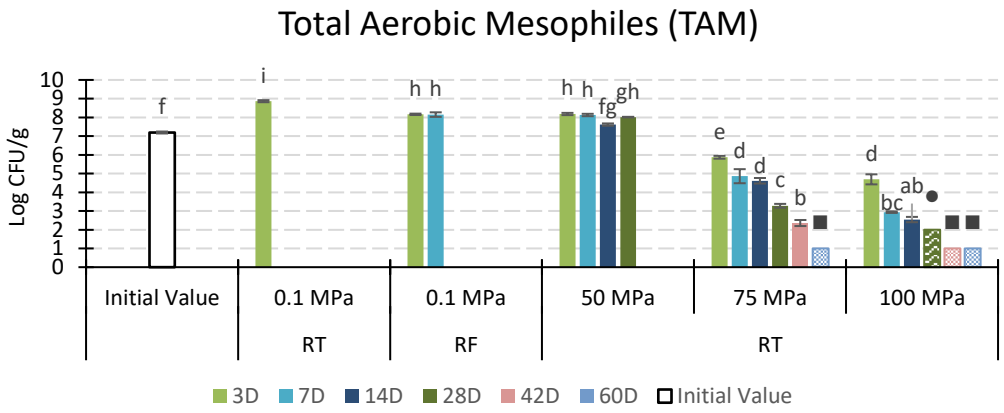
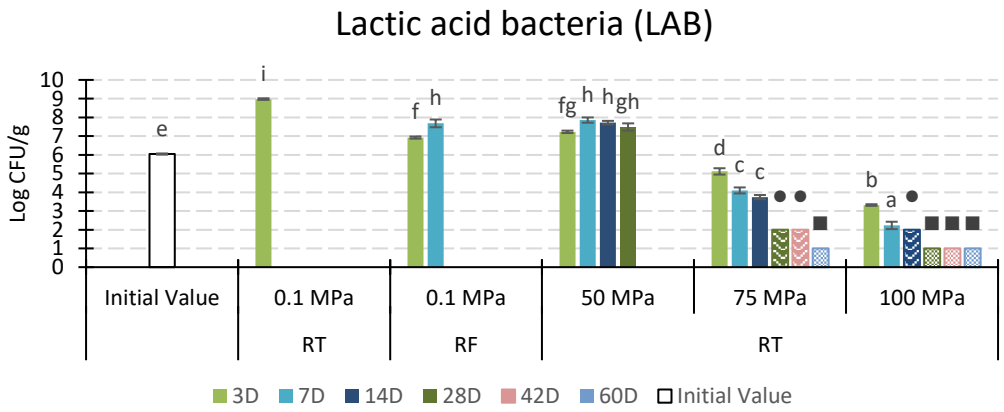


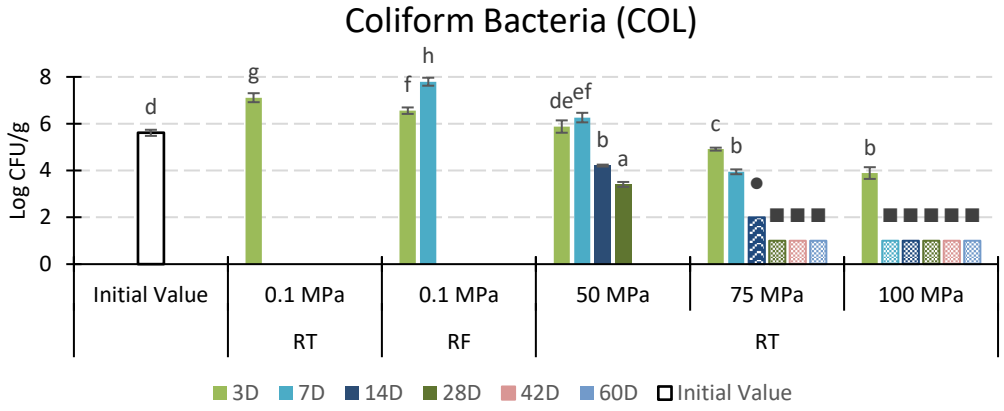
Figure 6.1 - TAM, LAB, COL, ENT, YM and TEL microbial growth during HS at room temperature (RT) of cow's fresh cheese, and comparison with storage under refrigeration (RF) and RT and atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$), where • and ■ represent counts below the quantification (2 log CFU/g) and detection limit (1 log CFU/g), respectively for all microbiological groups, except for TEL, to which the quantification and detection limits, are 2.3 and 1.3 log CFU/g, respectively.

Goat's FC microbial load was overall more sensible to HS, comparatively to cow's FC, resulting in faster microbial inactivation throughout storage. Although initially storage under 50/RT allowed microbial proliferation ($p < 0.05$) of TAM and LAB until the 3rd and 7th days of storage to around 8 log CFU/g, respectively, on the following storage periods no increments in microbial counts were observed ($p > 0.05$). Also, TEL counts remained below the quantification limit throughout the storage, while COL, ENT and YM microbiological groups were inactivated ($p < 0.05$) over time, with corresponding D_p-values of 11.2, 5.4 and 3.9 days (*Annex B*, ***Figure B.2*** *and* ***Table B.2***). Storage under 75 and 100 MPa, promoted a faster inactivation effect in all microbiological groups ($p < 0.05$), especially for the more resistant ones, TAM and LAB. For instance, 75/RT resulted in microbial inactivation of both microbiological groups to undetectable counts at the 60th days of storage, while at 100/RT a faster reduction was achieved, resulting in undetectable microbial counts for TAM and LAB at the 42nd and 14th day, respectively. Differences in microbial inactivation rates are also visible by the corresponding D_p-values for TAM, 9.9 and 3.4 days, and LAB, 6.3 and 1.9 days, regarding storage under 75/RT and 100/RT, respectively (*Annex B*, ***Figure B.2*** *and* ***Table B.2***). The other microbiological groups were highly susceptible to these storage conditions, as a faster inactivation to undetectable counts was achieved comparatively to 50/RT, presenting D_p-values of 4.2 (COL), and 3.5 days (ENT) at 75/RT, while at 100/RT, D_p-value determination was not calculated, due to faster inactivation to levels below the quantification and detection levels, in the first sampling periods and so not enough quantifiable data points could be gathered. At 75 MPa and above, endospores were inactivated, similarly to what was observed for cow's FC under these conditions.

Overall, HS of cow's and goat's FC at 50/RT resulted in a better microbial preservation comparatively to storage at AP/RF, promoting the inhibition of TAM and LAB in the first days, while on the other hand, inactivating COL, ENT and YM throughout the storage. Increasing the storage pressure to 75-100 MPa allowed a much better preservation of both cheeses at RT, promoting not only microbial growth inhibition, but also considerable and progressively a faster additional inactivation effect, resulting in significant counts reduction over time, in most cases, to undetectable counts. And so, HS/RT under these pressure levels can contribute to an enhanced microbial safety and a significant shelf-life extension of these highly perishable dairy product, with no temperature control and so as a *quasi*-energetically costless food preservation methodology.


Total Aerobic Mesophiles (TAM)
Log CFU/g
Initial Value
0.1 MPa RT
0.1 MPa RF
50 MPa
75 MPa RT
100 MPa
3D 7D 14D 28D 42D 60D Initial Value


Lactic acid bacteria (LAB)
Log CFU/g
Initial Value
0.1 MPa RT
0.1 MPa RF
50 MPa
75 MPa RT
100 MPa
3D 7D 14D 28D 42D 60D Initial Value


Coliform Bacteria (COL)
Log CFU/g
Initial Value
0.1 MPa RT
0.1 MPa RF
50 MPa
75 MPa RT
100 MPa
3D 7D 14D 28D 42D 60D Initial Value

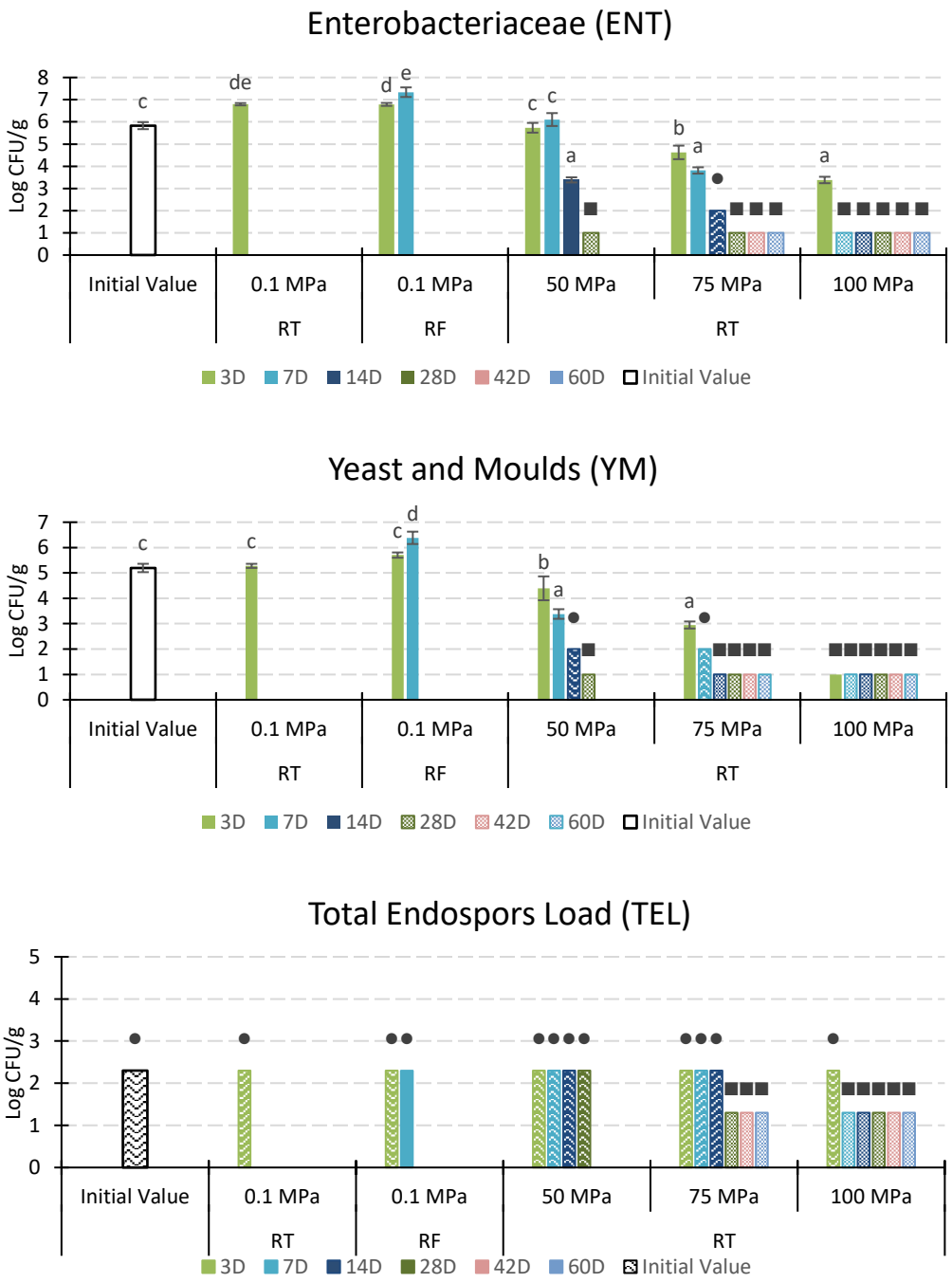


Figure 6.2 - TAM, LAB, COL, ENT, YM and TEL microbial growth during HS at room temperature (RT) of goat's fresh cheese, and comparison with storage under refrigeration (RF) and RT and atmospheric pressure (0.1 MPa). Different letters denote statistically significant differences ($p < 0.05$ where • and ■ represent counts below the quantification (2 log CFU/g) and detection limit (1 log CFU/g), respectively for all microbiological groups, except for TEL, to which the quantification and detection limits, are 2.3 and 1.3 log CFU/g, respectively.

6.3.2 Physicochemical parameters

Cow's and goat's FC presented an initial pH of 6.45 ± 0.01 and 6.58 ± 0.01, respectively, similar to the ones reported in the literature (Capellas, Mor-Mur, Gervilla, Yuste, & Guamis, 2000; Juan, Zamora, Quintana, Guamis, & Trujillo, 2013), with a pH value in the free whey similar to the ones observed in the cheeses, 6.43 ± 0.01 and 6.52 ± 0.03, respectively (**Table 6.1**). Overall, no considerable variations in the cheese and free whey pH values were observed under the same storage conditions, for both cheeses. In fact, storage at AP/RT after 3 days resulted a similar decrease in the pH values of both cheese and free whey, of 0.5 and 1.5 pH units ($p < 0.05$) in cow's and goat's FC, respectively. The differences in the decrease of the pH value can be attributed to the different overall microbial growth rate observed under this storage condition, as for instance TAM and LAB reached counts around 8 log CFU/g for cow's FC, while for goat's FC, reached counts around 9 log CFU/g. Microbial growth results in increased metabolic activity and metabolites production, which are responsible for lowering the pH (Buriti, da Rocha, & Saad, 2005). A slower decrease in the pH value at AP/RF over the storage period was observed in cow's FC ($p > 0.05$), while after 7 days, goat's cheese and free whey reached a minimum pH value ($p < 0.05$) of 6.06 ± 0.08 and 5.98 ± 0.07, respectively. Under HS conditions, the pH tended to decrease throughout storage under 50 MPa ($p < 0.05$), reaching values of 5.59 ± 0.01 and 4.63 ± 0.05 after 28 days in cow's and goat's FC, respectively, while at 75 and 100/RT, the microbial load inactivation throughout the storage, contributed to a more stable pH-value of both FCs and free whey ($p > 0.05$) throughout the entire storage.

Whey loss of cheeses throughout the storage was monitored and can be viewed in **Table 6.1**. Regarding cow's FC, refrigerated storage resulted in around 10% whey loss in the first 3-7 days, with storage at AP/RT showing losses of 24.0 ± 1.4%, while under HS, probably due to cheese matrix compression, 31-37% whey loss was verified at the 3rd day for all HS conditions ($p < 0.05$). Whey losses in FC immediately after high pressure processing (HPP), result from the direct compression effect of high pressure in cheeses, with several studies reporting also whey losses in HPP cheeses (200-600 MPa), between 10-17% (Capellas, Mor-Mur, Sendra, & Guamis, 2001; Van Hekken, Tunick, Farkye, & Tomasula, 2013). Throughout storage at 50/RT, whey loss increased continuously ($p > 0.05$), presenting these cheeses evident textural changes at the 28th day, with an apparent "musher" structure, and thus, whey loss was not analysed in that period for this storage condition. After the 3rd

day of storage under 75 and 100/RT, whey loss decreased towards values around 20%, remaining constant from the 14th day of storage until the end of the storage period ($p > 0.05$). The overall whey loss reflected decreases in moisture content of cow's FC, as initially cow's FC had a moisture content of 65.0 ± 3.1%, decreasing correspondingly to the increased whey losses in all storage conditions for day 3 of storage, especially in HS conditions ($p < 0.05$). After this period, the moisture content tended to increase in all studied conditions to values similar ($p > 0.05$) to the ones observed initially (65.0%), stabilizing around 65.9 ± 2.3 and 65.6 ± 1.5% for chesses at 75 and 100/RT at the end of the study.

Whey loss and moisture content of the goat's FC generally followed the same behaviour to the ones reported above for cow's FC, with slight changes. In the first 3 days of storage, whey loss of goat's FC was also affected by high pressure, being more pronounced under 100/RT (32.6 ± 2.3%) comparatively to storage under 50 and 75/RT (23.1 ± 1.5% and 22.4 ± 4.8%, respectively), while under AP/RF and AP/RT, 4.2 ± 0.7% and 14.6 ± 3.4% of whey was released, respectively. Throughout storage under 75 and 100/RT, whey loss fluctuated around 23-31%, achieving values of around 29% and 27% after 60 days of storage, respectively. Initially goat's FC moisture content was 61.6 ± 2.5%, decreasing in the first days of storage for all storage conditions, especially under 50/RT, to 50.2 ± 3.7%. On the following days, cheeses under 50/RT, similarly to cow's FC, exhibited also a clear textural disintegration, becoming softer, resulting in increased moisture content to 61.0 ± 2.1% after 14 days. Storage under 75 and 100/RT presented similar ($p > 0.05$) values between these two storage conditions, with the first one tending to present lower values, comparatively to the initial one, reaching values of 53.9 ± 1.2% ($p < 0.05$) at the end of storage, while 100/RT reached a final moisture content of 56.5 ± 2.1% ($p > 0.05$) at the end of the storage. In a study with cow's and goat's FC preserved at RF, conducted by Sant'Ana et al. (2013), whey loss naturally increased in all cheeses, up to 17% after 21 days, with decreases in pH attributed as one of the causes responsible for whey loss, since increases in hydrogen ions concentration and acidified medium, reduces repulsive forces between casein micelles, thereby promoting aggregation and whey expulsion. In HS samples, a clear effect of pressure was observed initially, without an increase over time, maybe because HS conditions also allowed a better pH stability, closer to the initial ones.

6.3.3 Colour

Results of cow's and goat's FC colour parameters L^*, b^*, a^* and ΔE^* are shown in **Table 6.1**. The initial values of L^*, b^*, a^* of cow's FC were 90.79 ± 0.26, 13.21 ± 0.11, and -0.85 ± 0.03, close to the ones found in the literature (Juan et al., 2013). b^* parameter presented a slight increase ($p < 0.05$) comparatively to the initial value in most of the storage conditions, remaining mostly constant after the 3rd day of storage ($p > 0.05$), except for AP/RF at day 3, which decreased to 10.92 ± 0.85 ($p < 0.05$). The only significant variations ($p < 0.05$) observed regarding a^*, was again under AP/RF at the 3rd day of storage, -1.31 ± 0.14, and under 50/RT at the 7th day of storage ($p < 0.05$), -0.47 ± 0.02. As for L^* parameter, all storage conditions presented lower values, which could be related to whey loss during storage and compression of the cheese microstructure (Van Hekken et al., 2013). Overall, ΔE^* of cheeses ranged from values of 2 to 5, which according to Drlange (1994) are considered to have distinct (1.5-3.0) to very distinct differences (3.0-6.0) perceptibility by consumers, nevertheless all storage conditions had a similar ΔE^* value comparatively with FC stored under refrigerated storage at the 3rd day (2.99 ± 0.96), except for FC under 75/RT at day 3 and under 100/RT at day 42, with respective ΔE^* of 5.40 ± 0.69 and 6.18 ± 0.77, as ΔE^* variance was mostly affected by the L^* parameter.

Goat's FC had initially L^*, b^*, a^* values of 85.69 ± 3.25, 8.83 ± 0.30 and -1.31 ± 0.20, respectively. Generally, L^* tended to increase in all storage conditions without great variance ($p > 0.05$), except for AP/RF after 7 days of storage ($p < 0.05$). Also, b^* parameter increased over time, reaching higher values ($p < 0.05$) after 60 days of storage under 75 and 100/RT of 11.43 ± 0.14 and 11.63 ± 0.88, respectively. Overall, no significant ($p > 0.05$) variations were observed in a^*, despite being detected fluctuations ($p < 0.05$) in some storage periods. Throughout the storage period, ΔE^* increased slowly under 75 and 100/RT, with both conditions achieving ΔE^* values below 3, even after 60 days, which are considered "distinct differences", while higher values were achieved at AP/RF and at 50/RT after 7 days, 5.55 ± 0.69 and 3.77 ± 0.64, respectively. Overall, storage under 75 and 100 MPa allowed to retain cheeses colour even after prolonged storage at RT, far better than storage at AP/RF.

Table 6.1 - Cheese and whey pH, whey loss (%), moisture content (%), colour, and lipid oxidation (µg MDA/g) parameters of cow's and goat's fresh cheese prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–g) indicate significant differences ($p < 0.05$) between the different conditions for each parameter.

	Condition	Initial	AP/RT	AP/RF		50MPa/RT				75MPa/RT						100MPa/RT					
	Days	0	3	3	7	3	7	14	28	3	7	14	28	42	60	3	7	14	28	42	60
COW'S FC	**Cheese pH**	6.45 defg	5.90 b	6.43 defg	6.36 cd	6.40 cde	6.40 def	6.29 c	5.59 a	6.40 def	6.46 defg	6.53 g	6.43 defg	6.42 defg	6.42 defg	6.45 defg	6.47 efg	6.51 g	6.46 defg	6.49 fg	6.43 defg
	Whey pH	6.43 cdefg	5.69 a	6.36 bc	6.37 bcd	6.37 bcd	6.37 bcde	6.31 b	*ND*	6.38 bcd	6.49 efg	6.49 g	6.43 cdef	6.45 cdefg	6.44 cdefg	6.37 bcd	6.47 defg	6.52 fg	6.45 cdefg	6.53 fg	6.46 cdefg
	Whey Loss (%)	0	24.0 bcde	8.6 a	9.1 a	30.9 ef	36.3 f	37.0 f	*ND*	30.0 def	33.0 f	24.0 bcde	21.4 bcd	19.0 b	22.2 bcd	29.3 cdef	21.7 bcd	20.9 bc	21.2 bc	21.0 bc	19.8 b
	Moisture Content (%)	65.0 efg	60.7 abcde	64.0 cdef	69.2 g	57.6 a	60.2 abcd	58.0 ab	*ND*	58.4 ab	62.0 cdef	61.8 cdef	65.7 fg	64.4 def	65.9 fg	59.3 abc	64.2 cdef	65.7 fg	66.0 fg	64.5 def	65.6 fg
	Colour ***L****	90.79 g	86.08 abcd	88.94 fg	*nd*	87.04 abcdef	88.33 defg	85.72 abc	*ND*	85.41 ab	88.07 cdef	88.11 cdef	88.64 defg	86.31 abcde	88.79 efg	87.93 bcdef	87.76 bcdef	86.11 abcd	87.11 abcdef	84.64 a	88.84 efg
	Colour ***a****	-0.85 bcde	-0.74 def	-1.31 a	*nd*	-0.70 ef	-0.47 f	-1.11 ab	*ND*	-0.77 cdef	-0.85 bcde	-1.07 abc	-0.97 bcde	-0.93 bcde	-0.99 abcde	-0.90 bcde	-0.90 bcde	-1.11 ab	-0.76 cdef	-0.98 bcde	-1.04 abcd
	Colour ***b****	13.21 bcd	11.85 ab	10.92 a	*nd*	14.42 def	15.17 f	13.92 cdef	*ND*	13.48 cde	14.25 def	14.30 def	14.75 ef	14.68 ef	13.98 cdef	14.38 def	13.68 cde	12.57 bc	14.37 def	13.76 cdef	14.68 ef
	Colour ***ΔE****		4.93 cde	2.99 abc	*nd*	4.07 abcde	3.26 abcd	5.16 cde	*ND*	5.40 de	2.96 abc	3.07 abc	2.68 ab	4.79 bcde	2.22 a	3.12 abc	3.07 abc	4.73 bcde	3.87 abcd	6.18 e	2.59 a
	Lipid oxidation (µg MDA/g)	1.20 a	1.30 ab	1.29 ab	1.63 abc	1.66 abc	1.62 abc	2.67 d	2.78 d	1.43 ab	1.21 ab	1.39 ab	1.77 abc	1.86 abc	2.21 bcd	1.50 ab	1.50 ab	1.58 abc	1.81 abc	1.89 bc	1.90 bc
GOAT'S FC	**Cheese pH**	6.58e	4.98b	6.54e	6.06d	5.87c	5.03b	4.80a	4.63a	6.53e	6.47e	6.42e	6.57e	6.49e	6.49e	6.54e	6.47e	6.46e	6.54e	6.46e	6.52e
	Whey pH	6.52 defg	4.98 a	6.56 fg	5.98 c	5.80 b	*nd*	*nd*	*ND*	6.53 efg	6.49 defg	6.43 d	6.57 g	6.46 de	6.48 defg	6.53 efg	6.49 defg	6.47 def	6.53 efg	6.47 def	6.51 defg
	Whey Loss (%)	0	14.6 bc	4.2 a	7.6 ab	23.1 cd	23.5 cd	*nd*	*ND*	22.4 cd	26.6 de	34.7 e	27.9 de	31.7 de	28.6 de	32.6 de	35.0 e	22.9 cd	26.4 de	30.5 de	26.8 de
	Moisture Content (%)	61.6 ef	58.5 bcdef	57.3 abcdef	59.1 bcdef	50.2 f	63.8 def	61.0 def	*ND*	60.1 cdef	52.4 ab	56.0 abcde	57.0 abcdef	53.5 abc	53.9 abcd	57.3 abcdef	54.7 abcde	55.7 abcde	57.1 abcdef	51.8 ab	56.5 abcdef
	Colour ***L****	85.69 bc	86.53 bc	85.60 abc	80.20 a	87.74 c	83.13 abc	81.13 ab	*ND*	85.97 bc	86.47 bc	87.91 c	87.30 c	86.16 bc	85.95 bc	85.19 abc	86.51 bc	87.94 c	87.27 c	87.60 c	85.40 abc
	Colour ***a****	-1.31 efg	-1.37 defg	-1.93 ab	-1.22 fg	-1.37 defg	-1.84 ab	-1.70 bc	*ND*	-1.32 efg	-1.27 efg	-1.35 defg	-1.15 g	-1.51 cde	-1.98 a	-1.46 cdef	-1.26 efg	-1.27 efg	-1.58 cd	-1.32 efg	-1.16 g
	Colour ***b****	8.83 a	9.13 abc	8.87 ab	9.51 abcd	10.07 bcd	10.15 bcde	9.70 abcd	*ND*	9.58 abcd	10.29 cde	10.37 cde	10.36 cde	10.79 def	11.43 ef	10.72 def	9.85 abcd	9.88 abcd	10.76 def	10.44 cdef	11.63 f
	Colour ***ΔE****		1.36 ab	0.70 a	5.55 d	1.76 abc	3.77 cd	2.80 bc	*ND*	1.33 ab	1.76 ab	2.71 bc	2.25 abc	2.23 abc	2.72 bc	2.39 abc	2.88 bc	2.49 abc	2.58 bc	2.51 abc	2.75 bc
	Lipid oxidation (µg MDA/g)	0.67 a	0.71 a	0.70 a	0.70 a	0.69 a	0.75 a	0.96 abc	0.93 abc	0.68 a	0.97 abc	0.87 ab	1.02 abc	1.27 c	2.13 d	0.70 a	0.86 ab	0.95 abc	0.99 abc	1.20 bc	1.15 bc

(*ND– stands for not determined*)

6.3.4 Secondary Lipid oxidation by-products

Regarding lipid oxidation, throughout storage under the different storage conditions it was clear an overall raise of TBARS values from 1.20 ± 0.11 µg MDA/g to a maximum of 2.78 ± 0.48 µg MDA/g and from 0.67 ± 0.07 to 2.13 ± 0.11 µg MDA/g, for cow's and goat's FC, respectively (**Table 6.1**). Lipid oxidation was more pronounced ($p < 0.05$) in cow's FC stored under 50/RT after 14 days of storage, reaching 2.78 µg MDA/g. Under 75 and 100/RT, lipid oxidation increased slowly up to 2.21 ± 0.16 (1.8-fold) and 1.90 ± 0.11 µg MDA/g (1.6-fold) at the 60th day of storage, respectively, but with values similar ($p > 0.05$) to the ones detected at the 3rd day for each of these two storage conditions. As for goat's FC, lipid oxidation was overall stable in most of the storage conditions, while at 75/RT a strong increase ($p < 0.05$) in TBARS values was observed mainly from the 42nd day of storage, reaching 2.13 ± 0. 32 µg MDA/g (3.2-fold) at the 60th day of storage. A significant slower lipid oxidation rate was achieved under 100/RT throughout storage, reaching 1.15 ± 0.05 µg MDA/g (1.7-fold) after 60 days of storage, which was like the one observed on the 7th day of storage (0.86 ± 0.11 µg MDA/g). Lipid oxidation can be affected by several factors such as the presence of light, oxygen or enzymes, promoting the formation of several volatile compounds, giving rise to off-flavours, with increasing rate over the storage period (Van Hekken et al., 2013). In fact, increase in lipid oxidation by products in cow's FC stored under AP/RF were reported by Zamora, Juan, and Trujillo (2015), observing increases of 2.5-fold after 13 days, while Ercan, Soysal, and Bozkurt (2019) observed increases around 3.4-fold after 21 days, both higher than the ones reported in the present work for both cow's and goat's FC even after 60 days under 100/RT, of 1.6 and 1.7-fold increase, respectively. Significantly higher increases in TBARS values under HS/RT were reported for fish (29-fold) and meat products (4.5-fold), but when a lower temperature (10 ºC) was combined with HS a slower decreasing trend in TBARS evolution was achieved, to 6.6 and 3.9-fold, for fish and meat products, respectively (Fidalgo et al., 2019; Fidalgo et al., 2018; Santos et al., 2020). However, results similar to the ones observed for FCs were obtained in HS of raw milk (*Chapter 5*), reporting a tendency to a more pronounce increase ($p > 0.05$) in TBARS values under 50-75/RT, while storage at 100/RT delayed lipid oxidation throughout the entire storage ($p > 0.05$).

6.4 Conclusions

As demonstrated in this study, fresh cheese highly perishable nature allowed microbial spoilage of most microbiological groups just after 3 days at AP/RT as well as at AP/RF, denoting the short shelf-life of this dairy product. On the other hand, both cow's and goat's FC kept at variable room temperature under HS conditions (≥75/RT) allowed a better microbial control with clear microbial loads reduction over storage, in some cases, to undetectable counts, remaining at constant low levels even after 60 days. In the beginning of the storage under 75 and 100/RT more pronounced changes were observed in whey loss and moisture content in both cheeses, that tended to decrease with the storage time. In contrast, even when comparing cheeses kept at AP/RF after 3-7 days with HS/RT (≥75/RT) after 60 days, lower differences in colour and pH were found, with 100/RT performing overall far the better, promoting a slower lipid oxidation development, when compared to 75/RT. These results points to HS/RT as more efficient, quasi energetically costless, environmental friendlier and more sustainable preservation methodology than AP/RF, without the need of constant energy supply and temperature control, presenting itself not only as a solution to the reduction of carbon footprint associated to the food sector, but also as an adequate strategy for prolonged food preservation/transportation.

6.5 References

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CHAPTER 7

Chapter 7.

This chapter is based on the fifth manuscript submitted for publication

Evaluation of cow's and goat's fresh cheese preservation under hyperbaric storage at room temperature up to 60 days versus refrigeration on nutritional, textural and physicochemical quality parameters

Ricardo V. Duarte, Susana Casal, José A. Lopes-da-Silva, Ana M. Gomes, Ivonne Delgadillo, Jorge A. Saraiva

7.1 Introduction

Hyperbaric storage (HS) is new a preservation methodology, based on storage under moderate pressure (between 25-150 MPa), that relies mainly on microbial growth slowdown/inhibition, similar to conventional refrigeration (RF) (Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). While the first studies regarding this new methodology, emphasized the combination of sub-zero or low temperatures with low pressure (Charm, Longmaid, & Carver, 1977; Mitsuda, 1972), when applied at room temperature (RT), HS arises as an environmentally friendlier food preservation methodology compared to RF, with the exponential extension of foods shelf-life and increased safety (Fidalgo et al., 2014; Santos, Castro, Delgadillo, & Saraiva, 2020). During HS at uncontrolled variable RT, energy employed to maintain the temperature is null, with energy only applied during the compression/decompression of the storage vessel, resulting in an 26-fold lower energy used by HS/RT, comparatively to RF (Bermejo-Prada, Colmant, Otero, & Guignon, 2017).

The feasibility of HS at and above RT was studied initially in fruit juices as case study, in more acid ones (strawberry juice) to low acidity juices, more perishable (watermelon and melon juice) (Fidalgo et al., 2014; Queirós et al., 2014; Segovia-Bravo et al., 2012), with the outcomes pinpointing to a possible shelf-life extension, due to microbial inhibition/inactivation during HS, with minimal physicochemical changes reported. Other non-liquid highly perishable food products were also evaluated under HS/RT as a case study, initially for short storage periods with promising results (Duarte et al., 2014; Fernandes et

al., 2015). One of these products was whey cheese, which has only a couple weeks of shelf-life at RF, presenting after 8 hours at 100/RT no pronounced changes in colour, pH, and water activity, showing a slightly increased in lipid oxidation values, with a clear microbial growth inactivation in all microbiological groups even at and above RT (25-37 ºC) (Duarte et al., 2014). One a second study, this product presented higher stability under HS (100 MPa) also during longer storage periods (10 days) at variable RT, retaining the pH, water activity, and fatty acid profile, while presenting fewer colour losses comparatively to RF, with an additional microbial inactivation effect to undetectable counts (1 log CFU/g) in all the studied microbiological groups (from the 3rd day of storage onwards) (Duarte et al., 2017).

Regarding meat products, the first studies embraced storage periods up to 24 hours, including cooked ham, bovine and minced meat, with an overall better preservation achieved in most of the physicochemical parameters (such as colour, pH, and fatty acid profile) under 100 MPa at and above RT comparatively to RF, despite the small rise in lipid oxidation values reported for cooked ham and minced meat, after 8 and 24 hours, respectively (Fernandes et al., 2015; Fernandes et al., 2018; Freitas et al., 2016). More recently, Santos et al. (2020) performed even longer HS periods in raw meat products (bovine and pork meat), up to 60 days under 75/25 ºC and 60/10 ºC. Although changes in moisture content, colour and lipid oxidation, tended to be reduced in the latter storage conditions (lower temperature), both storage conditions restrained and inhibited successfully the microbial growth, while under RF, meat samples surpassed the acceptable microbiological limit after 14 days. In Atlantic salmon, at HS/RT the first studies pointed to a minimal storage pressure of 75 MPa at 25-37 ºC during 10 days, to reduce the microbial proliferation, however significant colour losses, and rises in lipid oxidation (primary and secondary) parameters were reported under 75/RT (Fidalgo et al., 2019), with additional higher residual proteolysis being reported later by Fidalgo, Delgadillo, and Saraiva (2020) when compared with storage under 60/10 ºC during 50 days. Later, this combination (60/10 ºC) showed the capacity to restrain microbial growth during 30 days, with most of the physicochemical parameters remaining similar to the ones of the fresh salmon prior to storage, such as drip loss and water holding capacity, lipid oxidation, fatty acids, and volatile compounds, despite the decrease in hardness and resilience reported at the 30th day of storage, probably related to residual proteolytic activity,

however pointing to the potential of HS to increase fresh fish products shelf-life (Fidalgo et al., 2020).

Fresh cheeses (FC) are highly perishable dairy products, characterized by short shelf-life (a few weeks at RF), mainly due to its close to neutral pH, high water activity, and rich nutritional profile that promote microbial spoilage. In *Chapter 6*, FC stored under HS/RT conditions (75-100 MPa) resulted in increased microbial control, leading to an increased shelf-life, while also maintaining most of its basic physicochemical parameters at levels comparable to cheeses prior to storage. During that study significant hyperbaric inactivation (HI) was observed, gradually reducing total aerobic mesophiles counts more than 5 Log units throughout the 60 days of storage, (initial counts of ~7 Log CFU/g), reaching progressively values below the quantification and detection limits (2 and 1 Log CFU/g), depending on pressure level and storage time (*Chapter 6*). In the present study, the effect of HS/RT (50-100 MPa) for 60 days was studied on two FC (from pasteurized cow's and goat's milk), in vitro protein digestibility, total protein, free amino acids, fatty acids, volatile organic and textural profiles and compared with RF under atmospheric pressure. Additionally, with the data obtained from the volatile organic compounds a principal component analysis (PCA) was performed.

7.2 Materials and Methods

7.2.1 Fresh cheese samples preparation and storage

Two commercially fresh cheeses (FC) one made from pasteurized cow's milk and the other one made from pasteurized goat's milk, were acquired from a local supermarket. Temperature was kept low (3-8 ºC) during FC transportation being after packaged into low permeability polyamide-polyethylene bags (90 micron, IdeiaPack, Comércio de Embalagens, LDA, Abraveses, Viseu, Portugal), previously sterilized with UV-light, under aseptic conditions inside a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) and heat-sealed individually.

Fresh cheese samples were stored under different conditions, with the experiments performed under HS (50,75 and 100 MPa) at room temperature (RT) and sampling for analysis at 3, 7, 14, 28, 42 and 60 days, in a high pressure equipment SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), with a mixture of (40:60) propylene glycol and water

used as the pressurizing fluid. As a control, FC samples were also stored at RT (15 - 22 ºC) and refrigeration (4 ºC) at atmospheric pressure (AP, 0.1 MPa), with sampling, respectively, only at days 3 and 7, since samples were spoiled (*Chapter 6*). Samples at AP/RF and AP/RT were kept immerse in the same pressurizing fluid and in the dark, to mimic as much as possible the same environment of HS samples inside the HP vessel, except for pressure.

7.2.2 Textural profile analysis (TPA)

The evaluation of FC textural properties was determined by uniaxial compression test and texture profile analysis, carried out using an TA.HDi texture analyser (Stable Micro Systems, Surrey, England) equipped with a 5 kg load cell and with an aluminium plate (75 mm diameter). Cube-shaped samples (1 cm^3) of each cheese from the different storage conditions were cut with a device consisting of 1 cm parallel blades. Cubic shaped cheeses where then wrapped in aluminium foil and equilibrated at 20 ± 0.5 °C for 30 min before testing. In each test, cheeses were placed in the center of the bottom plate, at room temperature (18-22 ºC), and compressed to 50% of its original height two times, at a constant speed of 1 mm/s and with a gap of 10 seconds between the two compressions cycle. During the tests a force-time curve was generated, from which the TPA parameters were calculated: hardness (maximum force during the first compression (N)), cohesiveness (ratio between the positive total area obtained during the second and first compression), springiness (ratio of the time elapsed during the force input between the second and the first compression), adhesiveness (negative area obtained during the withdrawal phase of the first compression cycle (N/s)), gumminess (hardness multiplied by cohesiveness (N)) and chewiness (hardness multiplied by cohesiveness multiplied by springiness (N)) (Bourne, 2002; Koca & Metin, 2004).

7.2.3 Protein profile analysis and digestibility

Protein profile was assessed based on the determination of total nitrogen (TN) through the Kjeldahl method, free amino acids (FAA) employing the EZ:Faast Amino Acid Analysis Kit available for GC-FID and also by in vitro protein digestibility. Micro-Kjeldahl procedure was performed with a Kjeltec system 1002 Distilling unit (Tecator, Sweden) and the crude protein content determined by multiplying the total nitrogen content by 6.38 (AOAC Official Method 2001.14, 2002). For FAA determination and quantification, cheese samples were

homogenised in the same volume of 0.01 M HCl, centrifuged (17000 × g at 4 ºC for 5 min), and the supernatant was collected and centrifuged again. Then, 100 µL of the second supernatant was used for the analysis of free amino acids using the EZ:Faast Amino Acid Analysis Kit (GC-FID) (Badawy, Morgan, & Turner, 2008) being the results for individual FAA expressed in nmol per g of cheese. Protein digestibility was performed based on the method developed by Arte et al. (2015) with some modifications. Cheese samples were incubated with 1.5 mg of pepsin in 15 mL of 0.1 M HCl at 37 °C, at 150 rpm for 3 h, then neutralized with 2 M NaOH, 4 mg of pancreatin in 7.5 mL of phosphate buffer (pH 8.0) and 1 mL of toluene were added, followed by incubation for 24 h at 37 °C, at 150 rpm. The enzyme was inactivated with 10 mL of trichloroacetic acid (10%, wt/vol) and centrifuged (5000 × g at RT for 20 min) to separate undigested protein. Nitrogen in the supernatant was determined by micro-Kjeldahl method. Digestibility was performed in cow's and goat's FC stored under 100/RT for 60 days, and compared with the respective FC prior to storage, and expressed in % (**Equation 7.1**).

$$\text{Protein digestibility (\%)} = \frac{N\ Digested\ protein}{Total\ N} \times 100 \qquad \textbf{Equation 7.1}$$

7.2.4 Fatty Acids Profile

For the fatty acids profile determination, a similar method to the one described by Sobral, Casal, Faria, Cunha, and Ferreira (2020) was performed. Briefly, after cheeses fatty acids extraction and derivatization, determination was carried out by gas chromatography, as fatty acids methyl esters (FAMEs). FAMEs profile was analysed using a GC (Chrompack CP-9001 model, Netherlands) with flame ionization detection (FID). Fatty acids identification and FID calibration was accomplished with a certified reference standard mixture (TraceCert – Supelco 37 component FAME mix, USA) and the results were expressed in relative percentages of their FAMEs.

7.2.5 Volatile organic compounds

Volatile organic compounds (VOC) profile determination was based on the method described by Yue et al. (2015), through headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS). 2 g of cheese and 50 µL of cyclohexanone (25 µg/mL, internal standard) were added into the vial, then immediately

sealed with a polypropylene cap with silicon septum. Compounds were resealed at 50 ºC during 30 min, then the SPME fiber (DVB/CAR/PDMS; 50/30 µm; Supelco Inc.) was exposed during 30 min at 50 °C. After volatiles adsorption into the fiber, it was inserted in the injection port of the GC equipment, Agilent GC-7890 gas chromatographer equipped with a mass spectrometer Agilent 5977B, and a DB-5 MS Capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent, USA). Thermal desorption was achieved at 260 °C in splitless mode, with helium at a linear velocity 1 mL/min. The oven temperature was 35 ºC during 5 min, increasing to 100 ºC at a rate of 4 ºC/min, followed by an increase of 10 ºC/min until 225 ºC and held for 0.25 min (total of 33.5 min). The transfer line was maintained at 280 ºC and the ion source at 230 °C, with ionization energy of 70 eV. Mass spectra were scanned from 20 to 350 m/z in full scan mode. Identification of the volatile compounds was based on computer matching with the reference mass spectra library (NIST 11), retention times, retention index and with individual standards when available. Volatiles profile semi-quantitative determination was calculated using cyclohexanone as internal standard equivalents basis, and the results were expressed in µg per 100g of cheese.

7.2.6 Statistical analysis

All experiments were carried out in triplicate and all analyses were done in triplicate. The different storage conditions were compared using Analysis of Variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD test, at a 5% level of significance. Additionally, principal component analysis (PCA) was performed in order to identify statistical patterns using the VOC data set obtained for both cheeses.

7.3 Results and Discussion

7.3.1 Textural profile analysis

Due to Covid-19 restrictions and pressure vessel volume limitations, related with the high number of replicates and dimensions of FC samples required to properly perform textural profile analysis (TPA), the evaluation of these parameters was only conducted in in goat's FC (as a case-study), throughout the storage at the different storage conditions (**Table 7.1**), showing an initial profile of 2.11 ± 0.64 N, 0.56 ± 0.04, -0.05 ± 0.01 N·s, 0.56 ± 0.04, 1.17 ± 0.34 N and 0.66 ± 0.19 N, regarding hardness, cohesiveness, adhesiveness,

springiness, gumminess and chewiness, respectively, which are similar to values reported previously for this FC (Buriti, da Rocha, & Saad, 2005). Cheeses stored under AP/RT after 3 days presented increased ($p < 0.05$) hardness, gumminess, and chewiness of 1.9-fold for these three TPA parameters, while under AP/RF, cheeses maintained most of its textural characteristics similar to the initial ones ($p > 0.05$) at the 7th day of storage.

Storage under HS conditions, comparatively to FC prior storage, resulted in an overall increase ($p < 0.05$) in all TPA parameters at the 3rd day of storage, what might be due to the compression effect of HS on the protein matrix that forced free whey out from the cheese (*Chapter 6*), resulting in a more compact matrix. However, throughout storage the values of some TPA parameters (particularly for hardness, adhesiveness, gumminess and chewiness) decreased afterwards, reaching values closer to the initial ones. After the 7th day of storage hardness tended to decrease faster under 75/RT compared to 100/RT, reaching values of 3.57 ± 0.82 and 4.51 ± 1.07 N, respectively at the end of the storage period and a similar trend ($p < 0.05$) was also observed for cohesiveness, springiness, adhesiveness, gumminess, and chewiness. These TPA changes, could be associated with the decrease in whey loss and increases in moisture content, observed after the first storage periods under those HS conditions (*Chapter 6*). Decreases in TPA parameters of FC under AP/RF can also occur, as Sant'Ana et al. (2013) reported, in FCs made with milk from goat, cow and with a mixture of both, after 21 days, in hardness (to 52-59%), adhesiveness (to 23-30%), gumminess (to 35-69%), and chewiness (to 37-73%), which could result from changes in protein network structure and on the moisture and fat content, without considerable changes on the sensory properties. On the other hand, in Van Hekken, Tunick, Farkye, and Tomasula (2013) work (studying cheese treated by high pressure processing (HPP) in cheeses), although an increase of 3-fold in hardness (similar to HS samples at day 3) was reported immediately after processing (600 MPa for 3-10 min), hardness, fracture stress and rigidity presented no significant changes ($p > 0.05$), throughout a period of 84 days under 4 and 10 ºC. Interestingly, despite these textural changes induced by HPP in cheeses, sensorial untrained panellists that were familiar with fresh cheese consumption, valued more the new texture, assigning also similar flavour scores for HPP cheeses compared to control FC (Van Hekken et al., 2013). Anyway, changes in the textural properties of FC stored under HS conditions, could influence consumer acceptance of this product, and thus, further investigation on this subject should be conducted, such as sensory evaluation.

Table 7.1 - Textural profile analysis (TPA) of goats' fresh cheese stored up to 60 days at the different storage conditions, AP/RT, AP/RF, and under 50, 75 and 100MPa/RT. Different letters (a-j) indicate significant differences ($p < 0.05$) between storage conditions at each storage time.

Days	Condition	Hardness (N)	Cohesiveness	Adhesiveness (N·s)	Springiness	Gumminess (N)	Chewiness (N)
0	Initial	2.11a	0.56e	-0.05bcd	0.56de	1.17a	0.66ab
3	AP/RT	4.01bcd	0.56e	-0.04abd	0.56de	2.23bc	1.26c
	AP/RF	3.20ab	0.56e	-0.05bcd	0.57e	1.76ab	1.00bc
	50MPa/RT	6.66gh	0.74h	-0.03a	0.76gh	4.92ef	3.84e
	75MPa/RT	6.26fgh	0.74h	-0.03a	0.76h	4.76e	3.84e
	100MPa/RT	6.61gh	0.73h	-0.03ab	0.75gh	5.14ef	3.82e
7	AP/RF	2.75ab	0.49d	-0.08e	0.49abc	1.35a	0.67ab
	75MPa/RT	8.06i	0.68gh	-0.04ab	0.70g	5.50fg	3.84e
	100MPa/RT	9.50j	0.63fg	-0.03ab	0.63f	5.85g	3.71e
14	75MPa/RT	5.81efg	0.62ef	-0.04abc	0.62ef	3.56d	2.21d
	100MPa/RT	9.71j	0.45cd	-0.05bcd	0.50cd	4.40e	2.21d
28	75MPa/RT	6.00fgh	0.41bc	-0.06def	0.50bcd	2.48c	1.24c
	100MPa/RT	7.43hi	0.34a	-0.06def	0.48abc	2.52c	1.22c
42	75MPa/RT	3.16abc	0.38ab	-0.07def	0.43ab	1.19a	0.52a
	100MPa/RT	5.15def	0.34a	-0.06cdef	0.44abc	1.73ab	0.66ab
60	75MPa/RT	3.57abc	0.38ab	-0.08ef	0.45abc	1.34a	0.61ab
	100MPa/RT	4.51cde	0.32a	-0.06cde	0.43a	1.49a	0.63ab

7.3.2 Protein profile

Cow's FC presented a total protein concentration slightly inferior compared to goat's FC (**Table 7.2** and **7.3**), 15.11 ± 0.49 g/100g and 16.99 ± 0.50 g/100g, respectively, similar to what is reported in the literature (Sant'Ana et al., 2013; Van Hekken et al., 2013). Overall, both cow's and goat's FC presented a similar behaviour under the same storage conditions, with no significant changes ($p > 0.05$) observed at AP/RF after 3 days, comparatively to values prior storage. On the other hand, storage at AP/RT and 50/RT tended to present lower ($p > 0.05$) total protein values at the end of each respective storage period, which can be due to the high microbial load observed in both conditions (*Chapter 6*). Storage at 75 and 100/RT maintained the protein content constant throughout the storage ($p > 0.05$), in the two kinds of FC even after 60 days, similarly to what was observed when raw milk was stored under the same HS conditions (75 and 100 MPa) at variable RT for 60 days (*Chapter 5*).

Regarding FAA, cow's FC was initially rich in glutamic acid, followed by aspartic acid, ornithine, leucine, and glycine with a total FAA of 1.1 ± 0.1 µmol/g (**Table 7.2**), while goat's FC had initially a total FAA of 0.9 ± 0.1 µmol/g (**Table 7.3**), mainly constituted by glycine, followed by ornithine, glutamine, glutamic acid, valine, and aspartic acid. Similar compositions in initial FAA were also reported for cheeses made with cow's and goat's milk (Atanasova et al., 2021; Teter et al., 2020).

At the 3rd day of storage, no significant variations were observed regarding individual FAA ($p > 0.05$) of cow's FC stored at AP/RF, comparatively to the initial ones, while cheeses at AP/RT exhibited ($p < 0.05$) a 12-fold increase in alanine and a 3-fold decrease in ornithine, while also several amino acids were now undetected such as glycine, isoleucine, threonine, proline and histidine that were initially present, which could have been used in microbial metabolism (Hoskisson, Sharples, & Hobbs, 2003). Despite these small variations in individual FAA, total FAA content remind similar ($p > 0.05$) to the initial ones for both storage conditions.

As for storage under 50/RT, initially at day 3, changes were only detected in ornithine (decrease of 0.5-fold) without significant variations in all the other individual and total FAA ($p > 0.05$). However, on the following storage periods a remarkable increase in the majority of FAA was detected ($p < 0.05$), with increments of 100-, 48-, 27- and 21-fold, for alanine, histidine, threonine, and valine, respectively, after 28 days of storage. At this sampling period, FAA were majorly composed of alanine, leucine, serine, glutamic acid, valine, and lysine (altogether representing 66% of total FAA), while being also characterized by the presence of serine, phenylalanine, cystine and threonine that were initially absent, resulting in an overall increase of 7-fold in total FAA. This might be due to residual activity of the enzymatic coagulant used for FC production, or plasmin residual activity, initially present in the pasteurized milk, that hydrolyse caseins into intermediate-sized peptides (Enright, Patricia Bland, Needs, & Kelly, 1999). Furthermore, these smaller peptides can be hydrolysed into amino acids by the microbial flora present in the FC, as high microbial loads were observed throughout the storage at 50/RT (around 6.6 and 6.4 log CFU, for total aerobic mesophiles (TAM) and lactic acid bacteria (LAB), respectively, *Chapter 6*), or by extracellular proteinases and peptidases released from that microflora (Abellán et al., 2012). Nevertheless, this proteolytic effect was lower ($p < 0.05$) for FC stored at 75 and 100/RT, comparatively to storage at 50/RT, with an increase rate of FAA per day of, 93.66, 85.12

and 254.52 nmol/g, respectively (*Annex C, **Figure C.1***), resulting in increases in total FAA of 5.9 and 5.7-fold, under 75 and 100/RT respectively, at day 60 of storage. Interestingly the 100-fold increase in alanine observed after 28 days under 50/RT was much higher than the ones observed for storage under 75 and 100/RT after 60 days, of 13 and 14-fold, respectively, which was associated by Eugster, Fuchsmann, Schlichtherle-Cerny, Bütikofer, and Irmler (2019) with the microbial activity of added starter cultures in cheese ripening. New FAA such as serine, phenylalanine, cystine and threonine were present in all three HS conditions, with samples stored under 75 and 100/RT showing a higher abundancy in leucine, glutamic acid, valine, and asparagine, reaching a total FAA after 60 days of storage of 6.3 ± 0.8 and 6.1 ± 0.5 µmol/g, respectively. Both storage conditions were able to gradually inactivate the microbial load present in FC samples, however in a faster rate for 100/RT (D_p-values for LAB of 23.9 and 10.9 days, for 75 and 100/RT, respectively, *Chapter 6*), which could potentially explain partially at least the results of lower FAA increase. Overall, 75 and 100 MPa/RT presented a similar increase in all FAA ($p > 0.05$).

Regarding goat's FC, at AP/RF no significant ($p > 0.05$) oscillations were observed in individual or total FAA at the 3rd day of storage, while a high proteolytic activity ($p < 0.05$) occurred on cheeses stored at AP/RT, resulting in increments especially in valine, leucine, glutamic acid, proline and serine, responsible for an overall increase of 20-fold in total FAA ($p < 0.05$), comparatively to the initial cheese, despite the considerable reduction ($p < 0.05$) in glycine (similar to what was reported for cow's FC under AP/RT).

Under HS conditions, generally goat's FC presented indications of proteolysis throughout the storage period, however at different rates. Storage at 50/RT resulted in an estimated raise of 641.63 nmol/g FAA per day (*Annex C, **Figure C.2)***, with significant increases ($p < 0.05$) observed in almost all FAA, except for glycine, aspartic acid, ornithine, and glutamine that remained in similar concentrations ($p > 0.05$) as the initial ones. A more prominent abundance ($p < 0.05$) in FAA was observed for alanine, leucine, valine, glutamic acid, and lysine, with leucine, histidine, methionine and valine showing a higher abundance after 28 days of storage, with increments of 380, 208, 65 and 61-fold, respectively. Goat's FC had an initially high microbial load (around 6 log CFU, for LAB, *Chapter 6*), that increased under 50/RT (reaching almost 8 log CFU/g after the 7th day of storage), as LAB are well known to promote proteolysis in cheeses (Abellán et al., 2012), resulting in an increase of 30-fold in total FAA after 28 days. While under 75 and 100/RT, this increase in

total FAA was lower (16 and 8-fold increase, respectively), resulting in a proteolysis almost 2-times slower, with increases of 151.57 and 71.73 nmol/g FAA per day (*Annex C,* ***Figure C.2***), respectively, reaching values of 9.5 ± 0.9 and 4.8 ± 0.4 µmol/g for total FAA after 60 days of storage, respectively. Interestingly, TAM and LAB counts were strongly inactivated under those conditions, however the inactivation rate was almost 3-times faster under 100 MPa (D_p-values for TAM of 9.9 and 3.4 days, and for LAB of 6.3 and 1.9 days, under storage at 75 and 100/RT, respectively, *Chapter 6*), and thus, residual proteolytic activity from microbial proteases seem to be the main factor responsible for the proteolysis observed. This proteolytic activity, could have weaken the protein network structure of the cheese and result in the decrease in hardness, reported in the TPA analysis, especially at 75/RT that presented a faster decrease in this parameter, comparatively to storage at 100/RT. Despite the almost half concentration in most FAA between cheeses stored under 75 and 100/RT, both presented a greater abundance ($p > 0.05$) in leucine, valine, aspartic and glutamic acid, also similarly with storage at 50/RT new amino acids were now present, such as isoleucine, phenylalanine, serine, and tryptophan. Increased proteolysis of FC in prolonged storage also occurs under RF, as reported by Sant'Ana et al. (2013), observing an increased proteolysis in fresh cheeses stored at AP/RF after 21 days, around 1.8-fold, attributed mainly to the action of LAB, extracellular proteases, and to a smaller degree to plasmin.

Globally, for both FCs, storage under HS at 75 and 100/RT resulted over time in an increased concentration in FAA, although even after 60 days, values were significantly lower than the ones reported by Abellán et al. (2012) for goat cheese at day 1 of maturation. Still, the possible impact of these increases should be further investigated in the sensory properties of HS cheeses.

Regarding protein digestibility, cow's (**Table 7.2**) and goat's (**Table 7.3)** FC presented values prior to storage of 81.2 ± 2.1 and 75.8 ± 1.1%, respectively. Under 100/RT, after 60 days no significant variations were observed for cow's FC (81.4 ± 2.7%), while an increase ($p < 0.05$) to 81.0 ± 1.8% was detected for goat's FC. As mentioned previously, after 60 days of storage at 100/RT an increase in FAA of 5.7 and 8-fold was observed for cow's and goat's FC, respectively, indicating a higher proteolysis in goat's FC, which could be responsible for the increased protein digestibility.

Table 7.2 - Total protein (g/100g), protein digestibility (%), free amino acids (nmol/g) of cow's fresh cheese prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–g) indicate significant differences ($p < 0.05$) between the different storage conditions for each parameter.

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**			**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	3	14	28	3	14	28	60	3	14	28	60
Total Protein (g/100g)	15.11a	13.09a	1.46a	14.08a	14.47a	12.92a	14.57a	13.49a	15.39a	13.78a	15.32a	14.53a	14.85a	14.74a
Digestibility (%)	81.2a	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	81.4a
FAA (nmol/g)														
Alanine	16.7a	207.6bc	24.1a	19.9a	789.7d	1770.5e	24.7a	49.0ab	164.3abc	218.1c	34.0ab	98.2abc	256.3c	227.1c
Glycine	69.6a	*ND*	83.0a	116.9abc	154.4bcd	247.9e	106.7abc	156.9bcd	188.1de	172.1cd	89.0ab	162.8cd	203.0de	158.5cd
Valine*	23.3a	43.0a	11.1a	35.2a	176.5b	486.6e	25.1a	111.6ab	310.5cd	784.0g	26.5a	219.1bc	382.3de	668.7f
3-Aminoisobutyric acid	18.8 ab	*ND*	*ND*	12.6 ab	39.0 abc	69.4 abcde	15.0 ab	56.4 abcd	116.6 def	142.7 f	12.0 a	141.1 ef	88.8 bcde	108.0 de
Leucine*	85.6ab	303.8b	50.6a	66.4a	431.7bc	1333.1e	86.1ab	378.7abc	969.4d	1511.1e	145.8ab	650.7cd	1538.2e	1525.0e
Isoleucine*	4.8a	*ND*	*ND*	*ND*	33.7ab	96.2c	*ND*	31.7ab	104.0c	278.9e	*ND*	67.7bc	165.7d	297.2e
Threonine	5.0a	*ND*	*ND*	*ND*	30.2ab	136.6c	*ND*	*ND*	46.0abc	63.3abc	*ND*	*nd*	121.3bc	105.4abc
Serine	*ND*	20.2 a	*ND*	*ND*	166.4 bcd	605.9 e	*ND*	39.1 ab	137.9 abc	182.1 bcd	*ND*	66.5 ab	288.3 d	255.0 cd
Proline	19.8a	69.7abc	*ND*	*ND*	41.8abc	76.5abc	*ND*	104.2bcde	166.3de	185.9e	*ND*	36.5ab	117.1cde	87.9abcd
Asparagine	13.7ab	36.4abcd	11.7ab	21.6abc	26.4abcd	29.7abcd	10.2a	24.9abcd	48.1cd	57.3d	15.1ab	98.1e	41.5bcd	32.1abcd
Aspartic acid	181.8 abc	56.8 ab	37.4 a	94.6 ab	350.9 abcde	415.7 cde	128.0 ab	363.0 bcde	568.0 e	471.9 cde	113.7 ab	182.8 bcd	473.7 de	488.6 de
Methionine*	10.9a	38.0a	14.1a	*ND*	42.7a	108.1b	19.6a	43.1a	72.7ab	51.1ab	19.9a	111.6b	114.7b	62.6ab
Hydroxyproline	12.3ab	24.1ab	9.0a	9.6ab	30.3abc	41.3abcd	11.2ab	80.4e	65.8cde	74.1de	14.6ab	39.9abcd	42.5bcd	35.2abcd
Glutamic acid	292.3bcd	224.4ab	209.8abc	58.4a	173.6ab	496.6de	167.5ab	207.3ab	439.8cd	502.4de	255.8abc	481.9de	899.5f	653.8e
Phenylalanine*	*ND*	19.7a	*ND*	*ND*	34.8a	495.7e	*ND*	60.0ab	219.8bc	269.9cd	*ND*	92.4ab	269.9cd	390.9de
Glutamine	174.6 abcd	69.4 ab	58.3 a	74.5 ab	294.8 bde	286.6 bcde	117.7 abc	320.0 de	607.2 f	392.2 ef	101.6 ab	175.8 abcd	406.2 e	413.5 ef
Ornithine	167.5b	54.3a	201.2b	84.9a	40.5a	75.6a	54.5a	53.2a	42.8a	72.8a	45.1a	45.1a	49.5a	48.6a
Lysine*	14.1a	14.2a	15.1a	32.4ab	166.0bc	493.9e	30.7ab	155.8b	298.6cd	348.0de	33.6ab	121.1ab	164.0bc	94.4ab
Histidine*	5.7a	*ND*	*ND*	*ND*	94.8bc	272.2d	*ND*	57.1ab	144.7c	307.6d	10.1a	110.5bc	265.6d	300.7d
Tyrosine*	2.3a	2.0a	3.3a	4.4ab	6.6ab	4.7ab	2.9a	12.2ab	15.4ab	32.5cd	10.1ab	49.3d	21.3bc	11.7ab
Tryptophan*	*ND*	33.4ab	4.0a	*ND*	65.6ab	309.3e	*ND*	43.4ab	109.2bc	240.2de	*ND*	63.8ab	170.2cd	270.3e
Cystine	*ND*	*ND*	*ND*	*ND*	39.7a	168.4c	*ND*	11.3a	101.1ab	73.3a	*ND*	*ND*	48.6a	80.3a
Total FAA (µmol/g)	1.1a	1.2a	0.8a	0.6a	3.2b	7.8c	0.8a	2.3b	4.4c	6.3d	0.9a	2.9b	6.1d	6.1d

ND and *NP* - stands for not detected and not performed, respectively * - *essential amino acids*

Table 7.3 - Total protein (g/100g), protein digestibility (%), free amino acids (nmol/g) of goat's fresh cheese prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–i) indicate significant differences ($p < 0.05$) between the different storage conditions for each parameter.

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**			**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	3	14	28	3	14	28	60	3	14	28	60
Total Protein (g/100g)	16.99 abc	15.36 a	17.02 abc	18.84 c	18.33 bc	15.35 a	18.30 bc	16.43 ab	16.83 abc	15.96 a	18.19 bc	16.75 abc	17.13 abc	15.81 a
Digestibility (%)	75.8a	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	*NP*	81.0b
FAA (nmol/g)														
Alanine	41.4 ab	486.1 g	12.1 a	183.4 cde	841.3 h	1011.1 i	25.1 ab	134.5 abcd	200.4 def	323.0 f	55.9 abc	107.8 abcd	151.0 bcde	265.5 ef
Glycine	202.6 bcde	8.6 a	133.0 b	136.7 bc	225.4 def	276.5 ef	189.2 bcd	285.4 ef	277.0 ef	289.0 f	217.2 cdef	241.2 def	203.8 bcde	215.6 cdef
Valine*	54.4a	1296.1d	17.1a	218.5a	2426.1e	3383.8f	70.0a	733.9bc	1282.0d	2469.5e	98.8a	325.7ab	719.9bc	1168.0cd
3-Aminoisobutyric acid	5.6a	89.2b	*ND*	16.8a	*ND*	*ND*	9.3a	*ND*	82.0b	195.1c	21.4a	*ND*	*ND*	*ND*
Leucine*	12.6a	1572.3e	14.5a	332.1ab	3175.8f	4799.8g	50.6a	776.9bc	1308.7de	2839.9f	84.1a	455.4ab	1023.1cd	1610.6e
Isoleucine*	*ND*	149.4bc	*ND*	28.4a	263.7d	398.0f	*ND*	86.2abc	118.8bc	355.4ef	*ND*	58.7ab	137.9bc	276.7de
Threonine	*ND*	95.4b	*ND*	*ND*	10.7a	*ND*	*ND*	12.5a	5.6a	*ND*	*ND*	5.5a	*ND*	*ND*
Serine	*ND*	1644.7d	*ND*	*ND*	566.9c	462.6bc	*ND*	31.4a	93.4a	219.7ab	*ND*	16.1a	27.8a	201.1ab
Proline	*ND*	1488.9c	*ND*	179.0a	547.9b	724.5b	*ND*	14.8a	29.7a	85.5a	*ND*	8.2a	*ND*	18.4a
Asparagine	13.0a	102.9e	12.6a	32.4abc	68.1d	51.7cd	16.1ab	54.6cd	76.7cd	173.7f	35.1abc	20.2abc	31.8abc	47.2bcd
Aspartic acid	34.6a	77.2a	42.2a	36.9a	51.5a	40.0a	38.9a	100.0a	193.0b	494.6c	93.9a	33.1a	35.0a	62.4a
Methionine*	6.0a	384.7c	*ND*	28.5ab	341.0c	389.6c	*ND*	35.6ab	52.0ab	84.5b	*ND*	24.1b	21.9ab	68.7ab
Glutamic acid	65.7a	1823.3b	*ND*	171.2a	2329.1bc	2889.7c	*ND*	86.2a	222.6a	489.7a	42.4a	109.5a	210.9a	456.1a
Phenylalanine*	*ND*	500.4d	*ND*	52.6a	831.4e	1108.0f	*ND*	60.5a	133.6ab	274.1c	*ND*	26.8a	75.0a	210.4bc
Glutamine	56.3a	176.1bc	50.7a	46.0a	104.8ab	81.4a	48.3a	112.0abc	178.2c	431.6d	85.2a	67.2a	63.1a	81.3a
Ornithine	83.6 bcd	178.3 e	101.3 cd	39.8 ab	58.5 abc	110.0 d	34.9 a	43.3 ab	36.7 ab	72.1 bcd	41.6 ab	50.5 ab	56.0 abc	68.9 bcd
Lysine*	23.1a	575.2c	24.1a	35.1a	999.6d	1208.3d	27.8a	235.3ab	303.6b	250.5ab	48.6a	103.0ab	106.3ab	164.0ab
Histidine*	2.6a	292.7b	*ND*	8.9a	576.9c	529.8c	*ND*	75.8a	57.3a	93.1a	*ND*	89.5a	132.7ab	282.0b
Tyrosine*	1.3a	324.4c	*ND*	11.0a	157.9b	71.3a	*ND*	22.9a	4.0a	3.0a	*ND*	5.3a	2.4a	2.3a
Tryptophan*	*ND*	367.0cd	*ND*	81.4ab	467.2d	340.9cd	*ND*	124.2ab	112.5ab	454.8d	*ND*	55.8a	77.2a	240.9bc
Cystine	*ND*	*ND*	*ND*	*ND*	*ND*	127.3b	*ND*	27.5a	32.8a	73.4ab	*ND*	*ND*	*ND*	56.2a
Total FAA (µmol/g)	0.6a	11.6de	0.4a	1.6ab	13.9e	17.4f	0.5a	3.1bc	4.8c	9.5d	0.8ab	1.7ab	3.0bc	4.8c

ND and *NP* - stands for not detected and not performed, respectively * - *essential amino acids*

7.3.3 Fatty acids profile

Cow's FC fatty acid profile is represented in **Table 7.4**, while goat's FC fatty acid profile is shown in **Table 7.5**. Cow's and goat's FCs had an overall similar fatty acid content, with slight variations, both with a higher composition in saturated fatty acids (SFA), followed by monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), overall similar to the composition described by Van Nieuwenhove, Oliszewski, and González (2009). Initially, cow's FC had a total SFA, MUFA and PUFA of 63.98 ± 0.52%, 31.12 ± 0.38% and 4.42 ± 0.12%, respectively, while goat's FC had initially total SFA, MUFA and PUFA content of 66.16 ± 0.93%, 27.94 ± 0.83% and 5.09 ± 0.18%, respectively. Regarding SFA, cow's and goat's FC were rich in palmitic acid (C16:0, 32.01 ± 0.11% and 27.00 ± 0.35%, respectively), myristic acid (C14:0, 11.49 ± 0.29%, 10.60 ± 0.19%, respectively), stearic acid (C18:0, 10.35 ± 0.19%, 9.37 ± 0.30%, respectively), with the major difference being related with a higher capric acid (C10:0) percentage observed for goat's FC (9.28 ± 0.82%) comparatively with cow's FC (2.83 ± 0.20%), similar to what is reported in the literature (Sant'Ana et al., 2013). As for MUFA content, cow's and goat's FC most abundant fatty acids were oleic acid (C18:1*c*, 22.80 ± 0.36% and 20.98 ± 0.71%, respectively) and elaidic acid (C18:1*t*, 2.72 ± 0.07% and 2.77 ± 0.12%, respectively), as for PUFA the most representative was linoleic acid (C18:2*c*, 2.44 ± 0.05% and 3.42 ± 0.13%, respectively).

Throughout the different storage conditions, cow's FC fatty acid profile presented some variations when compared to the profile prior storage. In general, longer HS periods tended to present increased ($p > 0.05$) values in SFA content, with storage at 75 and 100/RT reaching values of 65.46 ± 1.04% and 64.96 ± 0.65%, respectively. This tendency was more pronounced especially under 75/RT, presenting a tendency for higher amounts of palmitic acid ($p < 0.05$), stearic acid and myristic acid ($p < 0.05$). In accordance, for MUFA and PUFA, HS tended to present lower values, with the major differences ($p < 0.05$) being related to storage at 100/RT after 7 and 14 days, presenting values similar to the initial ones on the following storage periods ($p > 0.05$). Despite the fluctuations ($p < 0.05$) detected regarding oleic, linoleic, and α-linolenic acids (C18:3*c*6,*c*9,*c*12), overall, the majority of MUFA and PUFA content was not affected during HS ($p > 0.05$).

As for goat's FC storage, despite some variability in few individual fatty acids during the different storage conditions, under HS no significant changes ($p > 0.05$) were observed after 60 days, comparatively to the initial cheese, although the same tendency was observed

similarly to cow's FC storage, with cheeses at 75 and 100/RT presenting higher values ($p > 0.05$) regarding SFA, accompanied by a decrease ($p > 0.05$) for MUFA and PUFA content. Comparable results were also found in HS of raw milk for 60 days, with a more pronounced increase SFA content ($p < 0.05$) being observed especially for storage at 75/RT, while MUFA and PUFA contents decreased throughout the storage (*Chapter 5*).

Overall, storage under 75 and 100/RT was able to successfully keep a similar fatty acid profile of both cow's and goat's cheeses, throughout the duration of the study.

Table 7.4 - Fatty acids profile (% of total fatty acids) of cow's fresh cheese prior storage (Initial) and stored under the different storage conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT).

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**		**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
C8:0	1.27ab	1.24ab	1.41ab	1.38ab	1.33ab	1.56b	1.21ab	1.39ab	1.20ab	1.48ab	1.34ab	1.23ab	1.18a
C9:0	0.05	0.05	0.06	0.06	0.05	0.06	0.05	0.06	0.05	0.06	0.05	0.05	0.04
C10:0	2.83ab	2.78ab	3.05ab	2.99ab	2.91ab	3.33b	2.72ab	3.04ab	2.69ab	3.19ab	2.95ab	2.75ab	2.66a
C12:0	3.30	3.25	3.44	3.41	3.40	3.67	3.24	3.46	3.23	3.61	3.43	3.25	3.21
C14:0	11.49	11.38	11.67	11.66	11.62	12.02	11.58	11.74	11.68	11.94	11.83	11.52	11.55
C15:0	1.16ab	1.15a	1.17ab	1.17ab	1.18ab	1.19ab	1.18ab	1.17ab	1.19ab	1.19b	1.19b	1.17ab	1.18ab
C16:0	32.01 ab	31.87 ab	31.76 a	31.98 ab	32.05 abc	31.77 a	32.87 cd	32.08 abc	33.14 d	32.13 abc	32.62 bcd	32.43 abc	32.88 cd
ai-C17:0	0.66aba	0.67ab	0.66ab	0.66ab	0.67ab	0.66a	0.68ab	0.66ab	0.68b	0.66b	0.67ab	0.67ab	0.68ab
C17:0	0.55abc	0.55abc	0.54a	0.55ab	0.55abc	0.54a	0.57bc	0.55ab	0.57c	0.55ab	0.56abc	0.56abc	0.57bc
C18:0	10.35 abc	10.32 abc	10.09 ab	10.22 abc	10.39 abc	9.88 a	10.72 bc	10.15 abc	10.78 c	10.08 ab	10.48 abc	10.50 abc	10.73 bc
C20:0	0.14c	0.13abc	0.13abc	0.13abc	0.13abc	0.12a	0.14c	0.13abc	0.14c	0.12ab	0.13abc	0.13abc	0.14bc
C21:0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.03
C22:0	0.08c	0.07ab	0.07ab	0.07ab	0.07ab	0.07ab	0.08bc	0.07ab	0.07ab	0.06a	0.07ab	0.07ab	0.07ab
C24:0	0.04abc	0.04abc	0.05abc	0.04abc	0.04abc	0.04abc	0.04abc	0.06d	0.05bcd	0.04a	0.04ab	0.05cd	0.05bcd
Total SFA	63.98ab	63.59a	64.18ab	64.38ab	64.46ab	64.96ab	65.14ab	64.63ab	65.52b	65.19b	65.41b	64.46ab	65.00ab
C10:1	0.28ab	0.28ab	0.31b	0.30ab	0.29ab	0.34ab	0.27ab	0.31ab	0.27ab	0.32aba	0.29b	0.28a	0.26ab
C14:1*t*	0.23ab	0.23ab	0.24b	0.23ab	0.23ab	0.24ab	0.23ab	0.23ab	0.23ab	0.24ab	0.24ab	0.23a	0.23ab
C14:1*c*	1.09	1.08	1.13	1.10	1.09	1.15	1.04	1.11	1.03	0.98	1.07	1.07	1.04
ai-C15:1	0.53ab	0.53ab	0.58b	0.54ab	0.54ab	0.55ab	0.53ab	0.46a	0.53ab	0.55ab	0.54ab	0.53ab	0.53ab
C15:1	0.26a	0.26ab	0.27c	0.26abc	0.26abc	0.26abc	0.27abc	0.26ab	0.27abc	0.26abc	0.27bc	0.26abc	0.26abc
C16:1*c*	1.91bcd	1.92cd	1.92d	1.90abc	1.89abc	1.91bcd	1.85ab	1.91bcd	1.84a	1.90abc	1.86abc	1.89abc	1.86abc
C17:1	0.23ab	0.23b	0.23b	0.22ab	0.22ab	0.23ab	0.22ab	0.22ab	0.21a	0.22ab	0.22ab	0.23ab	0.22ab
C18:1*t*	2.72	2.82	2.73	2.72	2.75	2.64	2.73	2.72	2.73	2.67	2.71	2.77	2.78
C18:1*c*	22.80b	22.79b	22.47ab	22.34ab	21.92ab	21.76ab	21.90ab	22.12ab	21.69ab	21.55ab	21.43a	22.39ab	21.98ab
C20:1*c*9	0.14	0.14	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13
Total MUFA	30.88b	30.98b	30.68ab	30.42ab	30.02ab	29.87ab	29.83ab	30.14ab	29.60ab	29.48a	29.39a	30.45ab	29.97ab

*Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

Table 7.4 (cont.) - Fatty acids profile (% of total fatty acids) of cow's fresh cheese prior storage (Initial) and stored under the different storage conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT).

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**		**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
C18:2*t*	1.19abcd	1.33d	1.24bcd	1.15abcd	1.28cd	1.13abcd	1.06abcd	1.24bcd	1.10abc	1.03a	1.03a	1.16abcd	1.23abcd
CLA*c*9,*t*11	0.52	0.52	0.51	0.51	0.51	0.50	0.50	0.51	0.51	0.50	0.49	0.52	0.51
CLA*t*10,*c*12	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
C18:2*c*	2.44bc	2.46b	2.40abc	2.41abc	2.36abc	2.35abc	2.36abc	2.38abc	2.29a	2.31ab	2.30ab	2.38abc	2.33abc
C18:3*c6,c9,c12*	0.09ab	0.11b	0.09ab	0.09ab	0.09ab	0.08ab	0.08a	0.09ab	0.09ab	0.09ab	0.09ab	0.09ab	0.09ab
C18:3*c*9,*c*12,*c*15	0.30c	0.30bc	0.29abc	0.29abc	0.28abc	0.29abc	0.28abc	0.29abc	0.28ab	0.28abc	0.28a	0.29abc	0.28abc
C20:2	0.04	0.04	0.04	0.03	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04
C20:3	0.13	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12
C20:4	0.18ab	0.18ab	0.18ab	0.17ab	0.18ab	0.17ab	0.18ab	0.18b	0.18ab	0.17a	0.17ab	0.18ab	0.18ab
C22:5	0.06ab	0.06ab	0.06ab	0.06ab	0.06b	0.06ab	0.06b	0.06ab	0.06ab	0.05a	0.06ab	0.06ab	0.06ab
Total PUFA	4.42bc	4.59c	4.42bc	4.32abc	4.40bc	4.27ab	4.29ab	4.41bc	4.14ab	4.22a	4.30a	4.36abc	4.32abc

*Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

Table 7.5 - Fatty acids profile (% of total fatty acids) of goat's fresh cheese prior storage (Initial) and stored under the different storage (AP/RT, AP/RF and 50, 75 and 100MPa/RT).

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**		**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
C8:0	2.78	2.86	3.03	2.97	3.15	3.13	3.43	3.32	3.25	2.89	3.02	3.02	2.97
C9:0	0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.06	0.06
C10:0	9.28	9.40	9.78	9.73	10.25	10.05	10.83	10.58	10.37	9.45	9.76	9.85	9.65
C12:0	4.57	4.67	4.79	4.74	4.82	4.83	5.09	5.01	4.96	4.67	4.75	4.80	4.73
C14:0	10.60	10.68	10.73	10.69	10.82	10.76	10.93	10.84	10.88	10.63	10.62	10.73	10.69
C15:0	0.77	0.78	0.77	0.77	0.76	0.77	0.77	0.77	0.77	0.77	0.76	0.77	0.77
i-C16:0	0.08ab	0.08b	0.08b	0.08b	0.07a	0.08b	0.08b	0.08b	0.08b	0.08b	0.08b	0.08b	0.08b
C16:0	27.00	26.90	26.66	26.88	26.48	26.55	26.33	26.23	26.42	26.86	26.42	26.72	26.69
ai-C17:0	0.69	0.69	0.68	0.68	0.66	0.68	0.66	0.67	0.68	0.69	0.68	0.69	0.68
C17:0	0.60	0.61	0.60	0.60	0.58	0.60	0.59	0.59	0.59	0.61	0.59	0.60	0.60
C18:0	9.37	9.27	9.17	9.22	8.94	9.07	8.78	8.83	8.94	9.27	9.10	9.16	9.16
C20:0	0.20	0.19	0.19	0.19	0.19	0.19	0.18	0.18	0.19	0.19	0.19	0.19	0.20
C21:0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
C22:0	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.09
C24:0	0.05c	0.04abc	0.04ab	0.04abc	0.06d	0.04abc	0.04ab	0.04a	0.04ab	0.05bc	0.04abc	0.04ab	0.04abc
Total SFA	66.16	66.34	66.71	66.75	66.97	66.92	67.89	67.32	67.34	66.33	66.20	66.82	66.45
C10:1	0.21	0.21	0.22	0.21	0.23	0.22	0.25	0.24	0.23	0.21	0.22	0.22	0.21
C14:1*t*	0.18ab	0.18ab	0.19b	0.18ab	0.18a	0.18b	0.19b	0.19b	0.19b	0.18ab	0.19b	0.18ab	0.18ab
C14:1*c*	0.18	0.18	0.18	0.17	0.17	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.17
ai-C15:1	0.42	0.43	0.43	0.43	0.42	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
C15:1	0.28	0.28	0.28	0.28	0.27	0.28	0.28	0.29	0.28	0.28	0.28	0.28	0.28
C16:1*t*	0.55b	0.55b	0.55b	0.55b	0.52a	0.54b	0.54ab	0.54ab	0.54ab	0.55b	0.55b	0.55b	0.55b
C16:1*c*	1.04	1.04	1.03	1.02	1.01	1.02	1.00	1.01	1.02	1.03	1.02	1.03	1.02
C17:1	0.33	0.32	0.31	0.32	0.34	0.32	0.32	0.31	0.30	0.32	0.33	0.32	0.31
C18:1*t*	2.77	2.70	2.63	2.74	2.62	2.61	2.64	2.64	2.67	2.71	2.81	2.76	2.78
C18:1*c*	20.98	20.95	20.50	20.61	20.64	20.40	19.74	19.91	20.12	20.82	20.60	20.52	20.92
C20:1*c*	0.04ab	0.03a	0.03ab	0.03ab	0.05b	0.03ab	0.04ab	0.04ab	0.03ab	0.03a	0.04ab	0.04ab	0.03ab
Total MUFA	27.94	27.62	27.31	27.49	27.42	27.15	26.53	26.68	26.91	27.66	27.57	27.45	27.85

*Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

Table 7.5 (cont.) - Fatty acids profile (% of total fatty acids) of goat's fresh cheese prior storage (Initial) and stored under the different storage conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT).

Condition	**Initial**	**AP/RT**	**AP/RF**	**50MPa/RT**		**75MPa/RT**				**100MPa/RT**			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
C18:2*t*	0.91ab	0.88ab	0.84ab	0.87ab	0.87ab	0.80a	0.83ab	0.82a	0.63ab	0.90ab	0.95c	0.86ab	0.86ab
CLA*c*9,*t*11	0.81	0.79	0.79	0.79	0.75	0.79	0.76	0.76	0.76	0.80	0.77	0.79	0.79
CLA*t*10,*c*12	0.06bcd	0.04ab	0.05bcd	0.06bcd	0.08cd	0.05abc	0.08cd	0.08cd	0.08cd	0.03a	0.07cd	0.08d	0.08d
C18:2*c*	3.42	3.36	3.34	3.37	3.41	3.33	3.23	3.29	3.33	3.41	3.35	3.34	3.44
C18:3*c6,c9,c12*	0.08c	0.08c	0.08bc	0.08bc	0.07a	0.08bc	0.07ab	0.07ab	0.08bc	0.08c	0.08c	0.08bc	0.08bc
C18:3*c*9,*c*12,*c*15	0.29	0.29	0.28	0.28	0.27	0.29	0.27	0.27	0.28	0.29	0.28	0.28	0.29
C20:2	0.03ab	0.03ab	0.03ab	0.03ab	0.03ab	0.03ab	0.03a	0.03b	0.03ab	0.03ab	0.03ab	0.03ab	0.03ab
C20:3	0.03abc	0.02a	0.03abc	0.03abc	0.03c	0.03abc	0.02ab	0.02ab	0.03ab	0.03bc	0.03abc	0.03ab	0.03abc
C20:4	0.25b	0.24ab	0.24ab	0.25ab	0.24ab	0.24ab	0.23a	0.23a	0.23ab	0.25ab	0.23ab	0.24ab	0.24ab
C22:5	0.08b	0.07ab	0.08ab	0.08ab	0.07ab	0.07ab	0.07ab	0.07a	0.07ab	0.08ab	0.07ab	0.07ab	0.07ab
Total PUFA	5.09	5.00	4.94	4.98	5.00	4.91	4.76	4.81	4.87	5.07	4.96	4.92	5.04

*Different letters (a–d) indicate significant differences ($p < 0.05$) between the different storage conditions.

7.3.4 Volatile organic compounds

Initially in cow's FC a total of 18 volatile organic compounds (VOC) were detected (**Table 7.6**) and consisted mainly of free fatty acids (FFA), esters, ketones, and aldehydes, with no alcohol compounds being detected initially, an overall similar composition to what is reported for this kind of dairy product (Tunick, Iandola, & Van Hekken, 2013). The composition in FFA consisted of butanoic, hexanoic, octanoic and decanoic acids, with sorbic acid ((2E,4E)-hexa-2,4-dienoic acid) also being detected, added in the form of potassium sorbate as a preservative by the producer, as stated in the product label. Ethyl butanoate and hexanoate were the main esters present, as for ketones, pentan-2-one and heptan-2-one were the most abundant compounds, and nonanal was the main aldehyde, which was only present in the cheese prior to storage.

After 3 days, cheeses under AP/RF presented a similar VOC profile ($p > 0.05$), regarding to the cheese prior storage, with a slight increase ($p > 0.05$) in most FFA, aldehydes, esters, and a decrease in ketones, with alcohol compounds such as pentan-2-ol, cyclohexanol and hexan-1-ol being now detected. Storage under AP/RT after 3 days, resulted in a clear distinguished VOC profile of cheeses, with increased concentrations ($p < 0.05$) of FFA, aldehydes, esters, and alcohols. An increase up to 10-fold was observed in almost all FFA and their respective ethyl esters after 3 days, with acetic and nonanoic acid, ethyl octanoate and dodecanoate being now present. As for aldehydes and alcohols the main increases resulted from 2-methylbut-2-enal and hexan-1-ol, respectively, with no significant changes observed regarding ketones ($p > 0.05$). High microbial or/and enzymatic activity can promote lipolysis and the release of FFA, as well as lactose and amino acids degradation, with ethyl esters formed by esterification of the FFA, and alcohols resulting possibly from reduction of aldehydes formed by amino acids degradation (Muñoz, Ortigosa, Torre, & Izco, 2003; Toso, Procida, & Stefanon, 2002).

After 7 days at 50/RT, cheese VOC profile presented overall an evolution similar to storage at AP/RF regarding esters, alcohols, and FFA, that continuously arose over storage ($p < 0.05$), while ketones and aldehydes decreased on the 28th day. An overall increase in all FFA was observed especially in hexanoic and octanoic acids, with the now detected acetic and nonanoic acids, contributing to an estimated increase of 52.01 µg/100g of FFA per day (*Annex C,* ***Figure C.3***). In parallel, esters increased around 21.49 µg/100g per day, mainly due to increases observed in ethyl decanoate, butanoate and hexanoate, and from ethyl

octanoate and dodecanoate that were initially undetected. Ketones presented an estimated reduction over time of 0.32 µg/100g per day, possibly due to reduction to alcohols, which increased around 0.67 µg/100g per day (*Annex C,* ***Figure C.3***), mainly due through the development of heptan-2-ol and butane-2,3-diol. Despite the initial increase in total aldehydes at the 7th day of storage, since these are transitory oxidation compounds, quick conversion into acids or alcohols can occur (Bezerra et al., 2017), resulting in the significant content reduction after 28 days of storage ($p < 0.05$). Changes in the VOC profile of cheeses stored at 50/RT can be attributed to the high microbial load under this condition (above 6 and 5 log units for TAM and LAB, respectively, *Chapter 6*), resulting in an overall quality loss of cheeses. Interestingly, storage under 75-100 MPa maintained total aldehydes, esters, ketones and alcohols at constants levels ($p > 0.05$) throughout the storage, with exception for FFA under 100/RT, that presented an estimated increase of 6.54 µg/100g per day (*Annex C,* ***Figure C.3***), which was more pronounced on the 42nd day of storage on forward. And thus, these storage conditions resulted generally in a more resembling cheese VOC profile to the ones prior to storage.

The conducted PCA present in **Figure 7.1**, resulted from multivariate statistical analyses of the volatile compounds detected throughout the storage of cow's FC. **Figure 7.1** shows the score plots of the different variables, with PC 1 and PC 2 accounting for 52.13% and 22.57% of total variability, respectively. As it can be seen, cheeses from storage at AP/RF, 75 and 100/RT at all storage periods are closer to the cheese prior storage (on the positive PC 1), while cheeses stored under 50/RT are more far apart as the storage period increased, with cheeses under AP/RT being more distant (negative PC 1) from the cheese prior to storage. In the loadings of the two principal components (**Table 7.7**), compounds more associated with cow's FC prior to storage, mainly ketones and aldehydes like pentan-2-one, heptan-2-one, hexanal and nonanal are scored on the positive loadings on PC 1, while the negative PC 1 is related to compounds associated with cheese spoilage, especially higher concentrations of FFA, esters and some alcohols. In fact, when the three major classes (FFA, esters and alcohols) were selected, a PCA that explains 97.91% of total variance was obtained (**Figure 7.2** and **Table 7.8**), with 92.09% of variance for PC 1 and 5.82% for PC 2 with a clear distinguish from cheeses stored at AP/RT, AP/RF and 50/RT from the other storage conditions that were closer to the volatile profile of cheeses prior to storage.

Table 7.6 - Volatile organic compounds (µg/100g) of cow's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–e) indicate significant differences ($p < 0.05$) between the different conditions.

Condition	Initial	AP/RT	AP/RF	50MPa/RT		75MPa/RT				100MPa/RT			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
Free fatty acids	182.10a	2076.29f	318.40ab	602.05d	1654.27e	177.03a	301.29ab	223.26ab	169.55a	252.42ab	247.83ab	402.03bc	572.93cd
acetic acid	*nd*	284.66d	4.69a	28.67b	137.33c	1.32a	0.69a	0.50a	1.19a	*nd*	*nd*	*nd*	*nd*
butanoic acid	19.01a	229.34d	32.79a	78.00bc	275.23e	20.73a	43.02a	29.82a	19.37a	39.71a	49.74ab	56.54abc	89.14c
hexanoic acid	28.90a	370.52c	56.60a	153.49b	494.08d	31.04a	86.10ab	56.84a	52.48a	62.86a	92.15ab	105.66ab	169.61b
(2E,4E)-hexa-2,4-dienoic acid	69.19ab	773.42e	115.25bc	135.18c	277.05d	46.35a	46.42a	32.38a	47.12a	48.40a	43.00a	43.57a	59.53a
octanoic acid	47.88ab	315.17e	65.27ab	132.93cd	357.54e	49.36ab	86.07abc	59.90ab	26.93a	60.71ab	82.21abc	120.13bcd	168.53d
nonanoic acid	*nd*	13.64c	*nd*	6.56ab	9.57bc	2.70ab	4.11ab	4.87ab	3.17ab	4.14ab	2.64a	2.87ab	3.51ab
decanoic acid	20.53a	96.63d	39.49abc	65.95bcd	102.98d	26.84ab	35.06abc	40.42abc	19.61a	36.65abc	51.14abc	76.06cd	68.75bcd
dodecanoic acid	*nd*	6.54bcd	4.31ab	7.82cd	10.07d	3.33ab	4.71abc	3.56ab	2.40a	4.09ab	4.28ab	5.05abc	4.82ab
Esters	26.60a	339.46c	63.34a	137.31b	617.04d	23.85a	34.64a	32.07a	22.51a	34.84a	45.17a	39.80a	35.51a
ethyl acetate	4.84ab	58.49d	8.77b	21.45c	19.47c	5.78ab	3.91a	3.45a	3.78a	6.33ab	4.72ab	3.45a	4.63ab
ethyl butanoate	9.97a	121.69c	17.56ab	32.71b	138.47c	7.21a	6.61a	6.18a	3.20a	11.32a	9.20a	8.10a	8.94a
ethyl hexanoate	11.68a	70.85b	16.62a	21.55a	119.34c	8.16a	8.46a	10.95a	5.86a	10.61a	15.08a	12.84a	13.94a
ethyl octanoate	*nd*	51.88c	14.17ab	37.44ab	128.71d	*nd*	8.58a	8.45a	9.80a	*nd*	10.03a	11.18ab	6.64a
ethyl nonanoate	*nd*	*nd*	*nd*	*nd*	3.13	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
ethyl decanoate	2.58a	34.65c	7.93a	22.64bc	200.89d	2.70a	5.81a	3.05a	1.15a	6.10a	6.13a	4.41a	3.23a
ethyl dodecanoate	*nd*	1.90a	0.81a	1.52a	7.04b	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Alcohols	*nd*	12.84c	8.24b	8.95bc	20.05d	1.04a	ND	1.16a	2.30a	*nd*	*nd*	0.50a	0.80a
pentan-2-ol	*nd*	*nd*	2.63	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
butane-2,3-diol	*nd*	*nd*	*nd*	5.02a	6.04a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
hexan-1-ol	*nd*	12.84d	1.42ab	3.93c	ND	1.04ab	ND	1.16ab	2.30b	ND	ND	0.50a	0.80ab
cyclohexanol	*nd*	*nd*	4.19	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
heptan-2-ol	*nd*	*nd*	*nd*	*nd*	14.00	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Aldehydes	14.57abc	127.40e	16.26bc	98.85d	19.10c	3.76ab	3.74ab	3.74ab	5.98abc	6.27abc	3.13a	3.39b	5.86abc
3-methylbutanal	*nd*	12.16b	2.42a	8.95b	*nd*	*nd*	*0.83a*	*nd*	1.06a	*nd*	*nd*	*nd*	1.28a
2-methylbut-2-enal	*nd*	90.90c	6.90a	82.63c	19.10b	1.85a	1.70a	2.35a	2.27a	1.19a	1.44a	0.61a	1.13a
3-methylbut-2-enal	3.01a	10.14b	3.13a	7.28ab	*nd*	*nd*	*nd*	*nd*	*nd*	2.84a	*nd*	*nd*	*nd*
hexanal	2.07a	*nd*	2.05a	*nd*	*nd*	1.91a	1.21a	1.39a	2.14a	2.24a	1.70a	2.14a	2.44a
heptanal	0.83a	14.20b	2.80a	*nd*	*nd*	*nd*	*nd*	*nd*	0.67a	*nd*	*nd*	0.84a	1.01a
nonanal	8.97	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

nd – stands for not detected

Table 7.6 (cont.) - Volatile organic compounds (µg/100g) of cow's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–e) indicate significant differences ($p < 0.05$) between the different conditions.

Storage condition	Initial	AP/RT	AP/RF	50MPa/RT		75MPa/RT				100MPa/RT			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
Ketones	15.51a	16.47ab	7.97a	12.42a	6.23a	10.02a	16.85a	16.04a	12.92a	19.05abc	30.60c	17.08ab	27.96bc
pentan-2-one	4.11a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	4.11a	6.45a	6.56a	*nd*
heptan-2-one	8.46a	9.51a	4.86a	7.07a	*nd*	7.46a	12.01ab	11.39ab	9.23a	11.57ab	18.40bc	8.00a	21.20c
nonan-2-one	2.94ab	6.96bc	3.11abc	5.35abc	6.23abc	2.56a	4.85abc	4.64abc	3.69abc	3.61abc	5.76abc	2.51ab	6.76c
Others	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
heptane	25.24c	42.67d	2.43a	13.25b	*nd*	3.16ab	7.94ab	9.99ab	12.00b	10.31ab	7.34ab	5.61ab	9.34ab
toluene	3.88b	11.86c	2.60ab	2.75ab	*nd*	2.16ab	1.11a	1.84a	2.23ab	2.60ab	1.75a	1.77a	1.72a

nd – stands for not detected

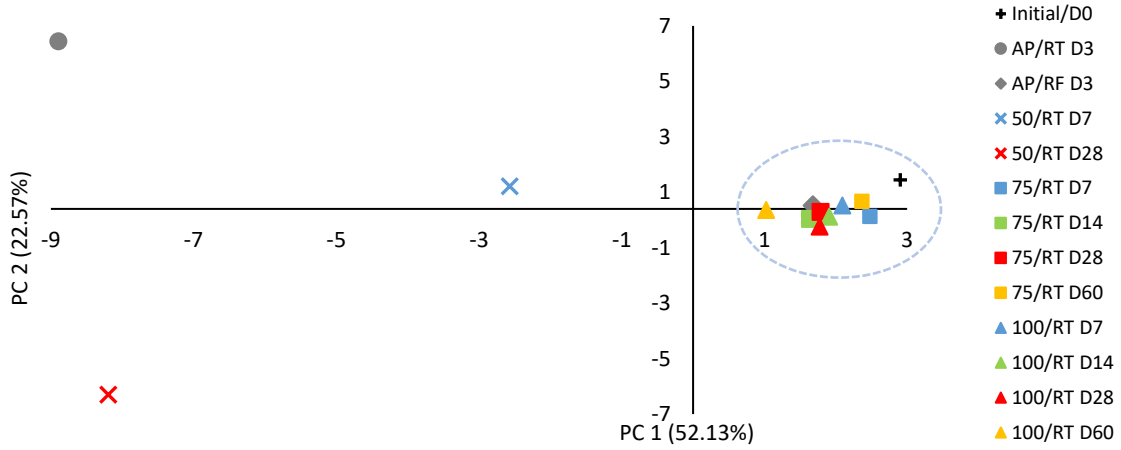

Figure 7.1 - Principal component analysis (PCA) score plot of the volatile compounds of cow's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) over time. Same storage periods have the same colour, while same storage conditions, have the same symbol.

Table 7.7 - Loadings of the variables in the first two principal component analysis of the volatile compounds in cow's fresh cheese.

Compounds	Principal Components	
	PC **1**	PC **2**
ethyl acetate	-0.861	0.496
pentan-2-one	0.349	-0.015
3-methylbutanal	-0.648	0.654
acetic acid	-0.917	0.309
heptane	-0.394	0.808
pentan-2-ol	0.126	0.013
2-methylbut-2-enal	-0.746	0.485
3-methylbut-2-enal	-0.562	0.682
toluene	-0.487	0.837
butane-2,3-diol	-0.620	-0.550
hexanal	0.865	0.006
ethyl butanoate	-0.972	-0.114
butanoic acid	-0.961	-0.169
hexan-1-ol	-0.653	0.728
cyclohexanol	0.126	0.013
heptan-2-ol	-0.612	-0.761
heptan-2-one	0.404	0.335
heptanal	-0.615	0.704
ethyl hexanoate	-0.912	-0.342
hexanoic acid	-0.950	-0.232
nonan-2-one	-0.676	0.061
nonanal	0.217	0.118
(2E,4E)-hexa-2,4-dienoic acid	-0.858	0.458
ethyl octanoate	-0.873	-0.470
octanoic acid	-0.945	-0.143
nonanoic acid	-0.916	0.165
ethyl nonanoate	-0.612	-0.761
decanoic acid	-0.845	-0.122
ethyl decanoate	-0.738	-0.651
dodecanoic acid	-0.795	-0.352
ethyl dodecanoate	-0.813	-0.550

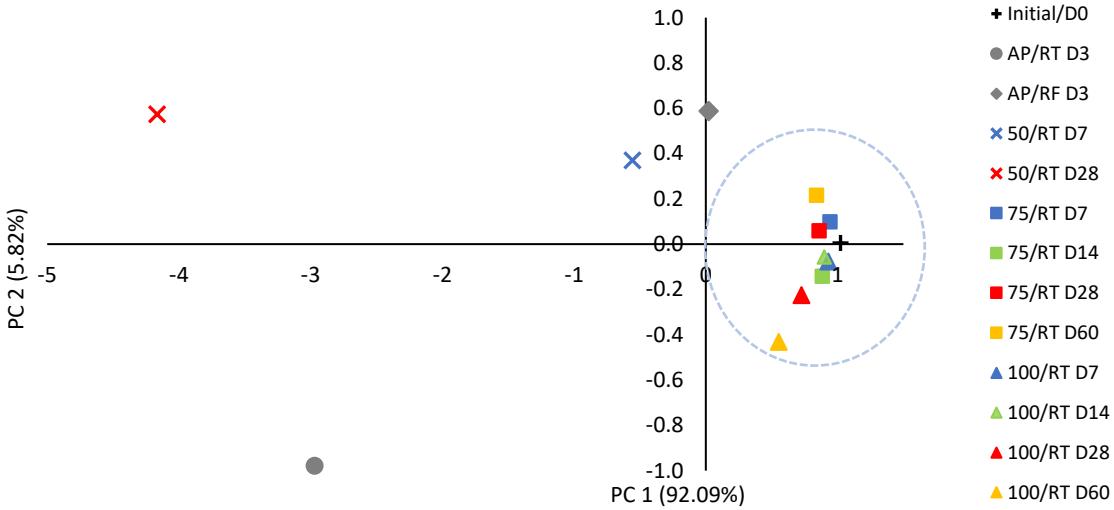

Figure 7.2 - Principal component analysis (PCA) score plot of the three major volatile compounds classes (free fatty acids, alcohols, and esters) of cow's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) over time. Same storage periods have the same colour, while same storage conditions, have the same symbol.

Table 7.8 - Loadings of the variables in the first two principal component analysis of the three major volatile compounds classes in cow's fresh cheese.

Compounds	Principal Components	
	PC 1	PC 2
Alcohols	-0.961	0.230
Esters	-0.976	0.097
Free fatty acids	-0.941	-0.335

In goat's FC initially a total of 24 compounds were detected (**Table 7.9**), most of the VOC belonged to FFA (n=6), followed by esters (n=5), alcohols (n=5), ketones (n=4), and aldehydes (n=1), resembling the ones reported by Quintanilla, Hettinga, Beltrán, Escriche, and Molina (2020).

Storage at AP/RT resulted in a higher VOC content in most major classes, with the exception for ketones and aldehydes that can be easily converted into acids or alcohols. This raise ($p < 0.05$) was almost up to 10-fold in alcohols, FFA and ethyl esters, resulting in a considerable increase in 3-methylbutan-1-ol, butane-2,3-diol, acetic, butanoic and octanoic acids, and in ethyl butanoate and hexanoate. Under AP/RF this evolution in cheese VOC profile was not so pronounced, despite the significant increases ($p < 0.05$) observed in acetic and nonanoic acids, ethyl esters remained within the values initially reported ($p > 0.05$), however with a higher alcohol abundance, mainly from 3-methylbutan-1-ol ($p < 0.05$), while ketones ($p < 0.05$) and aldehydes concentration were reduced after 3 days.

Under HS conditions, 50/RT promoted significant changes in cheese VOC profile, with an accentuated formation ($p < 0.05$) of FFA, ethyl esters and alcohols, while ketones and aldehydes were undetected just after 7 days of storage. Prolonged storage at 50/RT resulted in a rise of all FFA, esters and alcohols, contributing to a distinguished VOC profile comparatively to cheeses prior to storage ($p < 0.05$). Contrarily, storage under 75 and 100/RT promoted a more stable VOC profile over storage, with a reduction in ketones content slower under these storage conditions, while aldehydes increased slightly only after 60 days under 100/RT ($p < 0.05$). A greater alcohol formation was observed in the first 14^{th} days ($p < 0.05$), reaching values similar to the initial ones on the following storage periods, whereas FFA remained constant from the 7^{th} day on forward, without considerable changes ($p > 0.05$) being detected for esters over storage.

Changes in the VOC profile under the different storage conditions allowed the elaboration of a PCA considering the individual VOC, which could explain 71.02% of total

variance (**Figure 7.3**), with 55.42% and 15.60% corresponding from PC 1 and PC 2, respectively, with ketone and aldehyde compounds scoring on the positive PC 1 (**Table 7.10**) associated with unspoiled goat's FC like 3-methylbutanal, butane-2,3-dione, 3-hydroxybutan-2-one, heptan-2-one and nonan-2-one, while FFA, alcohols and esters compounds were more present in spoiled samples, with negative loadings on PC 1, such as heptan-2-ol, octanoic acid and ethyl butanoate. And thus, when only total FFA, esters and alcohols major classes were used in another multivariate statistical analyses, the elaborated PCA accounted for a total of 98.43% variance (**Figure 7.4**). Both PCAs aligned samples stored under AP/RF, 75 and 100/RT closer to the initial one (on the positive PC 1), while cheeses stored under AP/RT and 50/RT were dispersed on the negative PC 1.

For both cheeses, storage under HS at 75-100/RT, allowed a more stable VOC profile throughout the storage, and resembling more the VOC profile of cheeses prior to storage, even after 60 days at RT, with a better maintenance of FFA, esters and alcohols over storage, when compared to the other storage conditions. It is worthy to note, that even only after 3 days at low temperature (AP/RF), cheeses stored under HS (75-100/RT) after 60 days presented overall a more resembling VOC profile comparatively to cheeses prior to storage, additionally with negligence energy supply.

Table 7.9 - Volatile organic compounds (µg/100g) of goat's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT). Different letters (a–f) indicate significant differences ($p < 0.05$) between the different conditions.

Condition	Initial	AP/RT	AP/RF	50MPa/RT		75MPa/RT				100MPa/RT			
Days	0	3	3	7	28	7	14	28	60	7	14	28	60
Free fatty acids	166.38a	3170.90c	1017.31ab	3282.07c	4239.54c	1007.95ab	1264.32b	1121.78ab	1286.54b	799.95ab	1255.01b	700.41ab	1576.32b
acetic acid	21.64a	422.79d	179.00bc	173.23c	319.21d	74.99abc	71.31abc	46.02abc	48.22ab	56.39abc	56.84abc	40.51ab	37.81a
butanoic acid	14.34a	571.82e	107.32abc	344.72d	535.75e	94.99abc	124.43bc	96.47abc	181.96c	68.04ab	82.43abc	82.85abc	162.53bc
hexanoic acid	27.06a	1013.36c	223.33ab	1029.30c	1294.91c	254.32ab	380.46b	301.62ab	454.01b	193.04ab	267.10ab	260.93ab	470.72b
octanoic acid	61.41a	736.95cd	254.26ab	1062.65de	1198.72e	340.61ab	389.41abc	307.57ab	372.90abc	284.01ab	423.42abc	199.49ab	550.83bc
nonanoic acid	8.81ab	10.24abc	26.39d	21.55cd	17.44bcd	8.39ab	6.69a	8.71ab	5.25a	3.91a	7.88ab	8.62ab	13.65abc
decanoic acid	44.19a	396.63b	168.37ab	710.73c	690.81c	233.13ab	298.83ab	359.02b	122.28ab	164.69ab	336.46b	108.12ab	343.57ab
dodecanoic acid	2.71a	27.18c	7.07a	13.34ab	18.94bc	9.90ab	11.09ab	11.08ab	3.35a	7.77a	12.83ab	6.57a	10.86ab
Esters	55.03a	156.58bc	46.71a	225.24c	563.92d	29.50a	119.02abc	110.92abc	101.84ab	24.83a	107.46abc	64.91ab	117.70abc
ethyl acetate	10.16ab	26.43c	15.21b	9.56ab	10.00ab	8.91ab	7.83ab	5.00a	3.40a	10.14ab	10.96ab	4.88a	4.67a
ethyl butanoate	5.52a	52.20d	12.02ab	27.71bc	75.04e	11.45ab	18.60abc	20.04abc	28.80c	11.50ab	13.30ab	18.82abc	16.29abc
ethyl hexanoate	8.98a	30.22a	7.12a	40.56a	138.36b	8.82a	22.03a	17.46a	20.94a	7.02a	11.93a	12.90a	23.17a
ethyl octanoate	24.94a	41.05a	*nd*	40.56a	138.36b	*nd*	18.85a	13.83a	14.80a	*nd*	14.23a	10.98a	18.52a
ethyl decanoate	3.55a	10.02abc	8.43ab	95.92d	172.44e	*nd*	51.71c	54.58cd	33.89abc	*nd*	52.50cd	23.61abc	55.45cd
ethyl dodecanoate	*nd*	*nd*	*nd*	*nd*	4.43	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Alcohols	93.97a	834.88f	364.48d	649.46e	768.53ef	350.06d	329.98cd	175.64ab	166.17a	343.01bcd	362.75d	160.06a	156.15ab
2-methylpropan-1-ol	11.05ab	39.41e	18.99bcd	29.16de	24.53cd	12.78ab	11.48ab	8.77a	8.95a	16.05abc	13.73ab	8.39a	8.63a
3-methylbutan-1-ol	44.09a	482.63c	197.42b	424.03c	555.42c	239.00b	245.46b	103.44ab	105.37ab	212.59b	244.21b	99.50ab	87.33ab
2-methylbutan-1-ol	5.76a	40.86de	14.80ab	30.89cd	54.08e	23.28bc	24.69bc	17.54abc	17.84abc	23.09bc	24.87bc	13.92ab	12.70ab
butane-2,3-diol	29.87a	253.64b	79.79a	81.56a	80.82a	75.00a	48.35a	45.89a	34.01a	104.07a	77.42a	38.26a	56.26a
cyclohexanol	1.73a	15.24b	14.01b	*nd*	18.69b	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
heptan-2-ol	*nd*	4.66ab	3.66a	7.63ab	8.53b	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Ketones	98.39b	16.12a	27.54a	*nd*	*nd*	37.49a	15.67a	11.77a	19.17a	28.80a	29.96a	9.83a	14.59a
butane-2,3-dione	42.55	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3-hydroxybutan-2-one	59.97c	16.12ab	23.71ab	*nd*	*nd*	37.49bc	4.90a	3.92a	7.25ab	28.80ab	16.36ab	2.75a	*nd*
heptan-2-one	7.99a	*nd*	*nd*	*nd*	*nd*	*nd*	10.92ab	7.85a	11.20ab	*nd*	13.60b	7.86a	14.59b
nonan-2-one	5.00a	*nd*	*nd*	*nd*	*nd*	8.25abc	12.05bc	4.45a	5.47a	4.38a	7.10ab	3.71a	12.96c
Aldehydes													
3-methylbutanal	4.75a	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	3.87a	5.28ab	4.05a	3.30a	7.72ab	10.52b
Others	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
heptane	20.51abc	38.16bcd	24.55abc	51.21de	5.40a	44.97cde	4.63a	17.93abc	14.70abc	62.12e	10.01ab	6.30ab	42.90bcd
toluene	1.04a	5.85c	*nd*	5.20bc	5.26bc	2.83abc	1.91ab	3.82abc	1.42ab	3.05abc	4.85bc	3.61abc	2.66abc

nd – stands for not detected

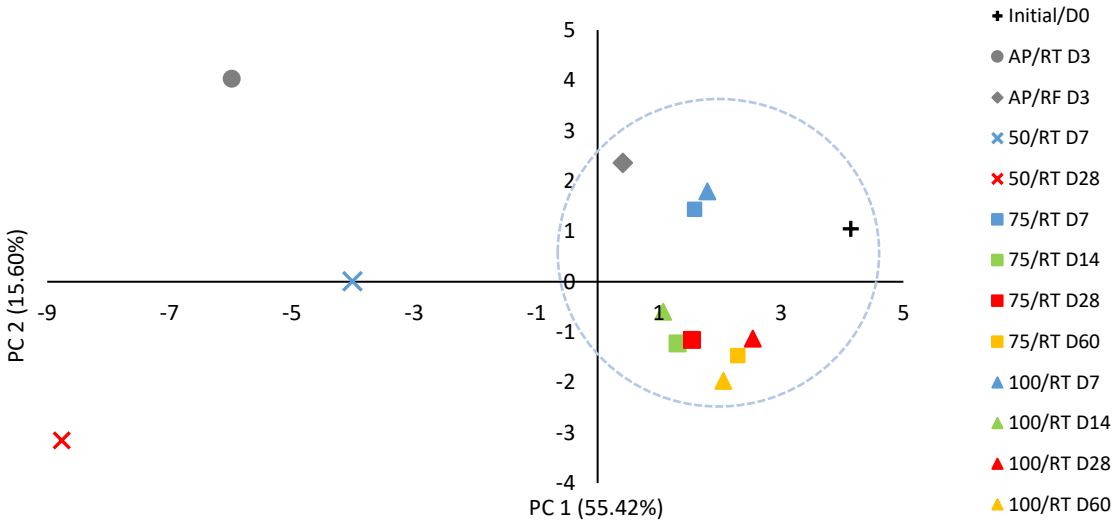

Figure 7.3 - Principal component analysis (PCA) score plot of the volatile compounds of goat's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) over time. Same storage periods have the same colour, while same storage conditions, have the same symbol.

Table 7.10 - Loadings of the variables in the first two principal component analysis of the volatile compounds in goat's fresh cheese.

Compounds	Principal Components	
	PC **1**	PC **2**
butane-2,3-dione	0.328	0.157
ethyl acetate	-0.530	0.772
2-methylpropan-1-ol	-0.839	0.496
3-methylbutanal	0.571	-0.375
acetic acid	-0.909	0.331
heptane	-0.023	0.530
3-hydroxybutan-2-one	0.397	0.587
3-methylbutan-1-ol	-0.954	0.111
2-methylbutan-1-ol	-0.937	-0.070
toluene	-0.686	-0.077
ethyl butanoate	-0.906	-0.222
butane-2,3-dione	-0.603	0.663
butanoic acid	-0.947	0.036
cyclohexanol	-0.757	0.221
heptan-2-ol	-0.914	0.008
heptan-2-one	0.564	-0.540
ethyl hexanoate	-0.812	-0.483
hexanoic acid	-0.947	-0.169
nonan-2-one	0.604	-0.348
ethyl octanoate	-0.819	-0.413
octanoic acid	-0.908	-0.257
nonanoic acid	-0.463	0.062
decanoic acid	-0.829	-0.292
ethyl decanoate	-0.671	-0.704
dodecanoic acid	-0.831	0.191
ethyl dodecanoate	-0.694	-0.470
butane-2,3-dione	0.328	0.157

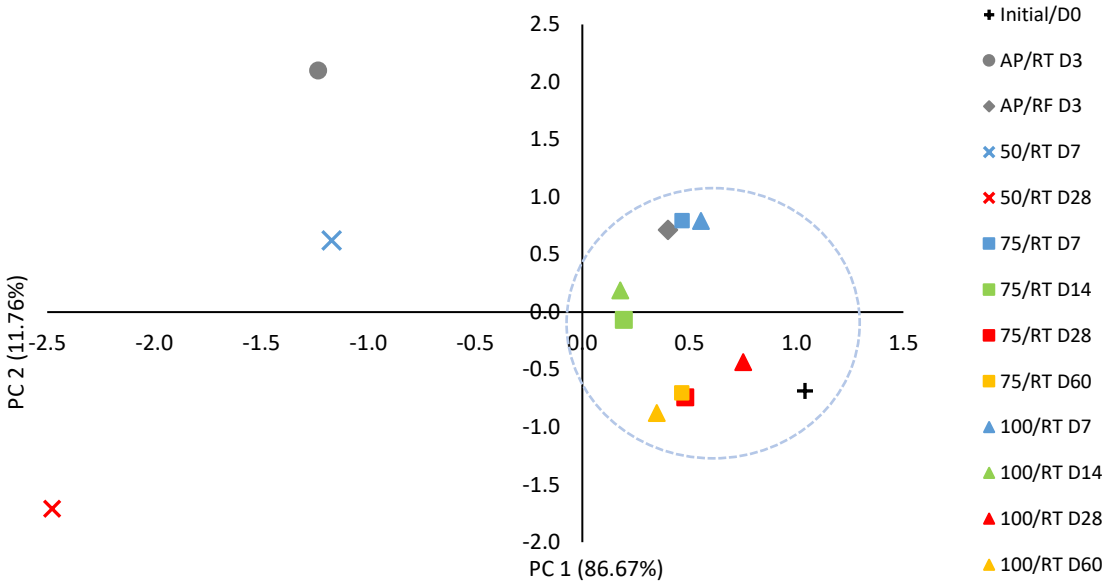

Figure 7.4 - Principal component analysis (PCA) score plot of the three major volatile compounds classes (free fatty acids, alcohols, and esters) of goat's FC prior storage (Initial) and stored under the different conditions (AP/RT, AP/RF and 50, 75 and 100MPa/RT) over time. Same storage periods have the same colour, while same storage conditions, have the same symbol.

Table 7.11 - Loadings of the variables in the first two principal component analysis of the three major volatile compounds classes in goat's fresh cheese.

Compounds	Principal Components	
	PC 1	PC 2
Alcohols	-0.907	0.408
Esters	-0.898	-0.431
Free fatty acids	-0.985	0.017

7.4 Conclusions

Overall, HS under 75 and 100 MPa at RT allowed a much better preservation of both cow's and goat's FC, during a considerably longer storage period, when compared with RF. The results at these two pressures are very interesting, as throughout storage especially under 100/RT, a more stable fatty acid profile and total protein was achieved after 60 days. Additionally, these two storage conditions retained the volatile organic profile similar to FC prior to storage, without noticeable formation of undesirable compounds associated with cheese spoilage, even after 60 days at uncontrolled variable RT, which was corroborated by the PCAs conducted for both cheeses. Although initially HS (75-100/RT) had a direct impact in goat's FC textural profile, throughout the storage this effect was reversed to TPA values overall closer to those prior storage, being verified also an increased FAA abundance, especially for the longest storage periods. The changes observed in HS cheeses for TPA and FAA should be further assessed to fully understand the real impact in the sensorial properties and consumers acceptability.

In conclusion, a much longer shelf-life can be achieved under HS/RT (up to at least 60 days, the maximum period studied in this work), opening new business opportunities, as FC has only few weeks of shelf-life under RF. In addition, HS would allow significant energy savings throughout the storage, being *quasi* energetically costless, due to the needless for constant energy supply to maintain temperature, thus being environmentally friendlier and more sustainable and suitable for example for longer transportation.

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CHAPTER 8

Concluding Remarks and Future Work

8.1 Concluding Remarks

In this work the feasibility to preserve two highly perishable dairy products (raw milk and fresh cheese) under hyperbaric storage (HS) conditions, ranging from 50 to 100 MPa, at naturally variable uncontrolled room temperature (RT), was evaluated comparatively to refrigeration (RF). In order to do so, several microbiological, physicochemical, rheological, textural, nutritional, and enzymatic parameters were evaluated under the different storage conditions throughout storage.

Regarding raw milk, the microbial loads under refrigerated storage quicky surpassed the acceptable limit within 7 days, while under 50 MPa/RT, microbial growth was slowed down, reaching unacceptable levels only on the 28th day. On the other hand, storage under 62, 75 and 100 MPa, continuously inactivated the overall endogenous microbiota, faster for Enterobacteriaceae, coliforms, yeast and moulds, while total aerobic mesophiles and lactic acid bacteria were more resistant to HS, reaching even though, counts bellow the detection limit under 100 MPa at RT at the 28th day of storage, remaining at this level till the end of the storage period studied (60 days). A similar behaviour was observed for raw milk with an initial higher microbial load (simulating a worst-case scenario), when stored under HS/RT (75 and 100 MPa), with total aerobic mesophiles and psychrophiles inactivation, reaching values below the detection limit after 130 days of storage (more than 6 log units reduction), with D_p-values of 12.8 and 13.0 days under 100 MPa, respectively. Moreover, this milk after HS/RT, presented an increased microbial stability when subsequently stored under RF (post-hyperbaric storage), with slower microbial growth. HS was also capable not only to restrain the microbial growth of inoculated surrogate pathogenic *Escherichia coli* and *Listeria innocua* as well as pathogenic *Salmonella senftenberg*, but also to inactivate these microorganisms even under 50 MPa. As for *Bacillus subtilis*, higher pressures were required (75 and 100 MPa) in order to inactivate both the vegetative and the endospore forms, resulting in an endospores load reduction of more than 4.5 log units after 31 days under 100 MPa/RT. In terms of the physicochemical, nutritional and enzymatic parameters evaluated, raw milk at RT under 75 and 100 MPa maintained mostly a profile comparable to milk prior storage, even after 60 days, without considerable changes in the pH, titratable acidity, total solids content, density, colour, and viscosity, although increased proteolytic activity was observed, with higher abundance of free amino acids mainly for the longest storage periods. Raw milk at HS/RT also retained the fatty acids and volatile organic profiles, avoiding the

formation of undesirable compounds that could impact the sensorial properties of milk, even after 60 days of storage, slowing down additionally lipid oxidation rate, while alkaline phosphatase and lactoperoxidase activities remained mostly stable throughout the storage, to values around 60-80% and 80-100% of residual activity, respectively, at 100 MPa/RT. Thus, these results point to a considerable microbiological and physicochemical shelf-life extension of raw milk, for up to at least 60 days (the longest storage period studied simultaneously for microbial and physicochemical quality), with additionally increased microbial safety, due to the microbial inactivation observed.

In the case of cow's and goat's fresh cheeses, storage under HS/RT required pressures of ≥75 MPa in order to achieve effective microbial control, with clear microbial loads reduction over storage being observed, especially under 100 MPa/RT, resulting in a reduction of total aerobic mesophile counts of about 4.8 and 6 log units in cow's and goat's fresh cheese, respectively. The other microbiota evaluated was inactivated faster, reaching values bellow the quantification and detection levels, some already in the first days of storage. Initially HS caused a compression effect of the cheeses matrix, resulting in whey expulsion, reduction in moisture content and increases in textural parameters, which tended to reverse to values closer to the initial ones throughout storage. Although an increase in free amino acids throughout HS/RT occurred, which could have contributed to the increased protein digestibility (5.2%) in goat's fresh cheese (after 60 days at 100/RT), the better microbial control observed in HS fresh cheeses resulted in a better quality maintenance of the cheeses, especially under 100 MPa/RT in terms of colour, lipid oxidation, total protein, fatty acids and volatile organic profile, even after 60 days, compared to those prior storage.

In can be concluded, that HS/RT of both dairy products studied in this thesis (raw milk and fresh cheese) can result in considerable shelf-life extension, as for instance, refrigerated controls reached considerable higher spoilage levels and pronounced physicochemical and quality losses, just after a few days, while HS/RT can achieve a much longer shelf-life, particularly under 75 and 100 MPa/RT, with no major changes in overall quality.

Additionally to these advantages, HS/RT can contribute to a significant lower carbon footprint associated to food preservation, being considered a *quasi*-energetically costless alternative to refrigeration, with no need of constant energy supply during storage, thus being environmentally friendlier and more sustainable. Anyway, although this work contributed to

expand and deepen the potential feasibility of HS/RT for food preservation, further studies and technological advances at equipment level are still required for a potential future implementation in the food industry.

8.2 Future Work

Promising results were obtained in this work, indicating an increased shelf-life, higher microbial safety, and better quality stability of raw milk and fresh cheese preservation under HS/RT compared to RF, pointing to the potential application of HS as a new preservation methodology for these products and food preservation overall.

Still, further research is required for a better and deeper evaluation of the feasibility of HS/RT for dairy products preservation, for example, longer storage periods should be evaluated, and other parameters could be better assessed, such as primary and tertiary lipid oxidation, lipid composition such as triglycerides, phospholipids and cholesterol, casein micelles and fat globule structures, lactose and other sugars, as well as vitamins. The effect of HS on other dairy foodborne microorganisms responsible for most of the dairy outbreaks, such as *Campylobacter jejuni*. *Staphylococcus aureus*, *Listeria monocytogenes*, *Brucella melitensis*, *Escherichia coli* O157:H7, among others, should also be evaluated. Milk and cheese enzymes, like lipoprotein lipase and plasmin can impact the quality of these products, and so, the effect of HS on their activity should also be studied. Since increase in free amino acids during storage was observed in the present work, it is important to further study the increment of proteolysis, mainly in fresh cheeses, attributable to the high initial microbial load present, and so, HS preservation studies should be carried out with fresh cheeses with a lower initial microbial load to verify this possibility.

In order to validate the possible shelf-life attainable under HS, sensorial evaluation needs to be carried out, as for instance the increase in free amino acids or secondary lipid oxidation products during storage, could impact the sensorial profile. If the changes reported under HS/RT impact the sensorial quality, the combination of HS with lower temperature (10-15 ºC), but still above refrigeration temperature, should be evaluated, since interesting results under these conditions were already reported for meat and fish products in two works of our research group.

The possibility to store other dairy products with different compositions and characteristics, such as cream, other cheeses, yogurts, as well as food in general that are highly dependent on refrigerated storage, under HS should also be further evaluated.

In this thesis, HS capacity to not only inhibit microbial growth but also to gradually inactivate both endogenous as well as inoculated microbiota, was highlighted, resulting in some cases in more than 6 log units reduction for both raw milk and goat's fresh cheese stored under 100 MPa. These results open the possible development of a new concept, called hyperbaric inactivation (HI), which is currently being studied in our research group during shorter exposure periods (hours) under pressures from 100 to slightly above 150 MPa, but substantially much lower than the ones applied in high pressure processing pasteurization, with interesting results, suggesting an effective pasteurization effect of several endogenous microbiological groups and inoculated pathogenic bacteria, with minimal impairments in food properties, which seems to be a new promising potential inactivation process. Such process would be a nonthermal microbial inactivation process, being *quasi*-energetically costless and would inactivate microorganisms while the food is being preserved by microbial growth control as in refrigeration.

ANNEXES

Annex A

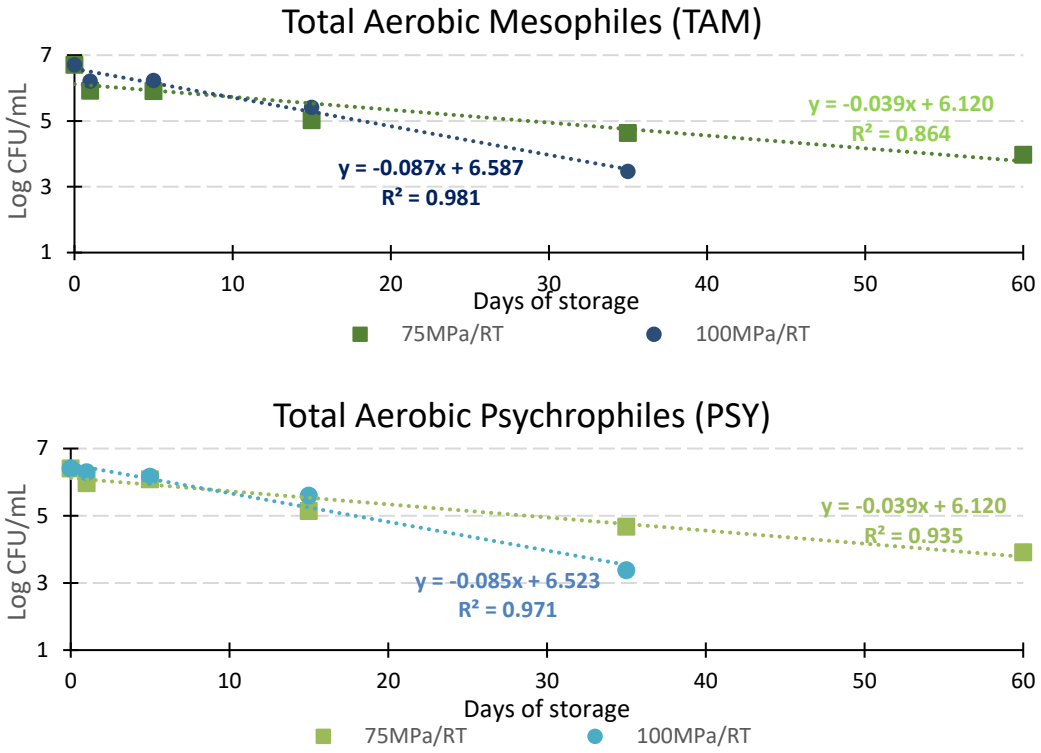


Figure A.1 - Log linear decreased of TAM and PSY (expressed in log CFU/mL), throughout the storage period under 75 and 100MPa/RT of raw milk used in the second study

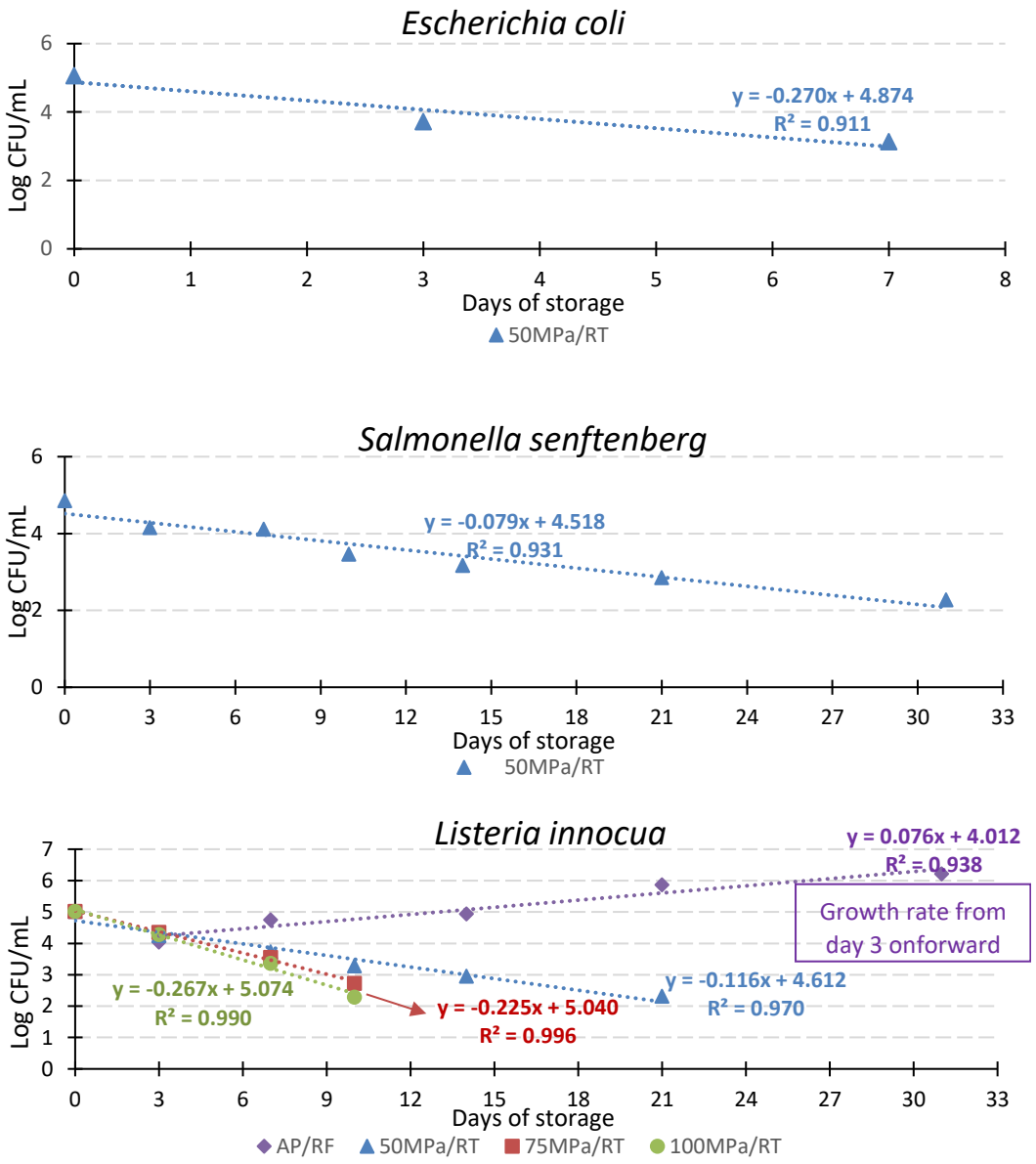


Figure A.2 - Log linear behaviour of inoculated *Escherichia coli*, *Salmonella senftenberg* and *Listeria innocua* (expressed in log CFU/mL), throughout the storage period under 50, 75 and 100MPa/RT and AP/RF of raw milk.

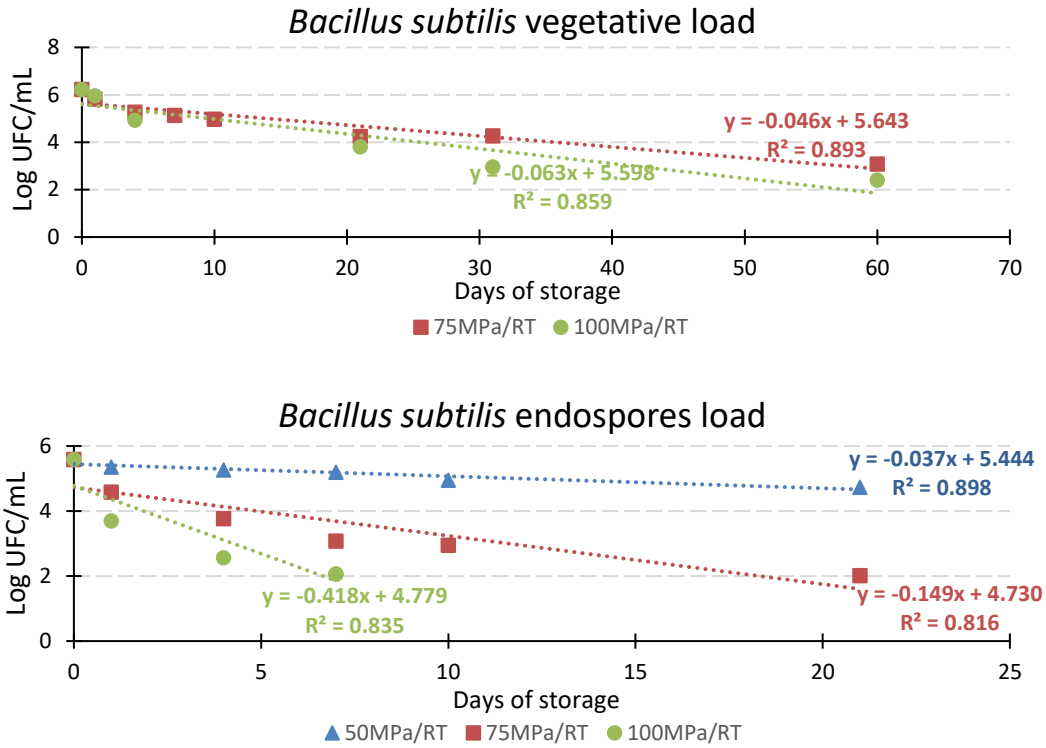


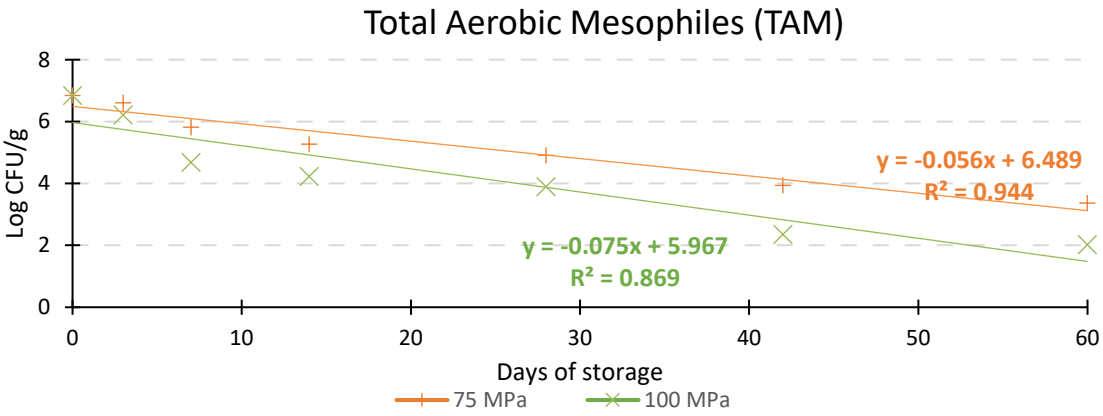
Figure A.3 - Log linear behaviour of vegetative and inoculated endospores of *Bacillus subtilis* (expressed in log CFU/mL), throughout the storage period under 50, 75 and 100/RT of raw milk.

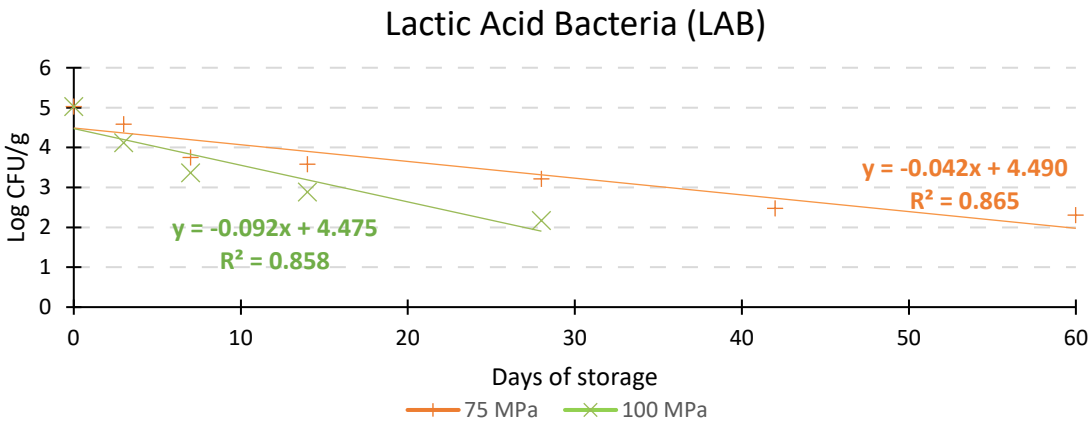
Table A.1 - D_p- (days) z_p-values (MPa) determined for TAM and PSY endogenous load, and in inoculated *Escherichia coli*, *Salmonella senftenberg*, *Listeria innocua* and in *Bacillus subtilis* vegetative and endospores load in raw milk, stored under 50, 75 and 100 MPa at RT.

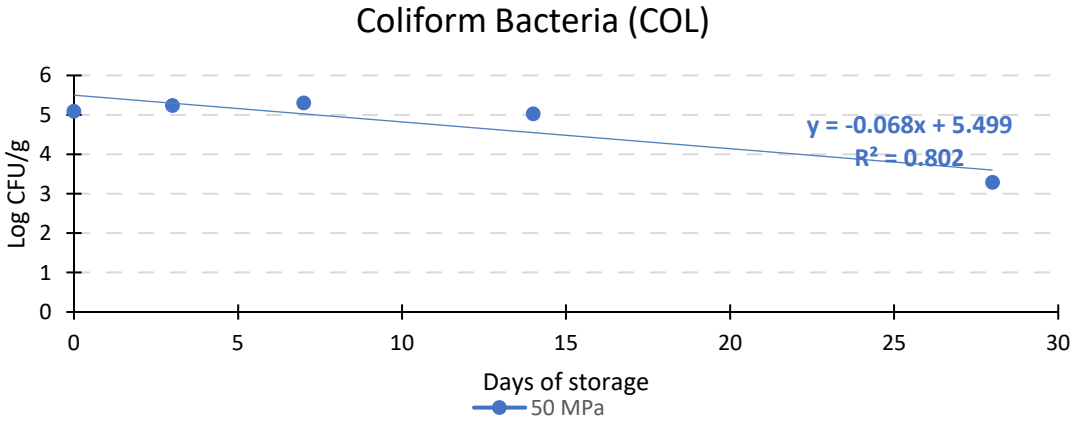
			D_p-values (days)				
Conditions	TAM	PSY	*E. coli*	*S. senftenberg*	*L. innocua*	*B. subtilis load*	
						vegetative	*endospores*
50MPa/RT	*nd*	*nd*	3.7	12.7	8.6	*nd*	27.0
75MPa/RT	25.6	25.6	*nd*	*nd*	4.5	21.7	6.7
100MPa/RT	12.8	13.0	*nd*	*nd*	3.7	16.0	2.4
z_p-value (MPa)	*nd*	*nd*	*nd*	*nd*	138.9 R^2=0.900	*nd*	47.6 R^2=0.993

nd – stands for not determined

Annex B


Total Aerobic Mesophiles (TAM)
Log CFU/g
y = -0.056x + 6.489
R² = 0.944
y = -0.075x + 5.967
R² = 0.869
Days of storage
75 MPa
100 MPa


Lactic Acid Bacteria (LAB)
Log CFU/g
y = -0.042x + 4.490
R² = 0.865
y = -0.092x + 4.475
R² = 0.858
Days of storage
75 MPa
100 MPa


Coliform Bacteria (COL)
Log CFU/g
y = -0.068x + 5.499
R² = 0.802
Days of storage
50 MPa

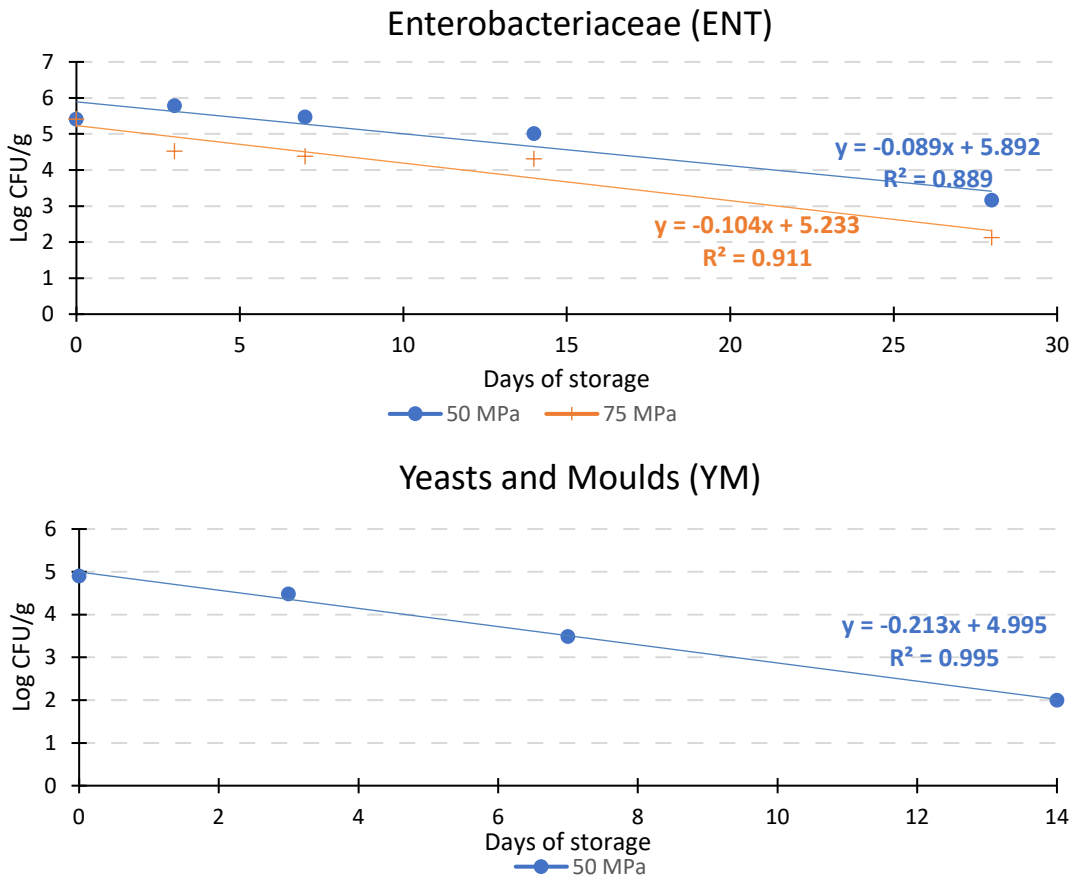
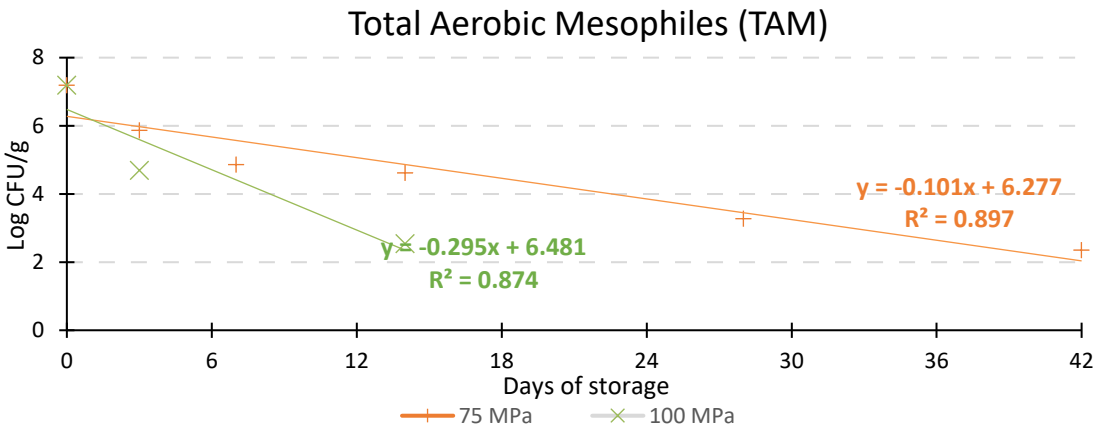


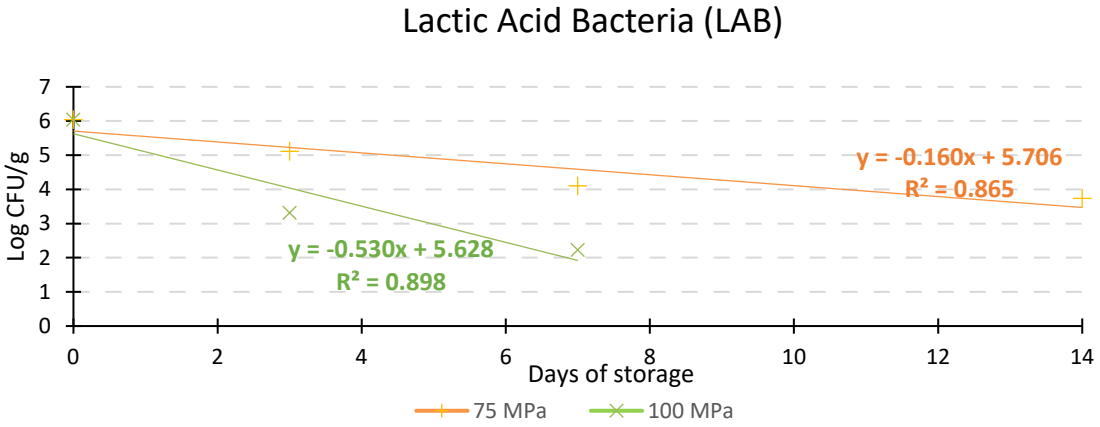
Figure B.1 - Log linear decreased of TAM, LAB, COL, ENT and YM (expressed in log CFU/g, throughout the storage period under 50, 75 and 100MPa/RT of cow's fresh cheese, from which D_p-values were calculated.

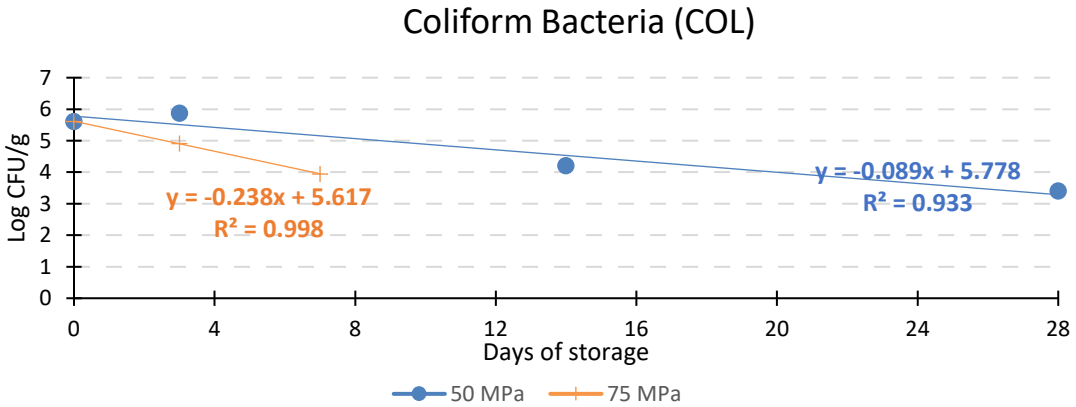
Table B.1 - D_p-values (days) determined for TAM, LAB, COL, ENT and YM in cow's fresh cheese, stored under 50, 75 and 100 MPa at RT.

	D_p-values (days)				
Conditions	TAM	LAB	COL	ENT	YM
50MPa/RT	*nd*	*nd*	14.7	11.3	4.7
75MPa/RT	17.8	23.9	*nd*	9.6	*nd*
100MPa/RT	13.4	10.9	*nd*	*nd*	*nd*

nd – stands for not determined


Total Aerobic Mesophiles (TAM)
Log CFU/g
Days of storage
y = -0.101x + 6.277
R² = 0.897
y = -0.295x + 6.481
R² = 0.874
75 MPa
100 MPa


Lactic Acid Bacteria (LAB)
Log CFU/g
Days of storage
y = -0.160x + 5.706
R² = 0.865
y = -0.530x + 5.628
R² = 0.898
75 MPa
100 MPa


Coliform Bacteria (COL)
Log CFU/g
Days of storage
y = -0.238x + 5.617
R² = 0.998
y = -0.089x + 5.778
R² = 0.933
50 MPa
75 MPa

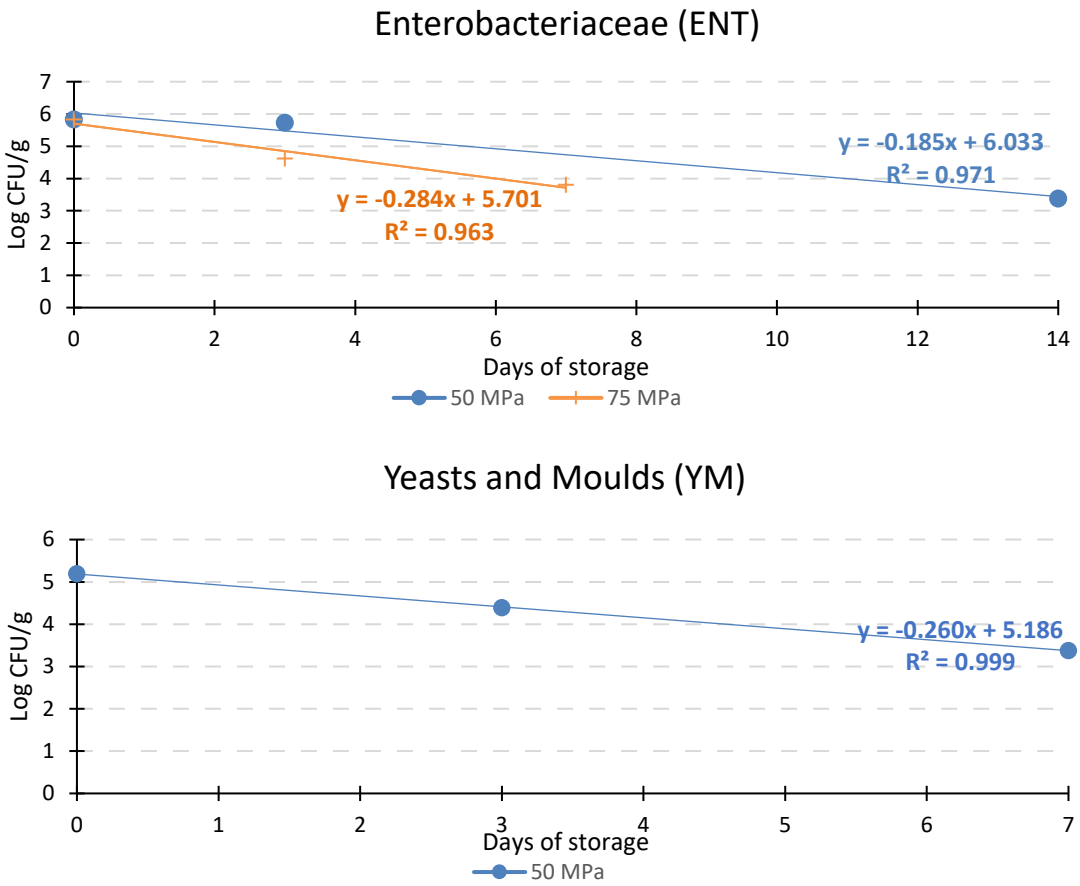


Figure B.2 - Log linear behaviour of TAM, LAB, COL, ENT and YM (expressed in log CFU/g, throughout the storage period under 50, 75 and 100MPa/RT of goat's fresh cheese, from which D_p-values were calculated.

Table B.2 - D_p-values (days) determined for TAM, LAB, COL, ENT and YM in goat's fresh cheese, stored under 50, 75 and 100 MPa at RT.

	D_p-values (days)				
Conditions	TAM	LAB	COL	ENT	YM
50/RT	*nd*	*nd*	11.2	5.4	3.9
75/RT	9.9	6.3	4.2	3.5	*nd*
100/RT	3.4	1.9	*nd*	*nd*	*nd*

nd – stands for not determined

Annex C

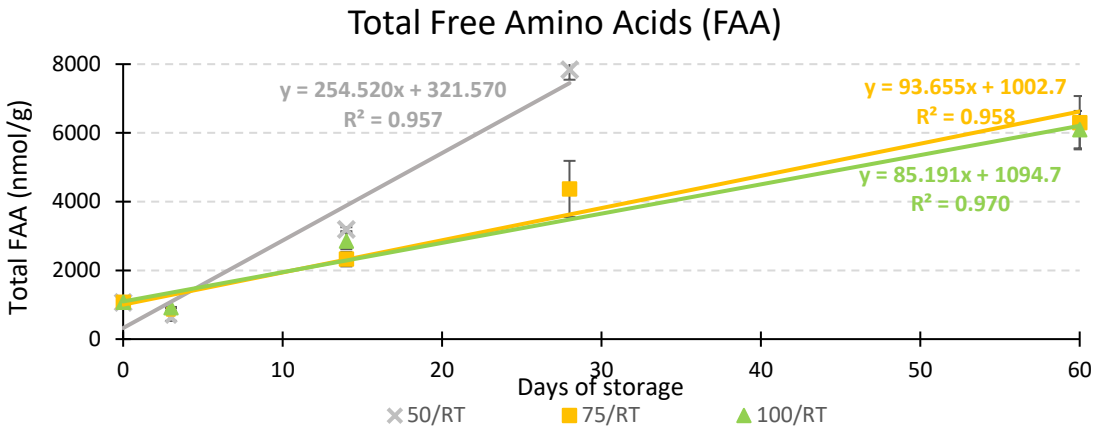


Figure C.1 - Evolution of the total free amino acids throughout the storage period under 50, 75 and 100MPa/RT of cow's fresh cheese.

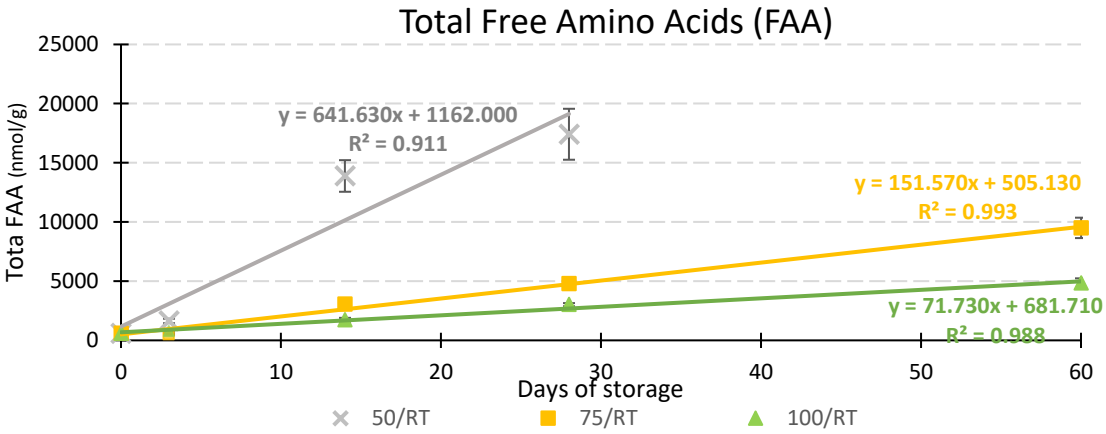


Figure C.2 - Evolution of the total free amino acids throughout the storage period under 50, 75 and 100MPa/RT of goat's fresh cheese.

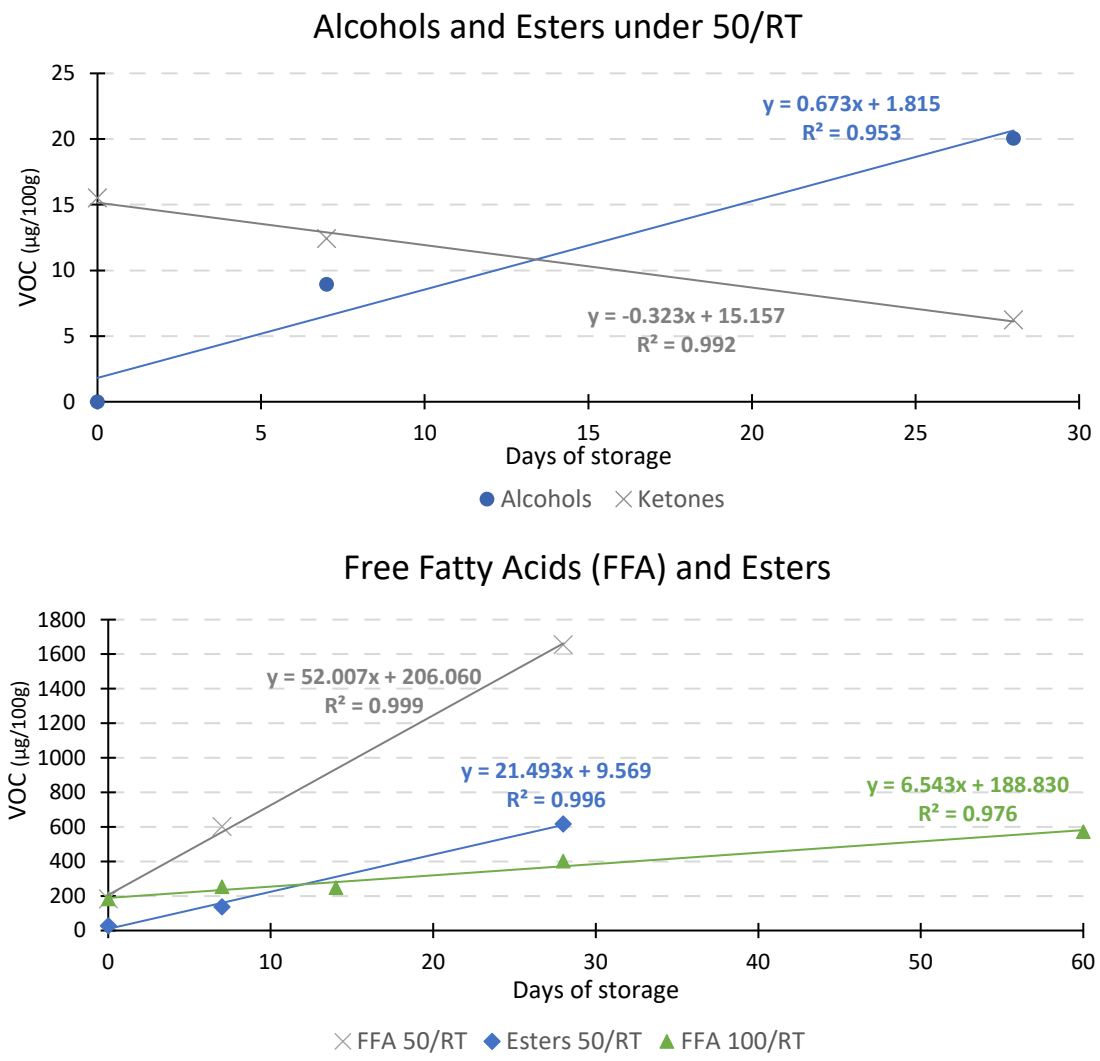


Figure C.3 - Evolution of the major volatile organic compounds classes (alcohols, esters, ketones, and free fatty acids) throughout the storage period under 50, 75 and 100 MPa at RT of cow's fresh cheese.