# EFFECT OF HIGH-PRESSURE AS A NON-THERMAL PASTEURISATION TECHNOLOGY FOR RAW EWES' MILK AND CHEESE SAFETY AND QUALITY: CASE STUDY ON *SERRA DA ESTRELA* CHEESE

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Food Science and Technology and Nutrition

By

Ana Rita Santos Inácio

November 2019

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

universidade de aveiro

Universidade do Minho

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Supervisor: *Professor Ana Maria Pereira Gomes*

Co-supervisor: *Professor Jorge Manuel Alexandre Saraiva*

November 2019

***"There is a cheese for every taste preference and a taste preference for every cheese"***

*Norman Olson (1990)*

Thanks are due to University of Aveiro, Universidade Católica Portuguesa and FCT/MCT for the financial support for the QOPNA (FCT UID/QUI/00062/2019) and CBQF (project UID/Multi/50016/2019) research units, through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement. Rita S. Inácio is also grateful for the financial support from FCT through the fellowship Grant SFRH/BD/96576/2013, as well as to Frulact S.A., through the Mentoring Program Comendador Arménio Miranda by Frulact Academy.

# Resumo

O queijo *Serra da Estrela* é um queijo tradicional produzido a partir de leite de ovelha cru, altamente reconhecido e referenciado, com um período de maturação de 45 dias, sendo posteriormente comercializado sob refrigeração. Sendo um queijo de leite cru, alterações nos parâmetros microbiológicos podem dificultar o armazenamento prolongado. O principal objetivo desta tese de doutoramento foi avaliar a possível contribuição que o processamento de alta pressão (AP), como uma tecnologia de pasteurização a frio, pode ter para melhorar a segurança microbiológica, mantendo a qualidade físico-química e sensorial (45 dias de maturação) durante armazenamento prolongado a 4 ºC. Uma vez estabelecidas a complexidade das relações entre os parâmetros de AP e o queijo, um segundo objetivo foi testar se a aplicação de AP para pré-tratar o leite de ovelha cru, isoladamente ou em combinação com o tratamento com AP dos queijos maturados resultantes poderia melhorar ainda mais a segurança microbiológica sem comprometer os seus atributos únicos de textura e sabor. Para tal, queijos com 45 dias de maturação foram submetidos a AP a 600 MPa/6 min (P1), 450 MPa/6 min (P2) e 450 MPa/9 min (P3). A contagem de células viáveis de lactococos, lactobacilos, enterococos e microrganismos mesófilos totais foram reduzidas em cerca de 4 log ufc/g nos queijos P1 e em 2 log ufc/g nos queijos P2 e P3, comparativamente aos queijos controlo (não processados, $Ch_C$). As contagens de *Enterobacteriaceae* e *Pseudomona*s spp. mostraram reduções >5 e >4 ciclos logarítmicos, respetivamente, para números abaixo do limite de quantificação durante o armazenamento. De uma forma geral, os índices proteolíticos foram mais baixos nos queijos AP do que nos $Ch_C$. Os queijos submetidos à AP nas condições P1 mantiveram o índice de extensão de maturação ao longo dos 15 meses de armazenamento (27-30%) próximo do valor quantificado para o $Ch_C$ aos 45 dias de maturação (29%), mantendo a textura característica, mas foram considerados mais duros que o $Ch_C$ do ponto de vista sensorial. O tratamento AP não causou grandes alterações nem no perfil lipídico do queijo *Serra da Estrela* (conteúdo total de triglicerídeos (65-66 TG/100 g) e de ácidos gordos) nem nas propriedades nutricionais (índices de aterogenicidade e trombogenicidade similares; ~2,3 e ~2,6, respetivamente). Um estudo complementar foi realizado para análise comparativa de dois sistemas de embalamento no embranquecimento da crosta do queijo: embrulhado em papel sem vácuo (P) e embalado a vácuo em plástico (V), na qualidade de queijos *Serra da Estrela* previamente pasteurizados por AP a 525 MPa/6 min. Queijos controle ($Ch_C$) e queijos AP ($Ch_P$) foram embalados nesses dois sistemas ($Ch_C$+V; $Ch_C$+P; $Ch_P$+V e $Ch_P$+P) e foram conservados sob refrigeração durante 10 meses. Os queijos $Ch_P$+V e

$Ch_P$+P continham um número de células viáveis de lactococos, lactobacilos, enterococos e mesófilos totais mais baixo do que os queijos $Ch_C$+V e $Ch_C$+P, i.e. entre 4 e 6 log ufc/g vs aprox. 6 log ufc/g, respetivamente. Os bolores e leveduras (>5 log ufc/g) proliferaram nos queijos embrulhados em papel. Os queijos $Ch_P$+V revelaram um índice de extensão de maturação constante ao longo dos 10 meses de conservação, com valores próximos aos quantificados para queijos ChC+V aos 0 meses. Sistema de embalamento em papel pode ser adequado para curtos períodos de conservação, enquanto o sistema a vácuo é preferível para longos períodos. A aplicação de AP ao leite de ovelha cru como pré-tratamento antes da produção de queijo pode levar a um aumento no rendimento do queijo e incremento da qualidade. Inicialmente, foi realizado um amplo desenho experimental (200–400 MPa; 5–30 min; 1–48 h de tempo de espera antes da AP; 1–24 h após AP), e os resultados alcançados permitiram identificar que a intensidade da pressão, o tempo sob pressão e o tempo de espera após AP foram os fatores mais importantes. A aplicação do modelo de superfície de resposta (100-300 MPa; 5-30 minutos, 24 horas antes e após AP), visando uma redução tão pequena quanto possível do número de células viáveis de lactococos, lactobacilos e enterococos, permitiu identificar como condições ideais de AP o tratamento de leite a 121 MPa/30 min. O modelo foi validado e um estudo de aumento de escala foi realizado em queijaria, resultando num incremento do rendimento em 10,4% com o leite pré-tratado ($M_P$) por AP em comparação com o queijo produzido com leite não tratado ($M_C$). O leite pré-tratado por AP revelou insignificantes reduções da carga microbiológica no leite e coalhada. Aos 60 dias de maturação, metade dos queijos produzidos com $M_P$ e $M_C$ foram tratados com AP a 525 MPa/6 min ($M_P$+$Ch_P$ e $M_C$+$Ch_P$, respetivamente) e a outra metade permaneceu como controlo ($M_P$+$Ch_C$ and $M_C$+$Ch_C$, respetivamente). Todos os queijos foram conservados sob refrigeração por 5 meses. A aplicação de AP em queijos maturados causou similares reduções da carga microbiológica às obtidas nos estudos anteriores (1-3 reduções logarítmicas para bactérias ácido lácticas, e abaixo do limite de quantificação para bolores e leveduras, *Enterobacteriaceae* e *Pseudomonas* sp.). O pré-tratamento com AP levou ao aumento do índice extensão de maturação, enquanto o tratamento AP no queijo manteve o mesmo. O pré-tratamento AP no leite parece melhorar as propriedades tecnológicas do queijo, enquanto a aplicação AP aos queijos maturados pode aumentar a segurança do queijo; o acoplamento de ambas manteve as características inalteradas e, poderá contribuir positivamente para a melhor promoção deste queijo junto dos produtores e consumidores.

**Palavras-chave:** Queijo *Serra da Estrela*, processamento por alta-pressão, leite cru, segurança alimentar, qualidade do queijo.

# Abstract

*Serra da Estrela* Cheese is a highly recognized and referenced raw ewes' milk cheese that is ripened for 45 days, being commercialized thereafter under refrigerated conditions. Being a raw milk cheese changes in microbiological quality may hamper desired prolonged storage. The major aim of this Ph.D thesis was to assess the contributions that high pressure processing (HPP), as a cold pasteurisation technology, could have on microbiological load and quality improvement while retaining optimum physicochemical, biochemical, textural and sensorial quality of *Serra da Estrela* cheese during extended storage at 4 ºC. Once the complexity of relationships were established between HPP and cheese parameters and associated stability, a second major objective was to test whether the application of HPP to pre-treat the raw ewes milk, alone or in combination with HPP treatment of resulting ripened cheeses before storage, could further enhance microbial safety without compromising their unique texture and flavour attributes. For this, cheeses ripened for 45 days underwent HPP at 600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3) in order to select the most favourable binomial pressure intensity/holding time. Lactococci, lactobacilli, enterococci and total mesophilic microorganisms were reduced ca. 4 log cfu/g in P1 cheeses and ca. 2 log cfu/g for P2 and P3 cheeses, comparatively to control cheeses ($Ch_C$). *Enterobacteriaceae* and *Pseudomonas* spp. counts showed > 5 and > 4 log cycle reductions, respectively, to numbers below the quantification limit during storage. The proteolysis indices were, in general, lower in HPP cheeses in comparison to $Ch_C$. P1 cheeses kept the ripening extension index (27-30%) along the 15 months of storage closer to that reported for $Ch_C$ (29%) at 45 days of ripening, retaining the characteristic texture, yet being considered harder than $Ch_C$ from a sensorial point of view. HPP did not cause major changes in *Serra da Estrela* cheese lipid profile in terms of total triglycerides content (65-66g TG/100 g), esterified and non-esterified fatty acids and similar atherogenicity and thrombogenicity indices (~2.3 and ~2.6, respectively) were obtained; a high total conjugated linoleic acid content (1.29-1.65 g FA/100g fat) was quantified in all cheeses along storage. Given the whitening of cheese surface colour as a consequence of its vacuum packaging, performed in order to apply HPP, a complimentary study was implemented to comparatively assess the impact of two packaging systems: paper wrapping package without vacuum (P) and packaging in plastic film under vacuum (V), on the quality of *Serra da Estrela* cheeses previously pasteurized by HPP at 525MPa/6 min. Control ($Ch_C$; untreated) and HPP treated ($Ch_P$) cheeses packed in either of the two systems (i.e. $Ch_C$+V; $Ch_C$+P; $Ch_P$+V and $Ch_P$+P) were stored for 10 months under refrigerated conditions. $Ch_P$+V and $Ch_P$+P

cheeses carried lactococci, lactobacilli, enterococci and total viable bacteria at lower viable cell numbers than $Ch_C$+V and $Ch_C$+P cheeses i.e. between 4 and 6 log cfu/g vs approx. 8 log cfu/g, respectively. Yeasts and moulds proliferated (> 5 log cfu/g) in paper wrapped cheeses. $Ch_P$+V cheeses maintained a stable ripening extension index throughout storage, with values close to those reported for $Ch_C$+V cheeses at 0 months. The non-vacuum paper wrapping was shown adequate for short storage periods (< 3 months), but for long periods the vacuum-packaging in plastic film method is preferable. The application of HPP to raw ewes' milk as a pre-treatment prior to cheesemaking could prompt an increment in cheese yield and contribute to a better quality standardization. Hence, a broad screening experimental design was initially performed (200–400 MPa; 5–30 min; 1–48 h waiting time before HPP; 1–24 h after HPP), and results allowed to pinpoint that the pressure intensity, the holding time under pressure, and the time after HPP were the most important factors. The application of a response surface model (100–300 MPa; 5–30 min, 24 h before and after HPP), targeting a small as possible reduction of lactococci, lactobacilli, and enterococci viable cell numbers, identified as optimum HPP conditions the treatment of milk at 121 MPa/30 min. The model was validated and a scale-up experiment was performed. In such a real cheese production facility, cheese yield increased by 10.4% with HPP pre-treated milk ($M_P$) in comparison to cheese manufactured from control untreated milk ($M_C$). HPP milk pre-treatment led to small, insignificant reductions in microbial viable cell numbers in milk and curd. At 60 days of ripening, half of the cheeses made from either $M_P$ or $M_C$ milks were HPP treated at 525 MPa/6 min ($M_P$+$Ch_P$ and $M_C$+$Ch_P$, respectively) and the other half remained as control ($M_P$+$Ch_C$ and $M_C$+$Ch_C$, respectively). All cheeses were stored under refrigeration for 5 months. HPP of ripened cheeses was determinant upon reduction of the viable cell numbers of the different microbial groups (between 1 and 3 log cycle reductions for lactic acid bacteria, and to below the quantification limit for yeasts and moulds, *Enterobacteriaceae* and *Pseudomonas* sp.). The HPP milk pre-treatment led to an increase of the ripening extension index, while the pressure treatment on cheese kept it constant. The HPP milk pre-treatment improves cheese technological properties, while HPP application to ripened cheeses can increase cheese safety; the coupling of both revealed few quality characteristics being changed and undoubtedly could contribute positively to producers' and consumers' awareness of such an important cheese.

**Keywords:** *Serra da Estrela* cheese, high pressure processing, raw milk, food safety, cheese quality.

# Acknowledgements

I would like to acknowledge the *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* and *Universidade de Aveiro*, for accepting me as Ph.D student and for providing the necessary conditions to carry out this work. Furthermore, I would like to thank to the two dairy facilities from PDO region for providing the cheeses, milk and allowing me to participate in the traditional process of cheesemaking. I would also like to acknowledge Frulact S.A. to through Mentoring Program Comendador Arménio Miranda by Frulact Academy, as well as the financial support from *Fundação para Ciência e Tecnologia* through the Ph.D grant.

The Ph.D was a huge challenge and a process of learning and personal development. It would not have been possible without the help, knowledge and support of many people. I could not imagine writing this thesis without you on my side, especially those mentioned below.

Firstly, I would like to express my gratitude to my supervisors, who accepted me and guided me through this journey. To Professor Ana Gomes, for the continuous support of my Ph.D research, for always saying "yes, and more and more…", for your patience outside the "normal" timetable, injections of motivation, and immense knowledge. To Professor Jorge Saraiva for believing in me, for inviting me to make this journey, for having the heavy task of reading and correcting for the first time the exhaustive reviews/research papers. Thank you to both!

To the Engineer João Madanelo thank you for helping me to accomplish the work in the dairy, for giving me so much knowledge that is nowhere to be found. To Engineer Maria João Monteiro, the sensorial leader, thank you for helping me with sensorial training, creating test questionnaires and analysing data. To Professor Rui Barros, the real statistical doctor. To Professor José Lopes-da-Silva for his fundamental aid in textural analysis. To Luís Alcalá and Lígia who gave me knowledges concerning lipid profiles. To Joel Pereira who kindly simplified me the volatile profile study.

To the Innovate group, Liliana Fidalgo, Rui Queirós, Mauro Santos, Carlos Pinto Diana Isa, Silvia Moreira, Catarina Ribeiro, Ricardo Duarte and Álvaro Lemos for all the support and the sacrilegious coffee break, fun and joy to recover energies… and of course by all the high pressure processing cycles. To ESB colleagues on the 4$^{th}$ floor, for the support and provide a good working environment. Thank you my sensorial panel, Professor Cristina Freitas, Ana Oliveira, Anita Pintado, Débora Campos, Fátima Coelho, Filomena Raposo, Joana Odila, José Soares, Marta Coelho, Pedro Castro, Susana Teixeira and Telma Delgado.

Thank you, Jaqueline Rocha, much more than a train travel companion among the endless travels between Aveiro and Porto. Thank you too my oldest friends, Francisco Nunes, Ricardo Figueiredo, Joana Barbosa, Joana Cabral and Joana Silva, who never asked for Ph.D-related deadlines.

Thank you to my family, especially to my parents. They are my base, my foundation, my support for everything. Finally, the first to suffer when things did not go as intended, my beloved Diogo, thank you for always believing in me and motivating me. Thank you to my dog Kiko, the best writing compassion.

I am sorry if I forgot anyone, but the journey was long…

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# Scope and outline

*Serra da Estrela* Cheese is a popular Portuguese traditional cheese with Protected Designation of Origin (PDO) manufactured from raw ewes' milk. Raw milk cheeses may be contaminated by food spoilage or eventually pathogenic microorganisms. High-pressure processing (HPP) has been increasingly applied for cold pasteurisation, mainly considering its capacity for producing microbiologically safe products, with additional nutritional and textural advantages for consumers and food processors over thermal processing. The application of HPP to raw milk cheeses may create an opportunity to produce a microbiologically safe and stable *Serra da Estrela* cheese leading to an extended shelf-life.

This Ph.D. research work has accordingly focused on the systematic elucidation of the multiplicity of effects of HPP on the microbial load and quality, as well as on the overall physicochemical composition, proteolysis, lipolysis, volatile compounds, texture profile and sensory attributes, upon treatment and throughout extended storage, of *Serra da Estrela* cheese - in attempts to evaluate HPP's ultimate role and impact upon cheese safety and organoleptic quality. Once the complexity of relationships were established between HPP and cheese parameters and associated stability, a second major objective was explored to test whether the application of HPP to pre-treat the milk used in *Serra da Estrela* cheese manufacture could further enhance microbial safety without compromising their unique texture and flavour attributes. *Serra da Estrela* cheese produced form ewe's HPP pre-treated milk was further treated by HPP before extended storage – the impact on microbiological, physicochemical, textural and sensory parameters was assessed.

Based on the above rationale, the following main objectives were established:

(i) To study the feasibility of HPP to pasteurize *Serra da Estrela* cheese (produced using raw milk) in order to achieve the best inactivation of potential pathogenic and spoilage microorganisms, while maintaining, as much as possible, the numbers and activity of the beneficial microflora and unique quality of the cheese;

(ii) To determine the best HPP conditions to be applied to milk as a pre-treatment for subsequent *Serra da Estrela* cheese production, envisaging cheese yield increment;

(iii) To study the impact of HPP pre-treated milk, using the optimum conditions obtained in (ii), on the overall quality of *Serra da Estrela* cheese throughout storage;

(iv) To study the effects of a double application of HPP –firstly, as pre-treatment of milk to be used in the manufacture of *Serra da Estrela* cheese and secondly to ripened cheese – on overall cheese quality during storage.

Taking into account the above considerations, this doctoral thesis is composed of four main parts, comprising eleven chapters in total, which interact logically with one another, and are closely related to the general objectives of this research work. The different chapters, distributed between two on state-of-the-art, seven related with research and two presenting major conclusions and future perspectives, are organized in a chronological manner, thus describing how the research work plan and design evolved throughout time.

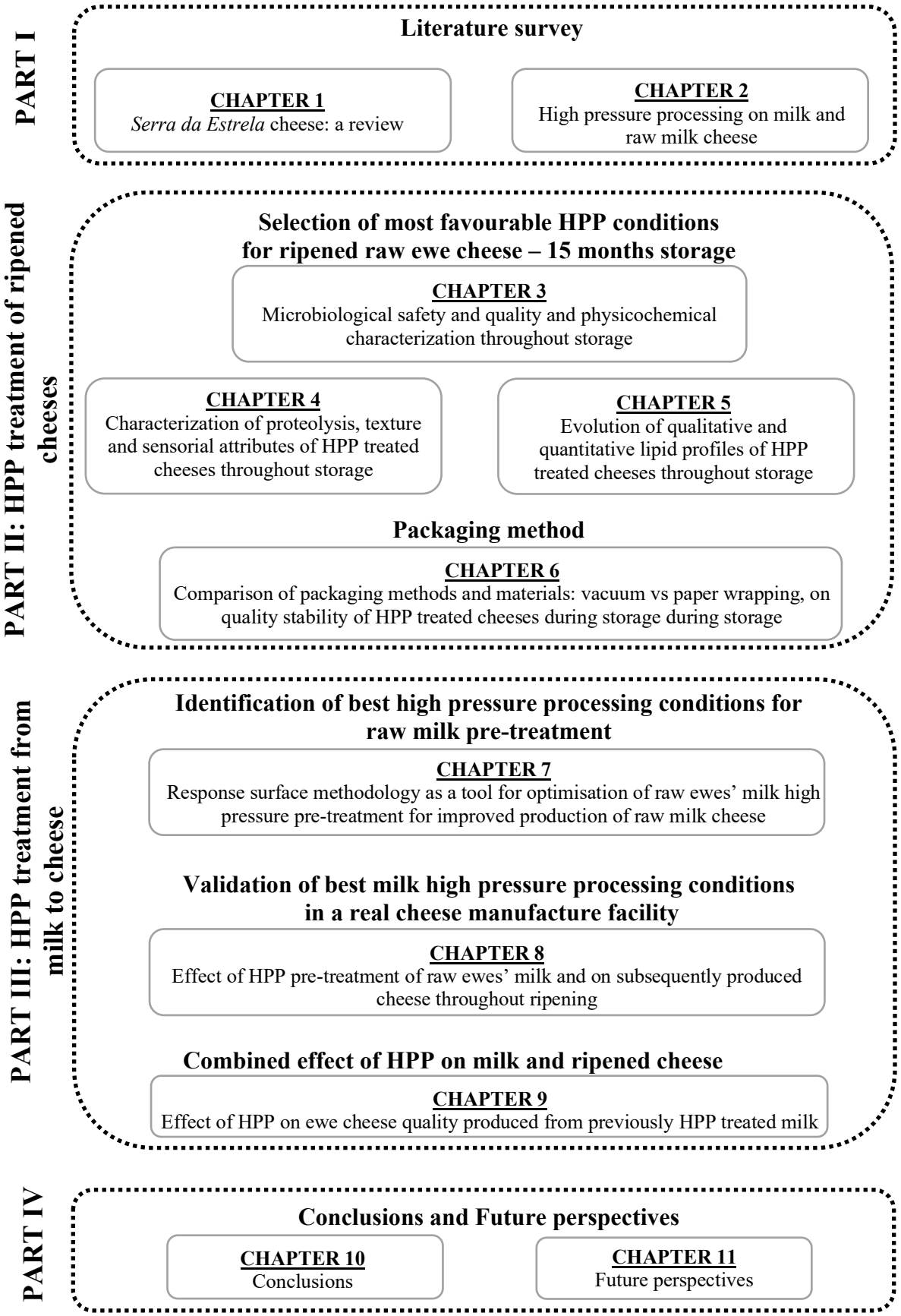
Part I includes Chapters 1 and 2, with Chapter 1 presenting a general updated literature review concerning *Serra da Estrela* cheese manufacture, composition, microbial profile, biochemical changes and possible innovation strategies to improve microbial safety, while in Chapter 2 HPP technology is presented, as a cold pasteurization procedure, providing also an overview of its application in raw milk cheeses (impact on natural microbiota and on cheese quality). Chapter 2 further presents a compilation of studies on the effect of HPP on milk, since HPP pre-treatment applied to milk for subsequent cheese production was also targeted in this thesis.

Part II includes Chapters 3, 4, 5 and 6, and is related with the reporting of results on *Serra da Estrela* cheese manufactured by commercially used conditions, submitted to HPP at the end of 45 days ripening stage. Cheese overall characterization, in order to define the most promising HPP conditions that could allow to increase the microbial safety of ripened raw milk ewes' cheese, with minimal impact on cheese characteristics, after HPP and during refrigerated storage, is covered in Chapters 3, 4 and 5. Specifically, Chapter 3 describes the effects of HPP on microbial and physicochemical changes, and the evolution of these parameters during refrigerated storage up to 15 months of storage,

while in Chapter 4, the effects of HPP on proteolysis, texture and sensorial characteristics during the same extended storage period are described and in Chapter 5 the impact that HPP has on the lipids profile throughout storage is reported. The results presented in Chapters 3 - 5, were obtained with cheeses packaged under vacuum and since it was observed that the cheeses became whiter over storage time, it was decided to explore alternative packaging conditions, to try avoid/minimize such unwanted effect. In this way, Chapter 6 describes the results obtained for HPP treated *Serra da Estrela* cheeses, which were stored under two packaging systems: paper wrapping package without vacuum and packaging in plastic film under vacuum for 10 months.

Part III presents the effects of HPP pre-treatment of milk for cheese production, and includes Chapters 7, 8, and 9. Chapter 7 contains the results of an experimental design to evaluate the effect of high-pressure processing on raw ewe´s milk (at microbial level and technological aptitude for subsequent cheese production), establishing as major goal the identification of the best HPP conditions to be applied in milk, enabling the most favourable trade-off between the best inactivation level of pejorative bacteria, with the lowest reduction in beneficial microbiota, and additionally with good cheese yield. Chapter 8 describes the evaluation of the effect of HPP pre-treatment of raw ewe milk on cheese, by validating the previously identified best predicted HPP conditions for milk treatment in Chapter 7 in a real dairy production facility. In this way, Chapter 8 presents the characterization of the pre-treated milk, the curd, the whey and the cheeses produced therefrom. Chapter 9 evaluates the combined effect of the best HPP condition obtained in Part II for cheese pasteurization and that identified and validated in Chapters 7 and 8, respectively, for pre-treatment of milk. Chapter 9 presents results for the effect of HPP on cheese pasteurized by HPP, produced from HPP pre-treated milk, throughout storage. Part IV is the final section of the thesis and includes the major conclusions from this research work (Chapter 10), as well as, future perspectives that may be explored (Chapter 11).

The following diagram provides a schematic outline of the arrangement of this thesis, as discussed in detail above.




General schematic flow diagram of the work performed in this Ph.D thesis.

The information presented in nine of the eleven chapters that constitute this dissertation, has either a been submitted to international peer reviewing and subsequently published in international scientific journals or as a book chapter, or is in the process of international peer reviewing, or being finalised for submission to international peer review – according to the following list:

**Chapter 1:**

Rita S. Inácio, Ana P. Gomes, Jorge A. Saraiva (2019). *Serra da Estrela Cheese: a review*. Submitted to Journal of Food Processing and Preservation. Under revision.

**Chapter 2:**

Mauro D. Santos, Rita S. Inácio, Liliana G. Fidalgo, Rui P. Queirós, Sílvia A. Moreira, Ricardo V. Duarte, Ana P. Gomes, Ivonne Delgadillo and Jorge A. Saraiva (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.

**Chapter 3:**

Rita S. Inácio, Ana P. Gomes and Jorge A. Saraiva (2018). *High pressure processing of raw ewe milk cheese promotes microbiological safety and quality during prolonged storage.* Submitted to LWT – Food Science and Technology.

**Chapter 4:**

Rita S. Inácio, Maria J. P. Monteiro, Jorge A. Saraiva and Ana P. Gomes (2019). *Characterization of proteolysis, texture and sensorial attributes of HPP treated cheeses throughout storage.* Submitted to Food Research International.

**Chapter 5:**

Rita S. Inácio, Luís Alcalá, Lígia Pimentel, Jorge A. Saraiva and Ana P. Gomes (2019).

*Evolution of qualitative and quantitative lipid profiles of HPP treated cheeses throughout storage.* Submitted to Journal of Agricultural and Food Chemistry.

**Chapter 6:**

Rita S. Inácio, Maria J. P. Monteiro, Ana P. Gomes and Jorge A. Saraiva (2019). *Comparison of packaging methods and materials on quality stability of high pressure treated cheeses during storage*. Submitted to International Dairy Journal.

**Chapter 7:**

Rita S. Inácio, Rui Barros, Jorge A. Saraiva and Ana P. Gomes (2019). *Optimisation of raw ewes' milk high pressure pre-treatment for improved production of raw milk cheese.* Submitted to Innovative Food Science and Emerging Technologies.

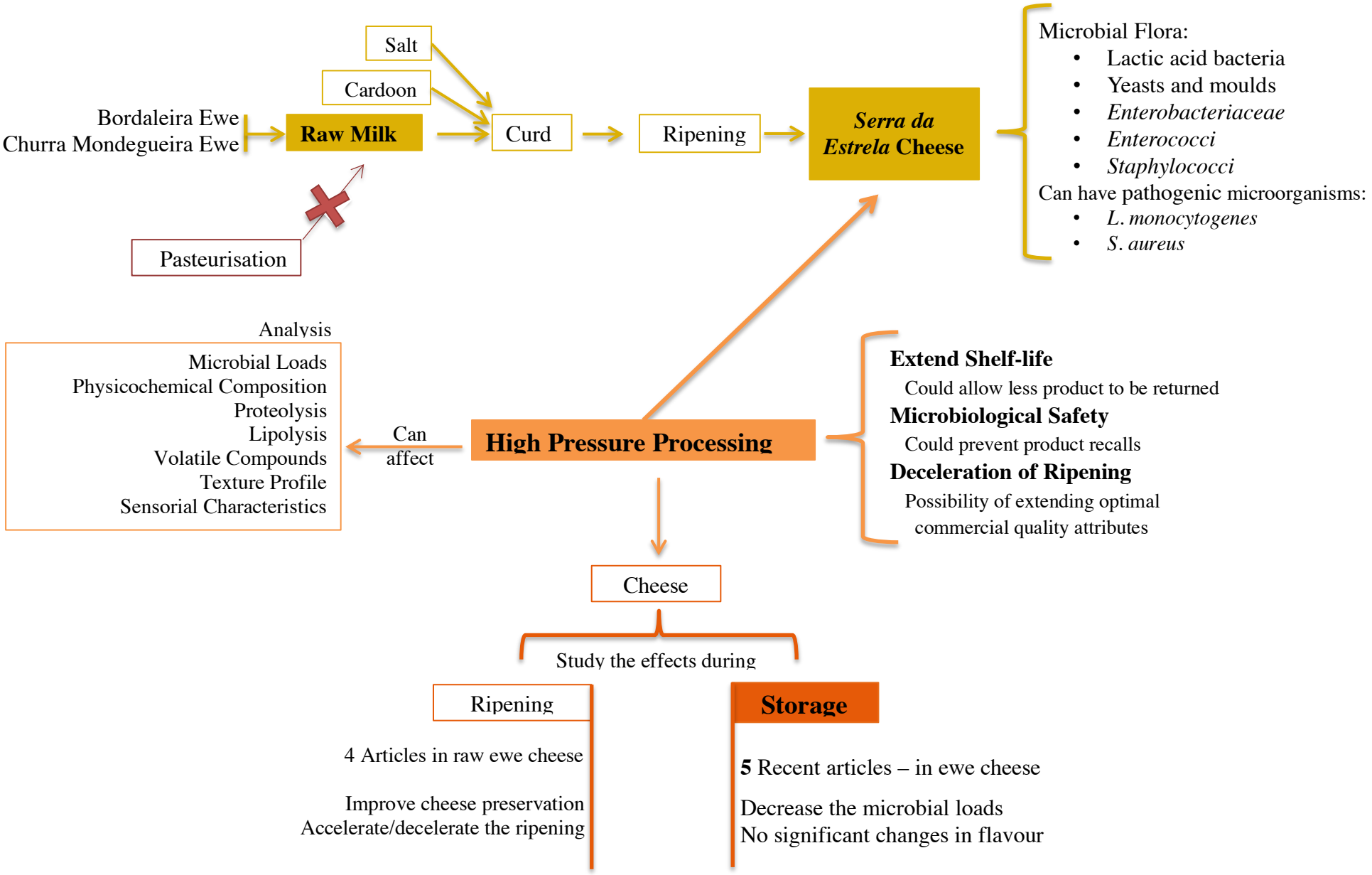
**Chapter 8:**

Rita S. Inácio, Carlos Pinto, Ana P. Gomes and Jorge A. Saraiva (2019). *Effect of high pressure pre-treatment on raw ewes' milk and on subsequently produced cheese throughout ripening.* Submitted to Journal of Food Science and Agriculture.

**Chapter 9:**

Rita S. Inácio, Maria J. P. Monteiro, Joel Oliveira, Maria Fátima Poças, Jorge A. Saraiva and Ana P. Gomes (2019). *Effect of high pressure processing on ewe cheese quality produced from previously high pressure processing treated milk.* Submitted to Food Chemistry.

# PART I – Literature survey




General schematic flow diagram of the work carried in Part I.

# CHAPTER 1 - *Serra da Estrela* cheese: a review

Rita S. Inácio [a,b], Ana M. P. Gomes [a] and Jorge A. Saraiva [b]

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*This chapter has been submitted for publication.*

## Abstract

*Serra da Estrela* cheese with Protected Designation of Origin certification is manufactured using only raw ewes' milk, salt and a crude plant rennet, from the dried flowers of *Cynara cardunculus* L., resulting in a cheese with peculiar sensorial features (without addition of starters or additives) that has existed over many centuries. More than forty scientific reports concerning this cheese have been published since 1992. The composition and the microbial profile have revealed certain variations among the literature. During ripening of *Serra da Estrela* cheese biochemical changes occur, which are reflected in its particular flavour and texture characteristics. An intense proteolytic activity takes place along ripening and storage, and volatiles are associated to typical flavour like "butter-cream texture". Innovation concerning *Serra da Estrela* cheese has been performed within milk quality, manufacture process and in ripened cheese.

## 1.1 Introduction

> *"Product obtained by slow draining of the curd, after coagulation of the raw pure ewe's milk, obtained from milking females of Bordaleira Serra da Estrela or Churra Mondegueira breed and the use of thistle."*(Planning and Political Office, 2011)

The previous citation is the definition of the *Serra da Estrela* cheese (Planning and Political Office, 2011). *Serra da Estrela* cheese is probably the best representative artisanal Portuguese cheese, with Protected Designation of Origin (PDO) certification (since 1985), manufactured using raw milk, salt and a crude plant rennet, from the dried flowers of *Cynara cardunculus* L. (Macedo *et al.* 1993), resulting in a cheese with a closed, moderately buttery, deformable when cutting, well connected, creamy and unctuous texture, with few or no eyes and sensorially smooth, clean and slightly acidic *bouquet* (Planning and Political Office, 2011), having these cheese features been published in the Portuguese Law (Dec. Reg. No. 42/85 of July 5$^{th}$, 1985). These cheese characteristics are related with the use of raw milk, imparting to the cheese several microorganisms, a native microbiota that plays an important role during cheese ripening (at least 30 days) (Macedo *et al.*, 1997). It has been produced for centuries in the interior of Portugal, in the *Serra da Estrela* region, from October to May (the typical lactation period of *Bordaleira Serra da Estrela/Churra Mondegueira* ewes) using unpasteurized milk immediately after collection (Macedo *et al*., 1995, 1996a; Planning and Political Office, 2011). In 2011, *Serra da Estrela* cheese was first place in the appetizer category, having been considered as one of the seven wonders of Portuguese gastronomy, due to its unique organoleptic characteristics. All these characteristics result in high commercial values +US$30/kg or €20/Kg. The production of *Serra da Estrela* cheese PDO has been around 120 thousand kilograms per year. Notable, these production quantities are related only to certified cheese that corresponds only to 10% of total *Serra da Estrela* cheese produced. The

scientific research on this cheese has been ongoing for decades, focusing all production and ripening stages, and continues to be active, as can be read in this chapter.

## 1.2 *Serra da Estrela* cheese manufacture

The manufacturing techniques used and the environmental conditions lead to cheeses with different characteristics (Guiné *et al.*, 2016; Macedo *et al.*, 1996a; Tavaria and Malcata, 2000). This traditional Portuguese cheese is made with only three ingredients: milk from *Bordaleira Serra da Estrela* and/or *Churra Mondegueira* ewes breeds, salt and a *Cynara cardunculus*, L. extract as rennet (Planning and Political Office, 2011), as illustrated in Figure 1.1. The detailed manufacture process was fully reviewed by Macedo *et al.*, (1993). Briefly, it is manufactured from raw ewes' milk, which is simply filtered through a clean, fine and white cloth to remove impurities like hair and dust. Milk coagulation is promoted by the addition of an aqueous extract of *C. cardunculus* L. thistle flower (cardoon), without the addition of any commercial starter culture (Tavaria *et al.*, 2006). The technological optimization of the *Serra da Estrela* cheese manufacturing process was performed by Macedo and Malcata (1997e), who identified the optimum conditions to obtain the best quality cheese as being 0.3 g/L of cardoon plant extract, 28º C for milk coagulation temperature and 0.05 g of salt per $cm^2$ of fresh cheese surface. Some cheese producers prefer to add the salt (20 g/L) at the beginning of the milk heating step, together with the rehydrated dry cardoon flower (0.15 - 0.3 g/L). In what concerns the rennet addition step some producers prefer to grind the coagulant, others prefer crushing it with salt using a mortar and pestle, and the coagulant is then put inside a cloth and milk is poured over it or in the cloth with closed ends (called "boneca"), followed by submersion in the milk, agitation, and squeezing. The coagulation time is approximately 45 – 60 min, after which it is manually stirred and cut (curd cutting). Portions of irregular shape and size curd are wrapped in white cloth, and they are pressed with both open hands in order to drain off the

whey. The drained curd is then placed into the mold and manually pressed to release as much whey as possible (molding). Pressing by stones, pressure springs or more recently pneumatic pressure were used to press the curd in the molds, step that took place for about 2:30 - 3 h at room temperature, being applied 1.5-2 Kg (per cheese when stones were used) or approximately 2 bar of pressure (pneumatic pressure), according to temperature and curd characteristics. After pressing, cheeses are removed from the molds, externally salted (0.5 to 0.9 g/cm$^2$ by some cheese producers, being the total salt added between 10 - 30 gr/L) and then fresh cheeses are embalmed with cloths to avoid deformation and left to ripen.

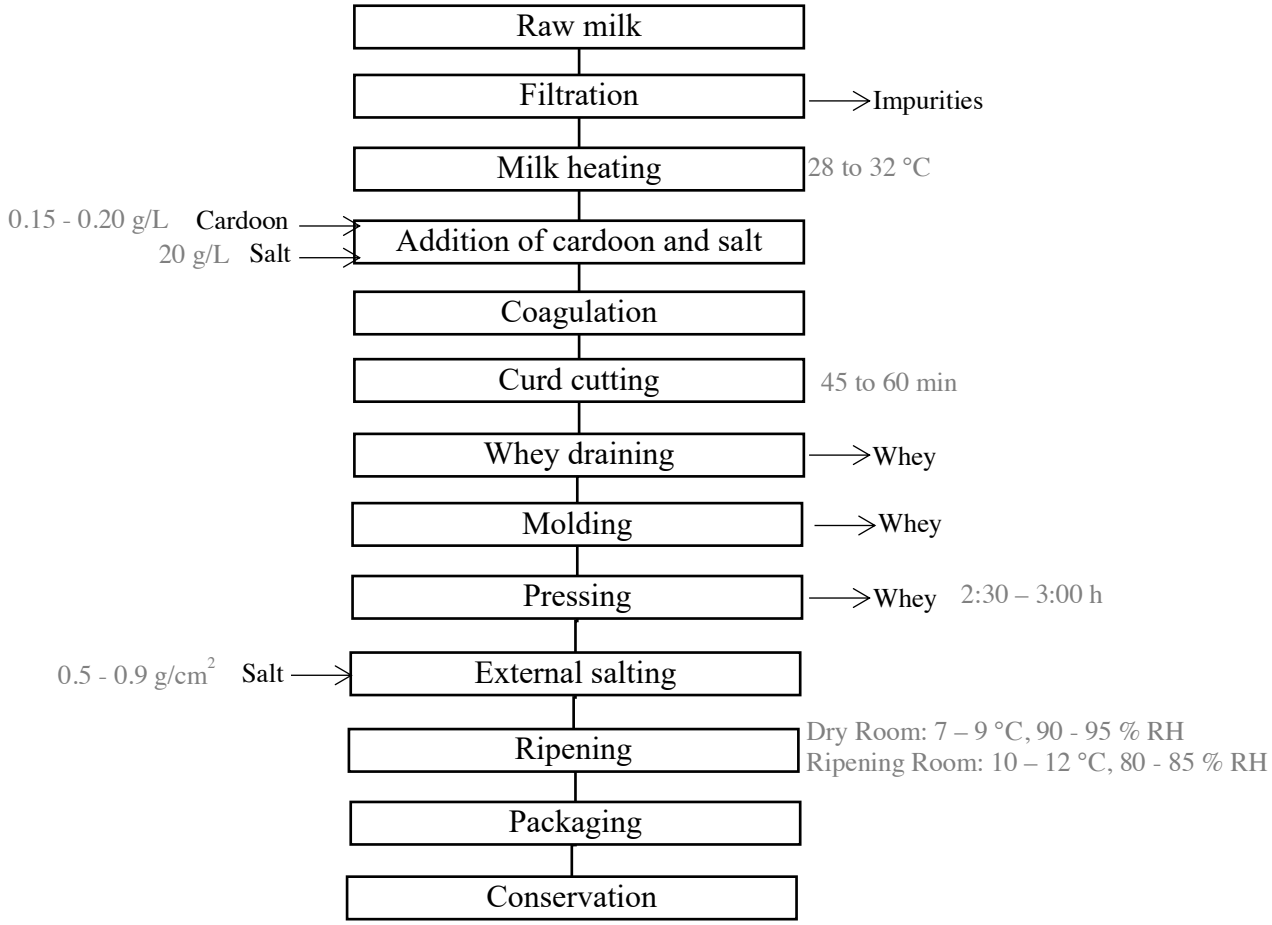


**Figure 1.1: Flowchart for *Serra da Estrela* cheese manufacture.**

The ripening process of *Serra da Estrela* cheese occurs in two controlled environmental chambers. The first is the dry chamber (*enxugo*), at 7 to 9 ºC and 90 to 95 % of relative humidity (RH), where lactic fermentation starts and simultaneously the *reima* occurs (Planning and Political Office, 2011). *Reima* is a white-reddish viscous smear, which is important to obtain a good cheese (Macedo *et al.*, 1993). In this phase, cheese loses humidity and allows for microbial growth favourable to ripening. The second chamber is the ripening chamber itself, at 10 to 12 ºC and 80 to 85 % RH. The ripening period depends on the type of cheese desired, buttery *Serra da Estrela* cheese ranging from 30 to 45 days (Macedo *et al.*, 1993) and old *Serra da Estrela* cheese needing a minimum period of 120 days. The main steps involved in the manufacture of *Serra da Estrela* cheese are summarized in Figure 1.1.

## 1.3 *Serra da Estrela* cheese composition

There are several studies that reveal the chemical composition of ripened *Serra da Estrela* cheese, but they show some differences, as shown in Table 1.1. This variation can be due to the different milk composition, the lack of standardized procedures for the manufacture (thistle ecotype, molding, pressing and salting operations), the geographical location and the different ripening times of the analysed cheeses. There is still an absence of raw milk standardization but, in fact, such is not possible because milk is collected from different geographical zones (among PDO region) where grazing conditions (hence nutritional components) to which ewes are exposed are different, from ewes at different lactation stages, and during different seasons and years, which reflect the different weather conditions. These factors influence the milk composition and subsequently the cheese composition.

The cheese chemical composition changes during the ripening process, being highly variable, as can be seen in Table 1.1. Among the different research studies, the moisture content

of ripened *Serra da Estrela* cheese varied between 34.9 and 49.8 %, however the Portuguese law considers the content to be between 61 and 69% based on cheese free of fat content (Ministry of Agriculture, 1985). In all studies, and as expected, the moisture content decreased during ripening (Guiné *et al.*, 2016; Macedo *et al.*, 2004; Sousa and Malcata, 1997), being affected by relative humidity conditions; the ripening conditions control the rate of water evaporation (Macedo *et al.*, 1997). A higher variation for fat content was observed, where results fluctuated from 25.15 to 53.57 %; in the meantime the law reports fat content to be between 45 and 60 % in dry residue (Ministry of Agriculture, 1985). As far as protein content is concerned mean values varied between 12.97 and 28.77 %; by law protein content should be between 26 – 33 % in dry residue (Ministry of Agriculture, 1985). Several studies reported slight fluctuations in fat and protein contents along ripening, in particular, reductions until 63 - 68 days were reported (Macedo *et al.*, 2004; Sousa and Malcata, 1997) whereas increase in values were reported at 52 and 177 days ripening (Guiné *et al.*, 2016). In the several studies the ash content fluctuated from 3.40 to 5.16 %, values which are lower than those required by law, namely, 5 to 6.5 % in dry residue (Ministry of Agriculture, 1985). During ripening the ash content increased (Guiné *et al.*, 2016; Macedo and Malcata, 1997b), being Na, Ca, P, K and Mg the main minerals found in cheese; Zn, Cu and Mg were only present at trace levels (Macedo and Malcata, 1997b). The salt content showed mean values between 1.10 and 3.05 %. The salt content in the centre and in the cheese surface change during ripening. Moreover, in the first 7 days, the salt content increases due to the diffusion of dry salt from the surface into the centre of the cheese (manufacturing step used by some producers), and also probably due to water evaporation from the surface of the cheese along with the decrease of the moisture content (Macedo and Malcata, 1997c; Sousa and Malcata, 1997). After 35 days of ripening, the average salt-in-moisture content is maintained during the ripening time (Macedo and Malcata, 1997c).

**Table 1.1: Chemical composition of *Serra da Estrela* cheese reported by several authors.**

| Moisture (%) | Fat (%) | Protein (%) | Ash (%) | Salt (%) | Ripening time (d) | Reference |
|---|---|---|---|---|---|---|
| 34.89 to 49.80 | 35.00 to 52.00 | 12.97 to 21.90 | 3.40 to 5.16 | 1.10 to 2.99 | 52 - 177 | Guiné *et al.*, (2016) |
| 43 ± 7 | 26 ± 2 | 28 to 33 ± 3 | 4 ± 1 | NA[#] | 30 | Carocho, *et al.*, (2016) |
| 48.43 ± 0.36 | 27.62 ± 1.99 | 28.77± 3 | NA | 1.41 ± 0.10 | 42 | Macedo *et al.*, (2004) |
| 45.41 ± 1.55 | 25.15 ± 2.33 | 22.10 ± 0.39 | NA | 3.05 ± 0.15 | 60 | Macedo and Malcata, (1997a) |
| 46.7 to 48.8 | 28.1 to 30.7 | 19.2 to 20.4 | 4.1 to 4.3 | 2.2 to 2.6 | NA | Barbosa (1990) |
| 48.8 | 28.8 | 19.9 | 4.4 | 2.6 | NA | Barbosa (1986) |
| 61 – 69* | 45 – 60* | 26 – 33* | 5 – 6.5 | NA[#] | >30 | Dec. Reg. No. 42/85 of July 5$^{th}$ (1985) |

[#]NA means not available; *Portuguese law expresses the moisture content on cheese free of fat content, and protein, fat and ash content in dry residue.

## 1.4 Microbial profile of *Serra da Estrela* cheese

Being a raw ewes' milk cheese with no added starter, *Serra da Estrela* cheese contains the native milk microbiota and other adventitious microbiota (Parker *et al.*, 1998), both of which play an important role during cheese ripening. There are 5 articles published between 1993 and 2003 (Dahl *et al*., 2000; Macedo *et al*., 1995, 1996a, 2004; Tavaria and Malcata, 2000) on the microbial profile of *Serra da Estrela* cheese during ripening, as shown in Table 1.2.

**Table 1.2: Summary of the different available studies in the literature on the microbial profile of *Serra da Estrela* cheese during ripening.**

| Publication | Study Date | Relevance |
|---|---|---|
| Macedo *et al.*, (2004) | 02/03 | Microbiological profile over 63 ripening days |
| Dahl *et al.*, (2000) | 98/99 | Microbiological profile between 60 and 180 ripening days |
| Tavaria and Malcata (2000) | 96/97 | Microbial profile over 60 days per dairy farms in 1996 and 1997 |
| Macedo *et al.*, (1996a) | 95/96 | Microbial profile over 35 days in October-November, January-February and May-June |
| Macedo *et al.*, (1995) | 93/94 | Microbial profile over 35 days ripening in Spring, autumn and winter |

Once again, results are not consistent and large differences in the counts of some microbial groups are observed (Figure 1.2).

Lactic acid bacteria (LAB) counts are the major fraction of total microbiota during ripening (Dahl *et al.*, 2000). Within the LAB microbial group, the lactococci and lactobacilli counts were studied, in particular (Macedo *et al.*, 1995). According to Figure 1.2 A, and independently of the research study, the viable cell numbers of lactobacilli increased $10^3$-fold over the first week, and 10-fold more in the second week. These values tended to remain stable for one week, decreasing slightly afterwards, but still present within $10^6$ to $10^7$ cfu/g at the end of the ripening period (Dahl *et al.*, 2000; Macedo *et al.*, 2004). This constancy is due to lactobacilli's tolerance to dehydrated environments and high salt concentrations. Analysis of

Figure 1.2 also reveals that lactococci are the most representative group among *Serra da Estrela* cheese microbiota, present at high values in all studies and throughout the ripening period (Figure 1.2 B). The quantitative profile of this group of microorganisms was similar in all publications (Dahl *et al*., 2000; Macedo *et al*., 2004, 1996a; Tavaria and Malcata, 2000) except that of Macedo *et al.*, (1995) where viable cell numbers were, in general 3 log cycles less. Similarly to the lactobacilli group, viable cell numbers increase 3 log cycles during early stages of ripening and remain up to two months at $10^8$ cfu/g. A slight decrease is observed by 4 months ripening but numbers remain relatively high between $10^7$-$10^8$ cfu/g until the end of the ripening period (Macedo *et al.*, 2004; Tavaria and Malcata, 2000). Enterococci were counted only in three publications (Dahl *et al.*, 2000; Macedo *et al.*, 2004; Tavaria and Malcata, 2000) (Figure 1.2 C) and were found at fairly high numbers from the day of manufacture ($10^7$ cfu/g) until the end of the 120 or 180 days ripening period. The most abundant LAB found in curd were *Lactococcus lactis* spp. *lactis* (Macedo *et al.*, 1995), which is homofermentative (Hui *et al.*, 2006) and *Enterococcus faecium* (Macedo *et al.*, 1995). The lactation period has a significant effect on the total number of microorganisms, which is higher in January to February and lower from May to June (Macedo *et al.*, 1996a), indicating that lower temperature and higher relative humidity during autumn and winter, favours LAB growth (Macedo *et al.*, 1995).

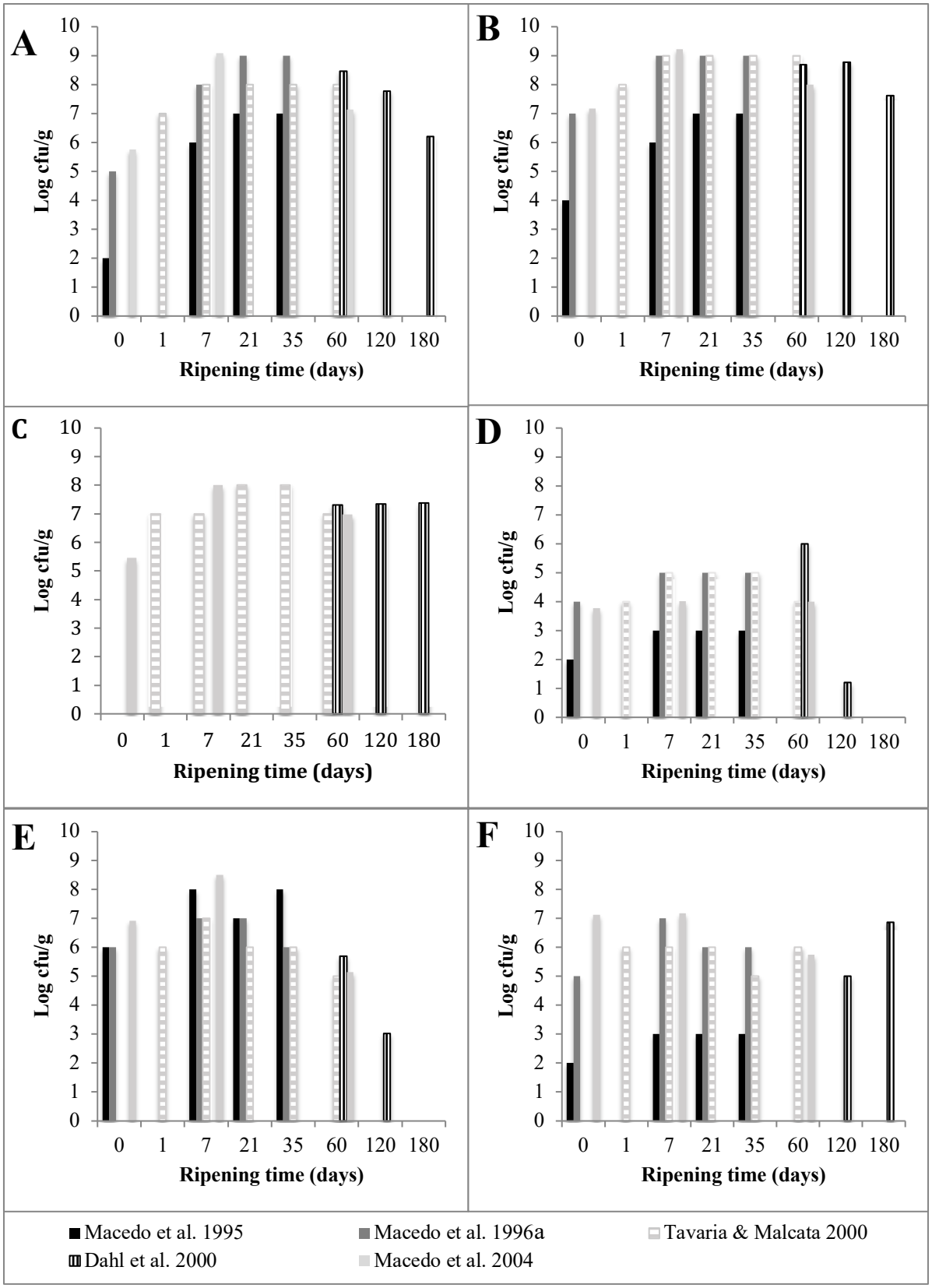


**Figure 1.2: Average of viable cell numbers (log cfu/g) of (A) lactobacilli, (B) lactococci, (C) enterococci (D) yeasts, (E) coliforms and *Enterobacteriaceae*, and (F) staphylococci during ripening of *Serra da Estrela* cheese, reported by several authors.**

Inside the cheese matrix, yeasts tend to slowly increase in the first 7 days, from $10^4$ to $10^5$ cfu/g, and then remain fairly stable (Figure 1.2 D). Between 60 and 180 days of ripening, a sharp decrease (about 4 log) was observed in viable numbers of yeasts, which became undetectable (below the detection limit) at 180 days (Dahl *et al.*, 2000). This highlights that the highest death rate of yeasts occurs upon 60 days of ripening (Dahl *et al.*, 2000). Rind samples showed viable cell numbers 3 logs higher than inner cheese matrix samples, possibly due to the fact that the rind is more exposed to environmental manufacturing conditions, so it can be easily contaminated (Macedo *et al.*, 1996a). The predominant yeasts in 35-days ripened cheese were *Leucosporidium scottail, Debaryomyces hansenii* and *Sporobolomyces roseus* (Macedo *et al.*, 1995).

The quantification of *Enterobacteriaceae,* coliforms and staphylococci is used as an indicator of the hygienic conditions under which a food product is made. Staphylococci were mainly found in the cheese rind as compared to the cheese inner matrix, because they require oxygen to survive and grow. Moreover, the rind is more easily contaminated due to manual washing during the ripening stage. Their bacterial counts did not decrease throughout ripening, registering viable cell numbers around $10^6$ cfu/g (Dahl *et al*., 2000; Macedo *et al*., 2004, 1996a; Tavaria and Malcata, 2000); nevertheless, Macedo *et al.*, (1995) reported viable cell numbers for this microbial group 3 log cycles lower Figure 1.2 E and F). These results do support the possibility of health hazards associated with *Serra da Estrela* cheese consumption specially when it was reported than 14% of the counted staphylococci belonged to the *S. aureus* spp., which are clinically relevant strains (Tavaria and Malcata, 1998). All authors, reported viable cell numbers for LAB, coliforms (Macedo *et al*., 1996a, 1995) and *Enterobacteriaceae* (Dahl *et al.*, 2000; Macedo *et al.*, 2004; Tavaria and Malcata, 2000) within the same order of magnitude ($10^6$ to $10^8$ cfu/g), except for Tavaria and Malcata (2000) who verified slightly lower viable cell numbers of about 1-2 log cycles. These results may be interpreted as a reflection of

poor sanitary conditions during milk collection and/or cheese manufacture at the time of sampling; interestingly, such conditions seemed to be more accentuated in 1995 (Macedo *et al.*, 1995) than in 2000 (Tavaria and Malcata, 2000), which may reflect the steady improvement of hygienic conditions over the years. The most abundant and proliferative coliform found in the curd was *Hafnia alvei* (Macedo and Malcata, 1997a), which is a psychrotrophic bacterium. *Escherichia coli* was found in all studied cheeses that had been ripened over winter of 1993/1994 (Macedo *et al.*, 1995). *Serra da Estrela* cheese is only consumed after 30-45 days, the minimum ripening time required for viable cell numbers of microbial contaminants to decrease to acceptable values, as discussed above. Indeed, Dahl *et al*., (2000) showed in their study that, after 120 days of ripening, the viable cell numbers of yeasts, *Enterobacteriaceae* and staphylococci suffered a pronounced decrease to almost non-detectable levels, making *Serra da Estrela* cheese a microbiologically safe product thereafter (Dahl *et al.*, 2000).

In summary, differences reported among microbial counts within the different studies are related with several factors, including the ripening time, the geographical location/producers (Tavaria and Malcata, 2000), the cheese manufacture from refrigerated or non-refrigerated milk (Dahl *et al.*, 2000) and the lactation season period (Macedo *et al*., 1995, 1996a).

## 1.5 Biochemical changes

### 1.5.1 Proteolysis

Proteolysis has been considered the most important complex biochemical process in cheese ripening, because it is responsible for the development of a number of organoleptic features (Fox *et al.*, 1993). The main proteolytic agents in cheese are: the indigenous milk proteinases (plasmin and cathepsin D), the enzymes present in coagulant and the enzymes released upon bacteria lysis (Sousa and Malcata, 1997). Each type of enzyme plays a specific

role, but given the synergism between all types, together they affect the final product more than each one individually (Pereira *et al.*, 2008a). The enzymes that are present in the aqueous extract of cardoon flower induce the clotting activity. Some enzymes are trapped in the curd and lead to protein breakdown during cheese ripening. Therefore, various peptides are released, having important biochemical, rheological and sensorial role in cheese (Planning and Political Office, 2011). From the standard variety of *C. cardunculus* L, two aspartic proteinases, called cardosins A and B, were isolated and characterized (Macedo and Malcata, 1997d), being responsible for the clotting activity of that plant (Verissimo *et al.*, 1995). Cardosin A acts in a similar way to chymosin. Cardosin B acts in a similar way to pepsin, a nonspecific and highly proteolytic enzyme, which can hydrolyse peptide bonds of $\alpha_{s1}$-, $\alpha_{s2}$-, and β-caseins. In general, $\alpha_s$-caseins are more susceptible to proteolysis than β-caseins given their secondary structure, being degraded in approximately 47.0% and 33.1%, respectively, in ewe milk cheese by proteinases of *C. cardunculus* (Sousa and Malcata, 1997). In another study, Macedo and Malcata (1997b) reported 82% and 76% degradation of the $\alpha_s$- and β-caseins, respectively at 35 days of ripening.

Cheese proteolysis can be evaluated by proteolytic indices. After quantification of the total nitrogen content (TN), the nitrogen soluble in water (WSN), the nitrogen soluble in 12% (w/v) trichloroacetic acid (TCA) and the nitrogen soluble in 5% (w/v) phosphotungstic acid (PTA), three proteolytic indices can be calculated: ripening extension index, WSN/TN; ripening depth index, TCA/TN; and free amino acid index, PTA/TN (Pereira *et al.*, 2008a). These indices were calculated for *Serra da Estrela* cheese in four different studies (Macedo *et al.*, 2004; Macedo and Malcata, 1997c; Reis and Malcata, 2011; Tavaria *et al.*, 2003), as can be observed in Table 1.3.

**Table 1.3: Proteolytic indices reported by different authors at different ripening time of *Serra da Estrela* cheese.**

| | WSN/TN | TCA/TN | PTA/TN | Ripening time (days) |
|---|---|---|---|---|
| Reis and Malcata (2011) | 9.62 – 23.33 | 1.48 – 3.71 | 0.56 – 2.60 | 1 - 60 |
| Macedo *et al*., (2004) | 10.74 – 59.17 | 1.51 – 6.77 | 0.71 – 0.74 | 1 - 63 |
| Tavaria *et al*., (2003) | 11 - 43 | 1.8 - 16 | 1 - 12 | 0 - 180 |
| Macedo and Malcata (1997c) | 9.5 - 36.9 | 2.2 - 5.5 | 0.93 - 1.24 | 0 - 35 |

WSN/TN - ripening extension index; TCA/TN - ripening depth index; PTA/TN - free amino acid index; WSN – water-soluble nitrogen; TCA - trichloroacetic acid soluble nitrogen; PTA - phosphotungstic acid soluble nitrogen; TN – total nitrogen.

The WSN/TN ratio has been used to follow the cheese aging process, being proportional to proteolytic activity. *Serra da Estrela* cheese showed an increase of WSN/TN index along ripening, from 9.5 – 11 % at 1 day to 23.33 - 59.17 % at 35-180 days of ripening (Macedo *et al.*, 2004; Macedo and Malcata, 1997c; Reis and Malcata, 2011; Tavaria *et al.*, 2003). These results indicate a strong proteolytic activity coming from the thistle enzymes (Tavaria *et al.*, 2003). Nevertheless, this index varied among dairy farms, refrigerated vs non-refrigerated milk (Tavaria *et al.* 2003) and with the cheesemaking season (Macedo and Malcata, 1997c). Within the same context, the concentration of water-soluble peptides also varied significantly with cheese manufacture location, lactation season and ripening time (Macedo *et al.*, 1996b). The 12%-TCA soluble nitrogen allows quantifying small peptides containing between 2 and 20 amino acid residues and free amino acids (FAA) (Sousa and Malcata, 1997), resulting from secondary proteolysis, brought about by the enzymes produced by the starter cultures and released thereby upon lysis. In *Serra da Estrela* cheese manufacture no starter cultures are added, thus, relatively low levels of TCA/TN are obtained at early ripening stage (Silva and Malcata, 2004). At 35 - 63 days of ripening a TCA/TN index varying between 3.71 and 6.77

% was reported for *Serra da Estrela* cheese (Macedo and Malcata, 1997c; Reis and Malcata, 2011; Tavaria *et al.*, 2003), although Tavaria *et al.*, (2003) calculated a TCA/TN index of 12.52 % at 42 days, which increased to 16 % at 180 days of ripening. The TCA/TN ratio has also been used to evaluate the action of lactic acid bacteria in the production of soluble nitrogen compounds in cheese (Macedo and Malcata, 1997c). In this context, Macedo *et al.*, (2004) showed a significant increase in TCA/TN in *Serra da Estrela* cheeses manufactured with the intentional addition of *L. lactis* or *Lb. plantarum* as starter cultures (strains previously isolated from *Serra da Estrela* cheese). The PTA/TN ratio represents the free amino acid index, i.e. the smallest peptides (that contain less than 6 amino acids residues, with a molecular weight lower than 600 Da) and FAA (Macedo and Malcata, 1997c), that are the final products of proteolysis (Pereira *et al.*, 2008a). In *Serra da Estrela* cheeses, at 35 and 63 days of ripening, this value was particularly low, 1.24% (Macedo and Malcata, 1997c) and 0.74% (Macedo *et al.*, 2004), respectively, which reveals that cardoon has little activity against peptides (Macedo and Malcata, 1997c). On the other hand, Tavaria *et al.*, (2003) showed that this index increased during the ripening up to 12% at 180 days.

The FAA content increased along cheese ripening reaching highest amounts at 180 days of ripening. The main FAA present in significant amounts in *Serra da Estrela* cheese were leucine, valine, proline, and glutamic acid (composing 56-70% of total FAA at 180 days) (Tavaria *et al.*, 2003); notably, a higher amount of FAA was quantified in cheese manufactured from refrigerated milk (exception for valine, glutamic acid and phenylalanine). Moreover, *Serra da Estrela* cheese compared to other cheeses, revealed a higher FAA content, revealing a strong microbial peptidasic activity (Tavaria *et al.*, 2003). Proteinase and peptidase activities were displayed by *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* and *Lactococcus lactis* ssp. *lactis*, lactic acid bacterium strains previously isolated from *Serra da Estrela* cheese with 35 days of ripening (Macedo and Malcata, 1997e).

### 1.5.2 Lipolysis

The extension of lipolysis in *Serra da Estrela* cheese was only evaluated, so far, by Macedo *et al.*, (1996a) who used fat acidity measurement for its assessment. Results revealed that lipolysis changes during ripening time, being more intense in the first week (the study analysed only 35 ripening days), having significant differences among cheese making season. The fatty acid profile was studied by Partidario *et al*., (1998) and Carocho *et al*., (2015 and 2016). Partidário *et al.*, (1998) studied along ripening, verifying an increase of fatty acids concentration from 625.6 to 1294 mg/kg (being registered 214.9 mg/kg in ewe's milk) during the first 21 days. Then, fatty acid content slightly decreased to 1012 mg/kg at 42 days (Partidário *et al.*, 1998). The most abundant fatty acids, in the curd, and in 3 and 6 weeks-old cheese samples, were C16 (124, 272 and 198 mg/kg, respectively) and C18:1 (142.8, 280 and 261.1 mg/kg, respectively). Other studies including those by Carrocho *et al*., (2015; 2016a; 2016b) detected the same FFA as the most abundant individual fatty acids, namely, C16:0 and C18:1, at 23.3 - 25 mg/100 g and 23 - 26.4 mg/100 g, respectively, at 30 and 35 days of ripening. The total percentages revealed 67.0 - 71% of saturated fatty acids (SFA), 24 - 25.5% of monounsaturated fatty acids (MUFA) and 4.8 - 5.4% polyunsaturated fatty acids (PUFA) (Carocho *et al.*, 2016b, 2016a, 2015). These results are in agreement with preferential release of short- and medium-chain fatty acid residues by *Leuonostoc. mesenteroides* ssp. *mesenteroides/ dextranicum,* strain isolated from *Serra da Estrela* cheese at 35 days of ripening that exhibited lipase activity (Macedo and Malcata, 1997e). As far as the ratio between the sum of short volatile fatty acids (C4-C10, value) and the sum of the medium and long chain fatty acids (C11-C20, value), resulting from lipolytic activity is concerned, it was smaller after 42 days of cheese ripening (0.24) than in milk (0.29). These results can be justified by several reasons: firstly, lower temperatures do not favour the lipolytic activity (Partidário *et al.*, 1998) and most cheesemakers kept the cheese under 12 °C (Planning and Political Office, 2011);

secondly, the short ripening period of *Serra da Estrela* cheese (30-45 days) does not allow for a higher lipolytic activity. In addition, *Serra da Estrela* cheese is manufactured only with the addition of plant rennet, which has a low lipolytic activity. Other cheeses ripen during more days and are made with the addition of other types of rennet that reveal higher lipoltytic activity (Partidário *et al.*, 1998).

### 1.5.3 Flavour

Cheese is not only distinguished by its physical features but also by its flavour, which depends on the milk used, the manufacturing methods and the conditions and duration of the ripening phase (Partidário *et al.*, 1998). Cheese manufactured with raw milk acquires more intense flavour than cheese made with pasteurized milk, due to the presence of high levels of metabolically active native lactic acid bacteria (Buchin *et al.*, 1998; Tavaria *et al.*, 2004). The aroma compound and the flavour profiles of *Serra da Estrela* cheese result mainly from the microorganisms and associated enzymes, thus being an enzymatic process with several and interdependent reactions during ripening (Fox *et al.* 1991). However, there is still little knowledge about the reactions that lead to flavour in the *Serra da Estrela* cheese matrix (Dahl *et al.*, 2000). In 2000, Dahl and his colleagues demonstrated that the predominant volatiles in *Serra da Estrela* cheese resulted from the degradation of sugars (mainly lactose), free amino acids (particularly valine and leucine) and lipids (free fatty acids) (Dahl *et al.*, 2000). The major families of volatile compounds in *Serra da Estrela* cheese found by gas chromatography mass spectrometry (GC-MS) analysis were ketones, pyrazines, alcohols, aldehydes, phenolic compounds and ethyl esters and FFA during 180 days of ripening (Tavaria *et al.*, 2004). FFA contribute direct or indirectly (as precursors) to the flavour, being the precursor for the formation of other compounds (Sousa *et al.*, 1997); however FFA did not seem to contribute to “off flavours” in *Serra da Estrela* cheese (Partidário *et al.*, 1998). Organic acids such as

acetic, propionic, iso-butyric, iso-valeric, butyric, valeric, caproic, caprylic and capric were detected below the odour threshold (Dahl *et al.*, 2000; Tavaria *et al.*, 2004). Tavaria *et al.*, (2004) reported the maximum impact of aroma of FFA at 90 days of ripening, being the isovaleric, the capric and the butyric acids those present with the highest values, 232, 113 and 88 odour activity values (OAV), respectively. These same organic acids were also found at high concentrations by Dahl *et al.*, (2000) in cheese at 60 (640, 53, 170 mg/kg, respectively) and 180 days of ripening (230, 45 and 490 mg/kg, respectively). Caproic and caprylic acids were also found at high concentrations (210 and 100mg/kg, respectively) at 180 days of ripening (Dahl *et al.*, 2000). The volatile compounds in *Serra da Estrela* cheese with 42 ripening days, that are present at low concentrations (in the order of ppm), yet of high relevance in the flavour profile are: ethanol (230.9 ppm), methanol (693 ppm), acid acetic (2980 ppm), and 2,3-butanediol (diacetyl, 28 ppm) (Partidário *et al.*, 1998). Acetic acid, that can be produced by deamination of glycine, alanine and serine, was associated with a positive flavour (Partidário *et al.*, 1998). Diacetyl, which could have derived from lactose and citrate metabolisms (Partidário *et al.*, 1998), contributes greatly to the typical flavour, "butter-cream texture" in cheese with soft consistency (Adda *et al.* 1982). Esters and ethanol were associated with fruity flavours (Urbach, 1997). Refrigeration status of the milk was shown to have impact on the quantitative and qualitative profiles of volatile compounds in *Serra da Estrela* cheese (Dahl *et al.*, 2000) and the aroma intensity varied significantly among different producers, but was not significantly influenced by geographical origin or thistle ecotype (Guiné *et al.*, 2016). The correlation between the volatile compounds and the associated microorganisms was duly described by Dahl *et al*., (2000).

## 1.6 Sensorial attributes and texture

*Serra da Estrela* cheese sensorial attributes, such as rind appearance and consistency, paste colour and texture, flavor and aroma, can be evaluated using as guideline the Portuguese Standard NP 1922 (1985), which describes the quantification procedures for the different sensorial characteristics of this traditional cheese. In what concerns the rating chart used, rind appearance must be smooth, well formed, thin and unbroken and of a uniform pale straw-yellow colour. The paste must be closed, well-connected, compact or with some eyes, medium buttery and uniformly ivory in colour. The flavor and aroma must be smooth, clean or reveal a slightly tangy bouquet (Henriques, 2008; NP 1922, 1985).

The presence of pin-hole eyes can be associated to growth of gas producing bacteria, such as coliforms (Macedo *et al.*, 1997). In the past, two decades ago, an approach used to improve the microbiological quality, was to manufacture *Serra da Estrela* cheeses with addition of distinct starter cultures isolated from the native microbiota of raw milk cheeses; however those cheeses exhibited a more acid and bitter flavors than the traditional cheese (Macedo *et al.*, 2004). More recently, the sensorial properties of *Serra da Estrela* cheeses were evaluated in regards to the thistle ecotype used, production location, dairy and ripening time having the cheese paste creaminess and the rind hardness presented significant differences in cheeses made by different producers and with different thistle ecotypes (Correia *et al.*, 2016; Guiné *et al.*, 2016).

Instrumental texture analysis can complement the texture evaluation. *Serra da Estrela* cheese texture is influenced by the ripening temperature and ripening relative humidity, where both variables contribute to the softening of the cheese matrix (Macedo *et al.*, 1997). Guiné *et al.*, (2016) was able to demonstrate that the firmness of inner paste varied significantly among cheeses from different producers, origins and thistle flower ecotypes, while the stickiness

varied significantly according to cheese geographical origin and thistle ecotypes used (Guiné *et al.*, 2016).

Being an artisanal cheese without addition of starters or industrial standard coagulant, several factors contribute to the development of different peculiar sensorial and texture attributes.

## 1.7 Innovation concerning *Serra da Estrela* cheese

As with all traditional products, improvements in *Serra da Estrela* cheese manufacture are constantly sought to improve quality and therefore consumer acceptance. Recently developed innovations span from raw materials to manufacturing process. Starting with innovation associated with raw ewe's milk: due to its high economic value, some producers can adulterate the milk or use milk from different ewes' races, which results in fraudulent *Serra da Estrela* cheese production. To avoid this situation, analytical techniques were recently adapted and proposed to screen the milk origin (Cunha *et al.*, 2016), like Random Amplified Polymorphic DNA (RAPD) for detection of adulterant breeds in milk mixtures and Sequence Characterized Amplified Regions (SCAR) markers for breed identification in processed dairy food. The combination of these methods, RAPD-SCAR can be used to prevent fraudulent *Serra da Estrela* PDO cheese production and be implemented as a quality control. Innovations are also associated with the possibility to manipulate the final texture/sensory profile of *Serra da Estrela* cheese mainly via the thistle ecotype selected (Correia *et al.*, 2016; Guiné *et al.*, 2016). The genotype evaluation of selected cardoons has been performed in order to improve the quality and standardization of cardosin profiles from cardoon flowers used for cheese production (Barracosa *et al.*, 2018b), having the different cardoon cultivars been morphologically evaluated (Barracosa *et al.*, 2018a). Other ways of innovation are the conversion of this traditional cheese into a functional food. For this purpose, decoctions and/or

dried extracts of chestnut flowers (Carocho *et al.*, 2015a, 2016), basil leaves (Carocho *et al.*, 2016b) and lemon balm plants (Carocho *et al.*, 2015a, 2016) were incorporated in the cheese curd before the pressing stage. The incorporation of these compounds confer functional properties, higher antioxidant activity mainly by lipid peroxidation inhibition (Carocho *et al.*, 2015, 2016b). The moisture loss was higher, while the protein content was higher in the cheese samples with plant extracts added (Carocho *et al.*, 2016a, 2016b). The incorporation of chestnut flowers and lemon balm dried plants showed better preservation capacity than decoctions (Carocho *et al.*, 2015, 2016b). On the other hand, basil decoctions were shown to be better to prevent lipid peroxidation, while the incorporation of dried basil preserved the proteins and the unsaturated fatty acids.

Like other raw milk cheeses, *Serra da Estrela* cheese can enable spoilage microorganisms' growth during ripening and cheese storage. To try avoiding this, a new food processing technology, i.e. high pressure processing (HPP) was tested for its efficacy to simultaneously reduce the risk of pathogenic and spoilage microorganisms development without jeopardising the desired LAB metabolic activity (Inácio *et al.*, 2014). *Serra da Estrela* cheese samples with 45 days of ripening were treated at 400 - 600 MPa pressures during 3 - 10 minutes and the effect of HPP after pressure processing and during storage (100 days at 5 °C) was studied. LAB were the least affected by HPP, having viable cell numbers been reduced, at maximum, by ~ 0.50 log cfu/g (samples treated at 600 MPa were the most affected). *Enterobacteriaceae* viable cell numbers showed ≥ 3.5 log cycle reductions, remaining unchanged during the storage period. These results boost further investigation in order to clarify other interesting parameters of the effect of HPP on *Serra da Estrela* cheese.

# CHAPTER 2 - High pressure processing on milk and raw milk cheese

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***Part of a Chapter: "Impact of High-Pressure Processing on Food Quality" in Effect of Emerging Processing Methods on the Food Quality, Springer, Switzerland: Editor Shahin Roohinejad.***

## Abstract

High Pressure Processing (HPP) is a non-thermal food pasteurization technique that makes use of elevated hydrostatic pressures (commercially up to 600 MPa) for a few seconds/minutes to inactivate vegetative spoilage and pathogenic microorganisms. Its application in the food industry has been of great interest as a possible alternative to the conventional heat pasteurization procedures. As HPP does not apply heat, the nutritional value of foods is kept, while ensuring food safety. This can be particularly interesting for heat sensible food products such as cheese and other dairy products.

There are many areas of interest for HPP application in dairy foods. Nevertheless, the application to milk for subsequent cheese making and the application directly to the pressed curd and/or during cheese ripening have been the main areas of study and are summarized in the present chapter.

## 2.1. Indroduction

*"High pressure processing is recognized as one of the most promising nonthermal preservation and pasteurization technologies"* (Lou *et al.*, 2015)

Conventional food processing uses thermal pasteurization treatment for food preservation (Huppertz *et al.*, 2002). This technology reduces microbial levels, however, it causes undesired effects in food, such as: loss of flavour, colour, texture, odour and nutritional value, that leads to the loss of final product quality (Hogan *et al.*, 2001). Consumers prefer more natural, preservative-free, shelf-stable, safety and tastier (Mertens and Deplace, 1993) foods, which has created the need of developing improved food processing technologies (Balasubramaniam *et al.*, 2015; Yaldagard *et al.*, 2010).

Since 1970, there was an interest in application of high pressure processing (HPP) in food technology, but only in 1990, a fruit jam was introduced in the market as the first HPP product (Huppertz *et al.*, 2002). The rapid evolution of this technology led to an exponential growth of industrial equipment in the industry (Figure 2.1).

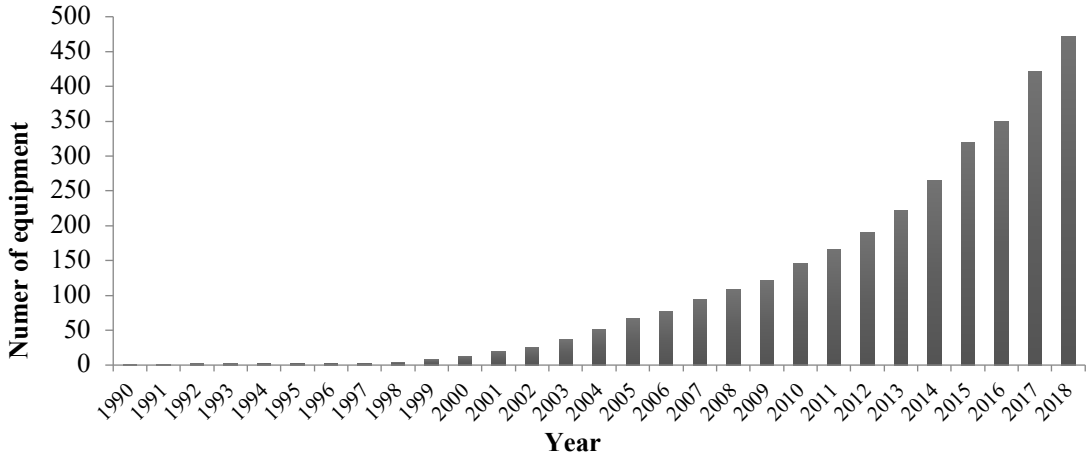


**Figure 2.1: Number of high pressure processing equipment operating worldwide. Courtesy of Hiperbaric S.A. (Burgos, Spain).**

HPP offers unique advantages over traditional thermal treatments, being recognized as one of the most promising non-thermal preservation and pasteurization technologies (Lou *et al.*, 2015). This technology ensures food safety and quality, as well as extends shelf-life, by eliminating pathogenic and spoilage microorganisms (Martínez-Rodríguez *et al.*, 2012) and is able to inactivate both surface and internalized pathogens, because pressure can penetrate into the entire food product (Lou *et al.*, 2015). Moreover, usually food matrices undergo minimal organoleptic and nutritional changes during pressurization (Martínez-Rodríguez *et al.*, 2012), because covalent bonds are unaffected by HPP (Lou *et al.*, 2015). Thus, there are many advantages of HPP for preservation of foods, such as: i) elimination or reduction of heating, ii) avoidance thermal degradation of some components in products; iii) retention of flavour, colour and nutritional value; iv) uniform and instantaneous transmission throughout food products, and v) reduced or no need of chemical additives addition (Yaldagard *et al.*, 2010). HPP is a key to maintain the quality attributes of processed food, while improving shelf-life and convenience.

These benefits are achieved due to a basic governing principle of HPP. The isostatic principle, presumes that the pressure applied in HPP is instantaneously and uniformly transmitted throughout the food, regardless of food geometry, shape and size (Lou *et al.*, 2015; Martínez-Rodríguez *et al.*, 2012). Thus, food is treated by uniform pressure from every direction and when pressure is released, the food returns to original shape.

In what concerns the equipment, a typical one consists in four parts: a high-pressure vessel and its closure, a pressure-generating system, a temperature-control device and a material-handling system (Balasubramaniam *et al.*, 2015). Nowadays, there are vessels with a volume up to 687 L (Lou *et al.*, 2015) and machines operating at pressures in the rage 100 to 1000 MPa (Huppertz *et al.*, 2002; Yaldagard *et al.*, 2010). But HPP for preservation of food typically uses pressure between 400 and 600 MPa using a batch

process. Food samples are packaged in flexible containers (foods decrease in volume under pressure and expand in volume during decompression) and loaded in a pressure vessel filled with pressure-transmitting fluid (water or oil) prior to operation. After, the vessel is filled, sealed and pressure is generated by pumping additional fluid into the vessel, being the pressure applied uniformly throughout the product. Thus, HPP is an energy-efficient process because it requires no additional energy once the desired pressure is reached (Lou *et al.*, 2015).

## 2.2. Application of HPP to dairy foods

There are many areas of interest for HPP application to dairy foods, being the possibility of shelf-life extension the main advantage (2-3 fold longer relatively to non-pasteurized products) (Dhineshkumar *et al.*, 2016). Milk, fresh cheese, ripened cheese, whey cheese, yoghurt, ice cream, and butter have been processed by this technology. However, the application on milk for subsequent cheesemaking and the application directly to the pressed curd and/or during cheese ripening have been the main areas of study (Martínez-Rodríguez *et al.*, 2012).

### 2.2.1. Application of HPP in raw milk

The first report concerning HPP treatment of milk was done by Hite in 1899, who explored effects of processing on milk (Hite, 1899). Thereafter, many authors have explored this strategy to understand better its impact on milk quality and safety as shown in Table 2.1.

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**Table 2.1: Summary of studies that have applied HPP on raw cow's (white rows), ewe's (dark gray rows) or goat's (light gray rows) milk.**

| Authors | Cheese variety | Raw milk source | Starts Addition | HPP (MPa) /time (min) | T (°C) HPP |
|---|---|---|---|---|---|
| **Study the effects in milk** | | | | | |
| Stratakos *et al.*, (2019) | | NA | - | 400-600/1-5 | 18 |
| Zobrist *et al.*, (2005) | | cow | - | 100-600/30 | 5, 10, 20 |
| Huppertz *et al.,* (2004a) | | cow | - | 100-600/30 | 20 |
| Huppertz *et al.*, (2003) | | cow | - | 100-600/0-60 | 20 |
| Gervilla *et al.*, (2001) | | ewe | - | 100-500/10-30 | 4, 25, 50 |
| Garía-Risco *et al.*, (2000) | | cow | - | 400/15 | 25-60 |
| **Study the effects in milk and/or curd** | | | | | |
| Huppertz *et al.*, (2006b) | | ewe | - | 100-400/30 | NA |
| Huppertz *et al.*, (2005) | | cow | - | 100-600/0-30 | 20 |
| Huppertz *et al.*, (2004c) | | cow | - | 100-800/0-60 | 20 |
| Needs *et al.*, (2000) | | NA | - | 200,400,600/15 | 20 |
| Trujillo *et al.*, (1999) | | goat | - | 500/15 | 20 |
| López-Fandiño and Olano, (1998a) | | cow | - | 100–400/15 | 25, 60 |
| López-Fandiño and Olano, (1998b) | | ewe and goat | - | 100–400/5-60 | 25 |
| López-Fandiño *et al.*, (1998) | | cow | - | 100–400/5-30 | 20 |
| López-Fandiño *et al.*, (1997) | | cow | - | 100–400/15 | 25, 60 |
| Felipe *et al.*, (1997) | | goat | - | 500/5-10 | 25, 50 |
| López-Fandiño *et al.*, (1996) | | cow | - | 100–400/10-60 | 25 |
| **Study the effects in milk and/or cheese** | | | | | |
| Sukmanov and Kiiko, (2016) | | cow | NA | 300-600/0.1-1 | 40-45 |

| | | | | | |
|---|---|---|---|---|---|
| Voigt *et al.*, (2012, 2010) | Cheedar | cow | x | 400,600/10 | 20 |
| San Martín-González *et al.*, (2007) | Cheedar | cow | x | 483/5 and 676/5 | 10, 30, 40 |
| Buffa *et al.*, (2005) | | goat | x | 500/5 | 20 |
| Trujillo *et al.*, (2002a) | | goat | x | 500/15 | 20 |
| Buffa *et al.*, (2001a, 2001b, 2001c) | | goat | x | 500/15 | 20 |
| Molina *et al.*, (2000) | | cow | x | 500/15 | 22 |
| Trujillo *et al.*, (2000) | | goat | NA | 500/5 or 30 | 20 |
| Trujillo *et al.*, (1999a) | | goat | NA | 500/15 | 20 |
| Drake *et al.*, (1997) | Cheedar | cow | x | 345/15, 586/15, 586/1, 586/1 (3x) | NA |

NA means not available; - outside the article aim; x starts were added

HPP may influence the physico-chemical and technological properties of milk (Chawla *et al.*, 2011; Dhineshkumar *et al.*, 2016; Trujillo *et al.*, 2002b). HPP was tested in milk and effects reported included the inactivation of microorganisms, the reduction of rennet coagulation time and the increase of cheese yield (López-Pedemonte *et al.*, 2007; O'Reilly *et al.*, 2000). Cheese yield has been evaluated mainly in cows' milk (Drake *et al.*, 1997; Huppertz *et al.*, 2004c, 2005; Molina *et al.*, 2000; San Martín-González *et al.*, 2007), goats' milk (López-Fandiño and Olano, 1998a, 1998b; Trujillo *et al.*, 1999b) and few studies have been focused on ewes' milk (López-Fandiño and Olano, 1998a, 1998b). Many different HPP treatments were applied to cows' milk: 345 and 586/15 min (Drake *et al.*, 1997); 483 MPa/5 min and 676 MPa/5 min (San Martín-González *et al.*, 2007); 100 – 600 MPa/5-30 min/20 °C after heat treatment (90 ºC/10 min) (Huppertz *et al.*, 2005); 100-800 MPa/10-60 min/20 °C (Huppertz *et al.*, 2004c); 400 MPa/15min/22 °C (Molina *et al.*, 2000). In general, the HPP treatment enabled an increase of the cheese yield in about 4 – 23 % in comparison to untreated milk. Huppertz *et al.*, (2005) verified higher yield values (13 - 18%) for milk HPP treated at 100 and 250 MPa. On the other hand, one year before the same group (Huppertz *et al.*, 2004c) had verified lower values for HPP treated milk at 250 MPa (exception for treatment for 60 min with 4 % increase in yield) and higher values for HPP treatment at 400 and 600 MPa (4 - 23%). Furthermore, higher cheese yield values were verified after a holding time at 20 °C for 24 h post HPP treatment. The same study revealed that a longer holding time under pressure (from 5 to 30 min) also increased the wet curd yield, relatively to control untreated milk. In ewes' milk, López-Fandinõ and Olano (1998) studied HPP treatments at 100 – 400 MPa during 5-60 min (López-Fandiño and Olano, 1998b) and at 25-60 °C (López-Fandiño and Olano, 1998a). Ewes' milk treatments for 30 min at 100 MPa revealed a similar yield to untreated milk and an increase of about 5, 5 and 16 % for 200, 300 and 400 MPa, respectively

(López-Fandiño and Olano, 1998b). HPP treatments at 300 MPa with a holding time of 10, 20 and 30 min showed similar yields values, but lower values for 5 min. Similar results were verified in a further study by the same research group (López-Fandiño and Olano, 1998a), in addition to a higher yield after HPP at 40 °C than at 25 °C (about 23 % *vs* 9 % in comparison to untreated milk), but did not improve the cheesemaking properties of milk. A similar behaviour was achieved for goats' milk, but in general with lower yields than the corresponding obtained with ewes' milk (López-Fandiño and Olano, 1998a, 1998b). Trujillo *et al*., (1999) compared goats' milk thermal and HPP treatments (500 MPa/15 min/20 °C) on cheese yield and reported an increase of about 5% with HPP in comparison to pasteurized milk. When milk is subject to HPP, the casein micelles considerably differ from those in untreated milk, e.g., size, composition and hydration (Huppertz *et al.*, 2006a). These alterations also reduce the rennet coagulation time (RCT) (Considine *et al.*, 2007; Drake *et al.*, 1997; Gaucheron *et al.*, 1997; Needs *et al.*, 2000; Zobrist *et al.*, 2005).

Since, milk components, mainly proteins, are influenced by HPP processing, it is important to study the effect on technological properties of milk during manufacture. Although a huge number of factors influence the cheese yield, they do not all have the same significant impact. According to some authors (Huppertz *et al.*, 2002; O'Reilly *et al.*, 2000), the thermal pasteurization of milk can be replaced by HPP in cheese manufacture. Numerous studies have been carried out on the application of HPP to milk pasteurisation in order to promote milk microbial destruction, creating the need thereafter for starter cultures addition to promote the cheesemaking process (Buffa *et al.*, 2001b; Drake *et al.*, 1997; Trujillo *et al.*, 1999b) (Table 2.1).

Different literature reports have indicated that a HPP milk pre-treatment can increase cheese yield (Huppertz *et al.*, 2004c, 2005) opening the possibility to manufacture more cheese from an equal or oven less amount of milk. Moreover, HPP processing can reduce the microbial numbers without significant effects on flavour and nutritional components contributing to safer high quality cheese products.

### 2.2.2. Application of HPP in raw cheese manufacture

Until 2013, date of first study in *Serra da Estrela* cheese (Inácio *et al.*, 2014) (from a master thesis). HPP had been applied in very few cases to raw milk cheese (only 3 related with ewes milk) to improve its microbiological quality and increase cheese safety and shelf-life but only during ripening as shown in (Table 2.2 complied the works until now).

In the same year my Ph.D proposed was submitted to FCT to study the effects of HPP in *Serra da Estrela* cheese during prolonged storage upon ripening. Until that time, to our knowledge there were no articles studying the applications of HPP on raw ewes' milk cheese with analysis of changes during subsequent storage. However, over the last five years 8 research papers were published, thus revealing the interest in use of HPP for raw milk cheese, specially for ewe's milk (5 studies) - Table 2.2.

**Table 2.2: Summary of studies that have applied HPP on raw milk cheeses prepared from cow's (white rows), ewe's (dark gray rows) or goat's (light gray rows) milk.**

| Authors | Cheese variety | Raw milk source | Type of coagulant | HPP (MPa) /time (min) | T (°C) HPP | Moment of application | Conditions after HPP |
|---|---|---|---|---|---|---|---|
| Calzada *et al*., (2015) | NA | cow | animal | 400/5 600/5 | 14 | 2 and 3w | Ripening at 8 °C and 72 % RH (60d) and at 5 °C and 75 % RH (240d) |
| Delgado *et al*., (2015) | *Torta del Casar* | ewe | cardoon | 200/5 200/20 600/5 600/20 | 10 | 60d | Storage at 6 °C (240d) |
| Rodríguez-Pinilla *et al*., (2015) | *Torta del Casar* | ewe | cardoon | 200/5 200/20 600/5 600/20 | 10 | 60d | Storage at 6 °C (240d) |
| Calzada *et al*., (2014b) | *Torta del Casar* | ewe | cardoon | 400/5 600/5 | 14 | 3 and 5w | Ripening at 8 °C and 92% RH (60d) and storage at 4 °C (240d) |
| Calzada *et al*., (2014c) | NA | cow | animal | 400/5 600/5 | 14 | 2 and 3w | Ripening at 8 °C and 72 % RH (60d) and at 5 °C and 75 % RH (240d) |
| Calzada *et al*., (2014a) | *Torta del Casar* | ewe | cardoon | 400/5 600/5 | 14 | 3 and 5w | Ripening at 8 °C and 92% RH (60d) and storage at 4 °C (240d) |
| Calzada *et al*., (2013) | *Torta del Casar* | ewe | cardoon | 400/5 600/5 | 14 | 3 and 5w | Ripening at 8 °C and 92% RH (60d) and storage at 4 °C (240d) |
| Delgado *et al*., (2013) | *Ibores* | goat | animal | 400/7 600/7 | 10 | 60d | Storage at 6 °C (90d) |
| Inácio *et al*., (2014) | *Serra da Estrela* cheese | ewe | cardoon | 400/10 500/5 600/3 | RT | 45d | Storage at 4 °C (100d) |
| Delgado *et al*., (2012) | *Ibores* | goat | animal | 400/7 600/7 | 10 | 1, 30 and 50d | Ripening at 10 °C and 82 % RH (60d) |
| Delgado *et al*., (2011a) | *Ibores* | goat | animal | 400/7 600/7 | 10 | 60d | Storage at 6 °C (90d) |
| Delgado *et al*., (2011b) | *Ibores* | goat | animal | 400/7 600/7 | 10 | 1, 30 and 50d | Ripening at 8-12 °C and 80 % RH (60d) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Garde *et al*., (2007) | *La Serena* | ewe | cardoon | 300/10 400/10 | 10 | 2 and 50d | Ripening at 8 °C and 90 % RH (60d) |
| Arqués *et al*., (2007) | *La Serena* | ewe | cardoon | 300/10 400/10 | 10 | 2 and 50d | Ripening at 8 °C and 90 % RH (60d) |
| Arqués *et al*., (2006) | *La Serena* | ewe | cardoon | 300/10 400/10 | 10 | 2 and 50d | Ripening at 8 °C and 90 % RH (60d) |
| Shao *et al*., (2007) | Cheddar-type* | cow | NA | 250/5 300/5 350/5 | 10-50 | NA | NA |
| Rodríguez *et al*., (2005) | NA* | cow | NA | 300/10 500/5 | 10 | 2 and 50d | Ripening at 12 °C and 90 % RH (60d) |
| Arqués *et al*., (2005a, 2005b) | NA* | cow | cardoon | 300/10 400/10 | 10 | 2 and 50d | Ripening at 12 °C and 90 % RH (60d) |
| Messens *et al*., (1998) | *Gouda* | cow | NA | 100/30 200/30 300/30 400/30 | 20 | 1d | Ripening at 14 °C and 85 % RH (14d) |

NA means not available; RH means relative humidity; RT means room temperature; * Studies the effect of HPP on the survivability of inoculated microorganisms in cheese.

Some application examples of HPP to raw milk cheeses include *Torta del Casar* raw ewe milk cheese where authors studied HPP as a procedure to prevent over-ripening (Calzada *et al.*, 2014a), to minimize biogenic amine build-up (Calzada *et al.*, 2013) or off-odours (Calzada *et al.*, 2014b), to improve microbiological quality and increase cheese safety (Rodríguez-Pinilla *et al.*, 2015) or to extend the commercialization period (Delgado *et al.*, 2015); other raw ewe milk (*La Serena*, Castellano type, *Serra da Estrela* cheese) or raw goat (*Ibores* cheese) and cow milk cheeses were mainly studied for the impact of HPP to improve its microbiological quality and increase cheese safety and shelf-life (Arqués *et al*., 2006; Ávila *et al*., 2016; Delgado *et al*., 2012, 2013, 2015; Garde *et al*., 2007a; Inácio *et al*., 2014; Rodríguez-Pinilla *et al*., 2015). After HPP treatment the cheeses returned to ripening stage and/or were stored during short periods (between 14 and 240 days). The effects of HPP on cheese characteristics are dependent on the pressure level applied, the length of treatment and particularly the stage of ripening at which HPP is applied, as explained further ahead.

#### 2.2.2.1. Effects of HPP on pathogenic, spoilage and endogenous microorganisms

Microbial inactivation by HPP was shown to be influenced by microbial characteristics, process conditions and product parameters. As far as microbial characteristics are concerned, yeasts and moulds are microorganisms that are more sensitive to HPP than bacteria. Among bacteria, Gram-positive bacteria are more pressure-resistant than Gram-negative bacteria (Martínez-Rodríguez *et al.*, 2012). Concerning the process conditions, microbial inactivation increases with treatment intensity and treatment time. Cheese composition influences the susceptibility of microorganisms to inactivation by HPP, since it induces changes in the fluidity of the cell

membrane making the microorganisms more or less resistant to HPP (more fluidity leads to more resistance) (Martínez-Rodríguez *et al.*, 2012). HPP induces effects leading to cell death, which can be due to: protein and enzyme unfolding; cell membranes undergoing a phase transition and change of fluidity; disintegration of ribosomes in their subunits and intracellular pH changes related to the inactivation of enzymes and membrane damage (Georget *et al.*, 2015). HPP above 300 MPa induces enzyme and protein irreversible denaturation, alterations that had been shown which led to ribosomes dissociation which led to limit the cell viability (Abe, 2007).

In order to achieve preservation, HPP has been used in cheese to inactivate or reduce viable cell numbers of pathogenic strains such as, *Staphylococcus* (Arqués *et al.*, 2005a, 2006), *Listeria monocytogenes* (Arqués *et al.*, 2005b) and its surrogate (Inácio *et al.*, 2014), *Escherichia coli* (Rodríguez *et al.*, 2005; Shao *et al.*, 2007), as well as spoilage microorganisms (such as *Staphylococcus* spp. *Enterococcus* spp.), coliforms (Arqués *et al.*, 2006), yeasts and moulds (Inácio *et al.*, 2014). *Staphylococcus aureus* CECT 976 was studied by Arqués *et al*., (2005a), showing more than 5.3 log reductions after application of HPP at 500 MPa/5 min at 10 °C, at 50 days of ripening. Staphylococci in *La Serena* cheese were reduced by 0.49 and 1.45 log units after application of HPP at 300 and 400 MPa/10 min (Arqués *et al.*, 2006). In *Serra da Estrela* cheese, *Listeria innocua* was inoculated at 45 days of ripening, revealing more than 4.8 log reductions after HPP application (400 MPa/10 min 500 MPa/5 min 600MPa/3 min) (Inácio *et al.*, 2014). In raw cow's milk cheese inoculated with *Listeria monocytogenes* Scott A., HPP (500 MPa/5 min at 10 °C) applied at 2 and 50 days of ripening, showed 5.02 log reductions and complete inactivation (>6.34 log reductions), respectively (Arqués *et al.*, 2005b). In Cheddar cheese, *E. coli* K-12 was completely inactivated (>6.5 log reductions), after HPP application at 350 MPa/3 min at 50 °C (Shao *et al.*, 2007). Rodríguez *et al*., (2005) studied

the effect of HPP treatment at 2 and 50 ripening days (at 500 MPa/5 min at 10 °C) and the authors concluded that the treatment was more effective at 50 days, being reported complete inactivation of *E. coli* O157:H7 (> 5.11 log reductions). In *Serra da Estrela* cheese application of HPP (400 MPa/10 min 500 MPa/5 min 600MPa/3 min) at 45 days showed for *Enterobacteriaceae* counts ≥ 3.5 log cycle reductions, remaining unchanged during the 100 days of storage (Inácio *et al.*, 2014). Also, Rodríguez-Pinilla *et al.*, (2015) registered a significant decrease of 2.18 and 2.46 log units in *Torta del Casar* cheeses after application of HPP (200 MPa/20 min at 60 days of ripening) after 60 and 120 days of storage, respectively. In *La Serena* cheese application of HPP at 300 and 400 MPa/10 min at 10 °C, led to the reduction of enterococci by 2.05 and 2.68 log units when pressured on day 2, and 1.37 and 1.98 log units on day 50 (Arqués *et al.*, 2006). In the latter being reported 4.13 and 5.50 log reductions when HPP was applied on day 2, and 4.85 log reductions and complete inactivation, respectively for 300 and 400 MPa, when applied on day 50 of ripening. Yeasts are not associated to food-borne diseases, however they are responsible for cheese spoilage (Daryaei *et al.*, 2008). In *Serra da Estrela* cheese, yeasts and moulds counts exhibited ≥ 3.6 log cycle reductions after HPP treatment (400 MPa/10 min 500 MPa/5 min 600 MPa/3 min) at 45 days (Inácio *et al.*, 2014). According to these studies, the barotolerance of spoilage and pathogenic bacteria in cheese, follow the order: *S. aureus* > *L. monocytogenes* > *E. coli* > yeasts and moulds (Martínez-Rodríguez *et al.*, 2012), supporting the possibility of HPP being able to increase cheese food safety.

HPP does not affect only pathogenic and spoilage microorganisms, but also affects LAB. In *Serra da Estrela* cheese, LAB were reduced, at maximum, by ~ 0.50 log cfu/g after HPP treatment (400 MPa/10 min 500 MPa/5 min 600MPa/3 min) at 45 days of ripening. After 100 days of storage, it was verified that treatments at 500 and 600 MPa were more severe for LAB viable cell numbers (less ~ 2 log cfu/g), revealing significant

differences compared to control samples (Inácio *et al.*, 2014). A greater effect, 1.64 and 6.51 log units, was described by Calzada *et al.*, (2013) for *Casar* cheese treated at 400 and 600 MPa, respectively at 5 weeks of ripening. According to these studies, HPP treatments on cheese above 500 MPa cause more considerable reductions in beneficial microorganisms present in cheese.

Thus, the prokaryotes microorganisms (bacteria) show a higher resistance towards the pressure than eukaryotes microorganism (yeasts and moulds) (Georget *et al.*, 2015). Within bacteria, in general, Gram-positive bacteria were more resistant reflecting lower cycle reductions than Gram-negative bacteria (Smelt, 1998). This different resistance can be correlated with the thicker peptidoglycan layer in Gram-positive microorganism, which generally shows to be more pressure resistance (Considine *et al.*, 2008; Murchie *et al.*, 2005; Smelt, 1998), but it also suggests that gram negative cell membrane complexity causes also more susceptibility to HPP (Shigehisa *et al.*, 1991). Among LAB bacteria, the coccoid shape were in general more resistance to HPP than rod-shape bacteria (Huang *et al.*, 2014).

Hence, it is important to promote further studies to establish conditions that best compromise an efficient reduction of spoilage and pathogenic microorganisms together with a good maintenance of beneficial microorganisms.

#### 2.2.2.2. Effects of HPP on cheese quality during ripening and storage: proteolysis and volatile compounds

HPP can affect some biochemical characteristics, leading to their change during ripening and storage; thus, it is necessary to understand the effect of this technology on proteolysis, lipolysis, volatile compounds, physicochemical, rheological and sensorial properties. However, there are few studies concerning the effects of HPP treatment during raw ewes' milk cheese storage.

The effect of HPP on proteolysis, in cheese manufactured with raw ewes' milk, was studied during ripening in *La Serena* cheese (Garde *et al.*, 2007a); and during storage in *Torta del Casar* (Calzada *et al.*, 2014a; Delgado *et al.*, 2015) and *Serra da Estrela* cheese (Inácio *et al.*, 2014). *La Serena* cheese at 50 days of ripening was pressure treated at 300 and 400 MPa for 10 min at 10 °C and showed a similar proteolysis level compared to the control cheese after 10 days at conventional ripening conditions (Garde *et al.*, 2007a). During storage of *Torta del Casar* cheese, casein degradation was significantly retarded in HPP treated cheeses (600 MPa/5 min), being the α-casein concentration 48–52 % higher on day 60 and 30–33 % higher on day 240 than in the control cheeses; while β-casein concentration was 25–26 % higher on day 60 and 100–103 % higher on day 240 (Calzada *et al.*, 2014a). Also, Delgado *et al.*, (2015) treated *Torta del Casar* at 600 MPa at 60 days of ripening and verified a reduction of proteolysis of casein fractions (para-κ-casein; $\alpha_{s1}$-CN I, II, III, $\alpha_{s2}$-CN: $\alpha_s$-caseins; and $\beta_1$-CN, $\beta_2$-CN: β-caseins) relatively to control cheese at 240 days of storage; although at 120 and 180 days of storage authors found similar levels of $\alpha_s$-caseins and β-caseins to those of control cheeses (Delgado *et al.*, 2015). Thus, nitrogen fractions were affected by HPP; ratio water soluble nitrogen per total nitrogen (WSN/TN – ripening extension index) decreased during storage in cheeses treated at 600 MPa compared with control counterparts (Delgado *et al.*, 2015). In

a previous study of *Serra da Estrela* cheese, WSN content did not show significant differences between control and treated samples up to 100 days (Inácio *et al.*, 2014).

According to these studies, the HPP treatments, at the end of the ripening period, slow down or maintain the hydrolysis of casein and nitrogen ratio relatively to control samples during storage.

Regarding the effect of HPP on volatile compounds, there is very little information. HPP treated *Casar* cheeses showed lower concentrations of aldehydes, esters and, particularly, sulphur compounds than control cheese, which exhibited putrid and rancid off-odours from day 120 of storage onwards (Calzada *et al.*, 2014b). In *La Serena* cheese, the levels of some volatile compounds of cheeses treated by HPP on day 2 are affected but tended to disappear during the ripening period. On the other hand, HPP treatments on day 50 did not influence the volatile compound profile of 60-d-old cheese (Arqués *et al.*, 2007).

#### 2.2.2.3. Effects of HPP on cheese quality: texture and flavour/sensorial properties

Rheological properties influence texture, eating quality and physical behaviour and are dependent on composition, microstructure, macrostructure, and physicochemical state of cheese components (Guinee, 2011). *Torta del Casar* cheese revealed an effect on firmness and consistency just after HPP treatment (60 days), but these changes were reduced during storage. The treatments at 600 MPa (5 and 20 min) were those that caused most intense changes, reducing the firmness and consistency of the HPP treated cheeses in comparison with the control ones (Delgado *et al.*, 2015). Similar results were found by Garde *et al*., (2007) in *La Serena* cheese. Cheese pressure-treated at 300 or 400 MPa for

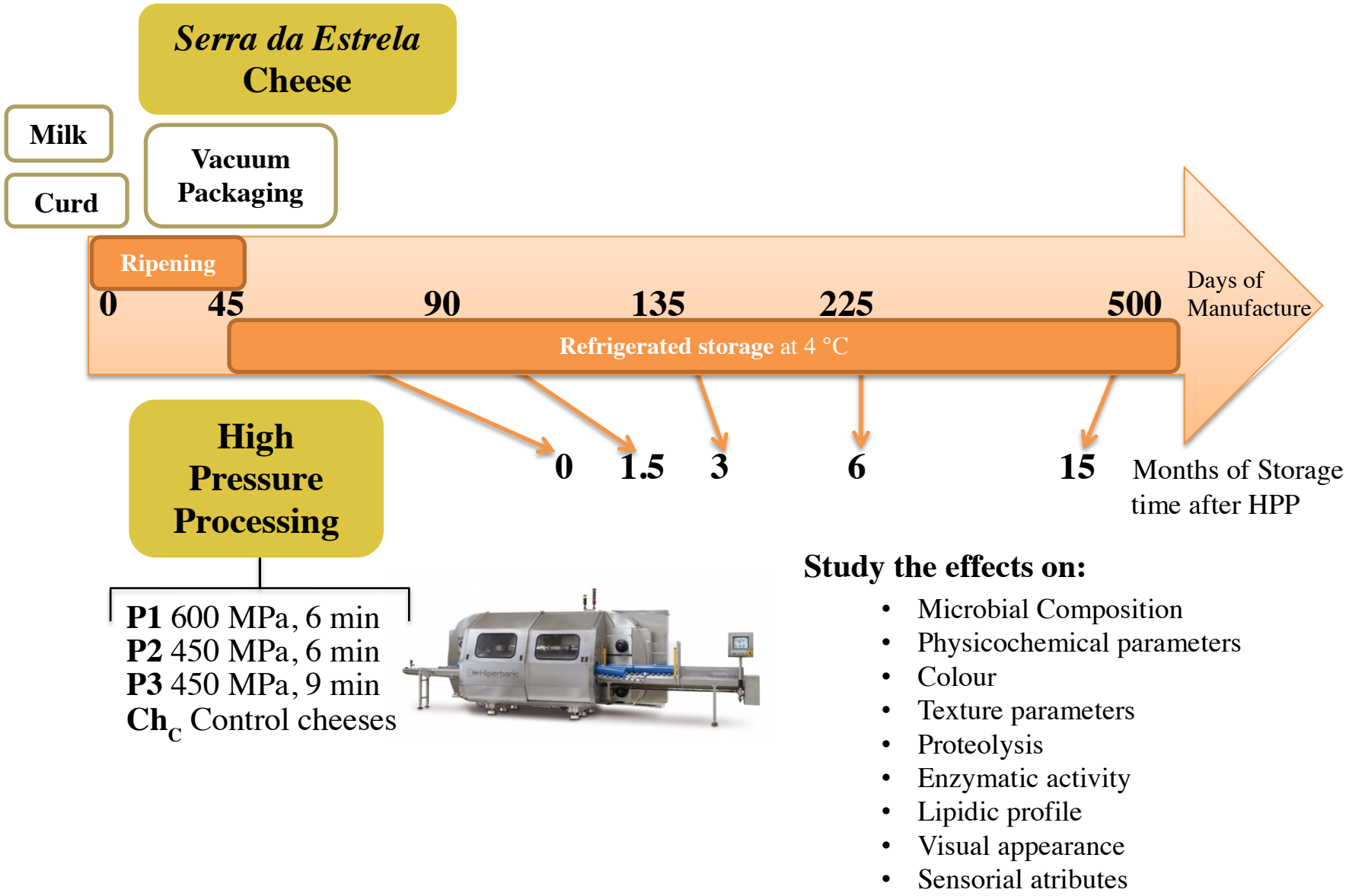
10 min on day 2 of ripening showed higher fracturability, hardness, and elasticity than control cheese and cheese treated at 50 ripening days, at 60 days of ripening. HPP treatment at 50 days did not influence the texture after 10 days of storage after the treatment. On the other hand, Calzada *et al*., (2014a) reported higher firmness values for 600 MPa processed *Casar* cheeses (treated at 3 and 5 weeks of ripening, and storage 240 days), which showed the highest levels of intact caseins that influences the stability of the cheese protein network.

Regarding sensorial analysis, HPP treated *Casar* cheese showed increase at a slower rate of flavour intensity scores than control cheese. However, HPP treatment prevented the dramatic decline in flavour quality recorded for the control cheese throughout the refrigerated storage. Until day 240, HPP treated cheeses (except cheese treated at 400 MPa after 3 weeks of ripening) retained the flavour quality scores, which did not differ from the respective 60-day-old control cheeses (Calzada *et al.*, 2014a). The authors concluded that HPP of cheese at 600 MPa may be recommended to maintain flavour quality during prolonged refrigerated storage (Calzada *et al.*, 2014a). Also, Calzada *et al*., (2014b) obtained significant beneficial effects of HPP at 400 or 600 MPa on the odour characteristics of *Casar* cheese stored for a long period. On day 240, HPP treatment led to cheeses with lower odour intensity and higher odour quality relatively to control cheeses on day 240 (possibly due to decrease the sulphur compounds some associated to the appearance of putrid odour and the loss of odour quality). On the other hand, *La Serena* cheeses treated with HPP on day 2 showed that odour intensity was scarcely affected, but aroma quality and intensity scores were lowered in comparison with control cheese of the same age. The same HPP treatment on cheeses with 50 days of ripening did not influence the sensory characteristics of 60-days-old cheese (Arqués *et al.*, 2007). Similar results were reported by Garde *et al*., (2007), where the cheeses treated

at 400MPa on day 2 were the cheeses that received the lowest scores for taste quality from panellists, whereas the rest of HPP-treated cheeses did not differ from the control cheese.

Thus, HPP retains cheese sensory quality or eventually leads to its enhancement, if the treatment conditions are not applied in an early ripening stage.

# PART II – High pressure processing treatment of ripened cheeses




General schematic flow diagram of the work carried in Part II – CHAPTER 3, 4 and 5.

# CHAPTER 3 - Microbiological safety and quality and physicochemical characterization throughout storage

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*This chapter has been submitted for publication.*

## Abstract

*Serra da Estrela* cheese (a raw ewes' milk) ripened for 45 days were treated at 600 MPa for 6 minutes (P1) and at 450 MPa during 6 (P2) and 9 minutes (P3) and kept under refrigerated storage for 15 months. Lactobacilli and lactococci viable cell numbers were reduced in 3.2-3.6 and 2.7-3.6 log cycle units, respectively. Lower reductions were verified for total aerobic mesophilic and enterococci viable cell numbers in cheeses treated at 450 MPa (2.4-2.5 and 1.2 log reductions, respectively). In HPP cheeses yeasts and moulds were below the detection limit up to 6 months of storage, but at 15 months 3.6-4.2 log cfu/g were quantified in all cheeses, while *Enterobacteriaceae* were inactivated to below the detection limit. The increment of pressure treatment caused a greater impact on microflora's viability, than the increase in time under pressure. Control cheeses ($Ch_C$) showed higher pH and titratable acidity values (but without significant differences compared to HPP at 450 MPa). During storage, minor total colour differences were determined for HPP P3 cheese surface relatively to $Ch_C$ cheeses at 0 months. HPP can thus be a good process to apply after cheese manufacture, since it offers a good potential to render raw milk cheese microbiologically safer, with minimal changes in quality.

## 3.1. Introduction

The origin of *Serra da Estrela* cheese, a traditional Portuguese raw ewes' milk cheese with Protected Denomination of Origin (PDO) certification, has been reported back to as early as the Roman occupation of the Iberian Peninsula (Macedo *et al.*, 1993), being the importance and relevance reported in CHAPTER 1- *Serra da Estrela* cheese: a review. The uniqueness of raw milk cheeses relies on the necessary dynamic interaction between diverse native microflora that together drive the necessary biochemical reactions during the ripening period toward the development of optimum and unique aroma, flavour and texture profiles. With a minimum ripening period of 45 days *Serra da Estrela* cheese is generally consumed as a semi-soft cheese.

Lactic acid bacteria (LAB), in particular lactobacilli and lactococci, have been reported as the predominant bacterial groups in *Serra da Estrela* cheese (Dahl *et al.*, 2000; Macedo *et al.*, 1993, 1995; Macedo and Malcata, 1997e; Tavaria *et al.*, 2006; Tavaria and Malcata, 2000), but other groups of microorganisms, such as coliforms/*Enterobacteriaceae*, staphylococci and yeasts have also been found (Macedo *et al.*, 1995, 1996a; Tavaria *et al.*, 2006; Tavaria and Malcata, 2000). Furthermore, when hygiene practices are insufficient, *Listeria monocytogenes*, *Staphylococcus aureus, Escherichia coli, Klebsiella oxytoca, Citrobacter freundii, Hafnia alvei, Staphylococcus xylosus, Staphylococcus epidermidis, Enterococcus faecium* may also be found in this cheese type (Guilherme, 2012; Macedo *et al.*, 1995).

Given its uniqueness, *Serra da Estrela* cheese is a symbol of national gastronomic heritage that has crossed borders and is sought by consumers worldwide; such status demands that its shelf-life be as prolonged as possible, on the one hand, and that its singular and unique organoleptic quality is not altered during transport and storage. The demand for such microbiologically safe, wholesome, tastier and minimally processed

foods, has led to the search of new production strategies, including novel food processing technologies. High pressure processing (HPP) has been highlighted as a minimum processing technology that is capable of producing microbiologically safe food products, with minimal changes on their characteristics, demonstrating clear advantages over thermal processing (Martínez-Rodríguez *et al.*, 2012). In cheese production HPP may be applied directly to milk for subsequent cheese production, after pressing of the curd and/or during cheese ripening. These applications have different objectives, including the improvement of cheese preservation or the deceleration/acceleration of the ripening process (Martínez-Rodríguez *et al.*, 2012; O'Reilly *et al.*, 2001; Trujillo *et al.*, 2002b). For example, HPP was applied to *Torta del Casar* raw ewes' milk cheese either as a procedure to prevent over-ripening (Calzada *et al.*, 2014a), to minimize biogenic amine build-up (Calzada *et al.*, 2013) or off-odours (Calzada *et al.*, 2014b), to improve the microbiological quality and increase cheese safety (Rodríguez-Pinilla *et al.*, 2015) or to extend the commercialization period (Delgado *et al.*, 2015). In the case of other raw ewes' milk (*La Serena*, Castellano type, *Serra da Estrela* cheeses), raw goats' (*Ibores* cheese) or cows' milk cheeses HPP was used mainly to improve cheese microbiological quality and increase cheese safety and shelf-life (Arqués *et al*., 2006; Ávila *et al*., 2016; Delgado *et al*., 2012, 2013, 2015; Garde *et al*., 2007a; Inácio *et al*., 2014; Rodríguez-Pinilla *et al*., 2015). In all these research studies, after HPP treatment the cheeses were ripened and/or stored during periods between 14 and 240 days. Taking advantage of the selective inactivation effect of HPP on microorganisms, its application to cheese, with minimal effects on quality and without changing the traditional manufacture procedure, and so with potential to keep the PDO status, as is the case of *Serra da Estrela* cheese, is of great interest. In a previous study, where HPP was applied to small 15-g portions of *Serra da Estrela* cheese, results revealed that LAB were the microorganisms least affected by HPP

(0.86 log cycle reductions), while *Enterobacteriaceae*, *L. innocua* (inoculated at 8.56 log cfu/g) and yeasts and moulds were reduced to below the limit of quantification during 100 days of refrigerated storage (Inácio *et al.*, 2014).

Based on the above rationale, where HPP can be potentially used as a non-thermal pasteurisation process of raw milk cheeses to assure microbial safety and increase shelf-life, the main objective of this work was to study the effect of HPP on 45 days (optimally organoleptic) ripened PDO *Serra da Estrela* cheese, immediately after HPP and during post-processing refrigerated storage. Three different HPP conditions, varying in terms of pressure intensity or application timespan (two factors that may affect HPP efficiency) (Sakharam *et al.*, 2014), were applied to study the microbiological, and physicochemical changes, and the evolution of these parameters during the refrigerated storage up to 15 months. To our best of knowledge, this is the first study where raw ewes milk cheeses subject to HPP are exhaustively evaluated during such a long refrigerated storage period, of great potential if economic valorization and internationalization is sought.

## 3.2. Materials and methods

### 3.2.1. Cheese manufacture

One hundred and fifty litres of ewes' milk were collected in the morning at two farms of the PDO region, Portugal. Milk was transported under refrigeration (30 min) and kept under refrigeration in a reservoir with constant mixing until cheese manufacture. Two batches of *Serra da Estrela* PDO cheese were manufactured, due to the limited production capacity, one in the morning (Batch A) and the other after lunch (Batch B); the milk was kept under refrigeration and agitation until manufacture. Fifty-six cheeses (of about 0.5 kg each) were manufactured and ripened (first 15 days at 8 ± 2 ºC and 95% RH and then at 10 ± 2º C and 85% RH) at the dairy during 45 days according to the

traditional procedures (Macedo *et al.*, 1993) in order to reach the optimum organoleptic quality. Upon ripening, the cheeses were placed into polyamide-polyethylene (PA-PE) bags (Plásticos Macar – Indústria de Plásticos Lda, Santo Tirso, Portugal) and vacuum sealed (vacuum packaging machine HenkoVac E-193, Aveiro, Portugal). The sealed cheeses were then transported, under refrigeration, to the laboratory at University of Aveiro for HPP (transport took about 1 h and 30 min).

### 3.2.2. High pressure processing

Treatments were performed in a 55-liter capacity industrial scale high pressure equipment (model 55, Hyperbaric, Burgos, Spain). Batches of 7 *Serra da Estrela* cheeses were subject to one of three HPP treatments (two batches per treatment (A and B): 600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3) (Figure 1.1). Average times to reach 450 and 600 MPa were 1.42 and 2.30 min, and depressurisation times 3 and 5 s, respectively. The initial temperature of the water used as transmitting fluid was 8 °C and remained under 24 °C throughout processing. The two manufactured batches (A and B) were processed into different high-pressure processing batches and used as sampling replica. Upon HPP, control and HPP-treated cheeses (56 in total) were placed under refrigerated storage (4 °C) and sampled at 0, 1.5, 3, 6 and 15 months of storage.

### 3.2.3. Sampling

In order to characterize the milk used for cheese manufacture and the resulting curd, two milk samples (one from the morning batch and another from the afternoon batch) were collected from the refrigerated reservoir (Figure 1.1). After coagulation, cutting and pressing of the curd, fresh cheese samples were collected (1.5 h after milk coagulation

initiated), one per cheese batch. In the case of the ripened cheeses, 4 cheeses were taken from each batch (one per treatment and one control) at each sampling time and were analysed; a total of 8 cheeses were analysed, plus 4 cheeses for sensorial analysis, per sampling point, as shown in Figure 1.1.

Aliquots of each cheese (≈35 g per sample) were stored at −80 °C until physicochemical analyses were carried out. Non-processed cheeses were used as controls ($Ch_C$).

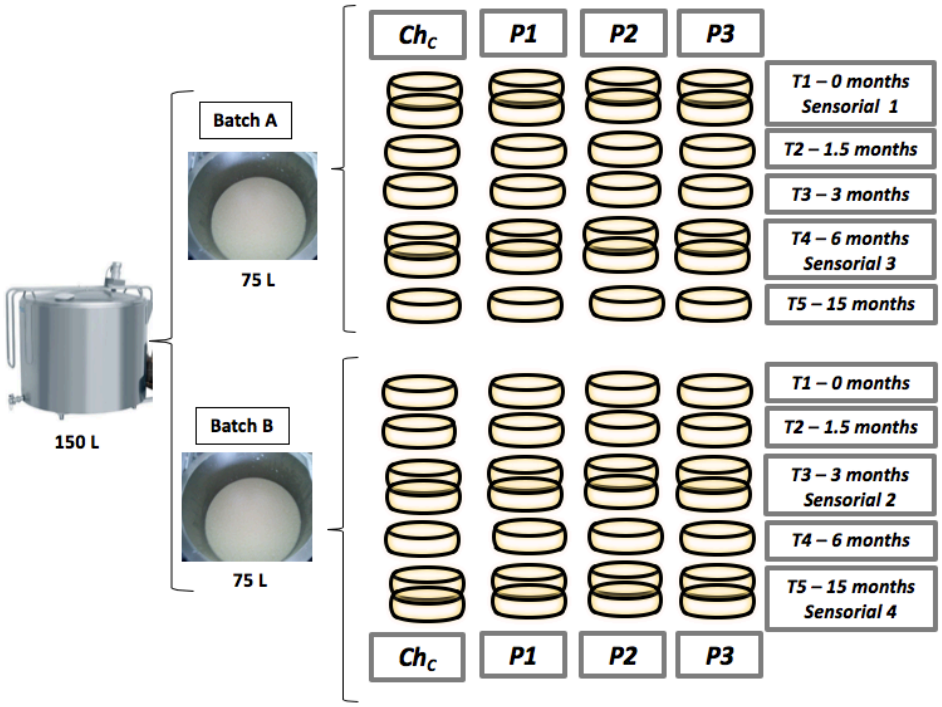


**Figure 3.1: Schematic representation of cheese production and sampling.**

### 3.2.4. Microbiological analyses

Cheeses were cut in half and a thin slice was cut through the innermost, the intermediate and the outermost layers of cheese, the rind was removed, and the combinations of all three of them mixed to obtain a single 10 g cheese sample. This sample was aseptically handled, and homogenised for 4 min using a 2 % (w/v) aqueous sodium citrate solution as extraction buffer in a Stomacher Lab-Blender 400 (Milano,

Italy). Aliquots of 1.5 mL were then taken and decimally diluted in 13.5 mL of sterile 0.1% (w/v) aqueous peptone, decimal dilutions were subsequently prepared and these were then plated in triplicate on several culture media. The following microbial groups were enumerated, using the pour plate method: *Enterobacteriaceae* on violet red bile dextrose agar (VRBDA from Merck, Germany); coliforms and *E. coli* on chromocult coliform agar (CCA from Merck) both incubated at 37 ºC for 1 d. The spread plate technique was used for enumeration of: *Enterococcus* spp. on kanamycin aesculin azide agar base (KAAA from Oxoid, United Kingdon) and incubated at 37 ºC for 1 d; *Lactobacillus* spp. on Man, Rogosa and Sharpe (MRS from Merck) and incubated at 30 ºC for 3 d; *Lactococcus* spp. on M17 (Liofilchem Italy) and incubated at 30 ºC for 3 d; and *Bacillus* spp. on HiChrome (from Fluka, India) and incubated at 30 ºC for 2 d. The Miles and Misra technique (Miles *et al.*, 1938) was used for enumeration of: total aerobic mesophilic microorganisms on plate count agar (PCA from Merck) and incubated at 30 ºC for 3 d; total anaerobic microorganisms on PCA and incubated at 37 ºC for 2 d in anaerobic jars (Merck) with Merck Anaerocult A (Merck); total psychotrophic microorganisms on PCA and incubated at 20 ºC for 5 d; yeasts and moulds on rose-bengal chloramphenicol agar (RBCA from Merck) and incubated at 25 ºC for 5 d; *Staphylococcus* spp. on Baird-Parker agar (BPA from Merck) with egg yolk tellurite emulsion (Liofilchem) and incubated at 37 ºC for 2 d; *Listeria* spp. on PALCAM agar selective agar base (Liofilchem), with selective supplement for PALCAM (Liofilchem) and incubated at 37 ºC for 2 d; and *Pseudomonas* spp. on pseudomonas agar base (PAB from Liofilchem) with glycerol and pseudomonas CFC supplement (CFC from Liofilchem) and incubated at 30 ºC for 2 d. Petri dishes containing 30-300 and 10–100 colony forming units (cfu) were selected for counting for spread plate, pour plate; and Miles and Misra, respectively.

The results were converted into logarithmic decimals of the number of cfu per g of cheese sample, and values were considered below the limit of quantification of 2.0 log cfu/g for spread plate and pour plate techniques and 3.0 log cfu/g for Miles and Misra technique. Less than 1 log/mL was considered for milk samples due to direct liquid sample plating.

### 3.2.5. Physicochemical analyses

The pH was measured at room temperature, randomly on the cheese, using a properly calibrated pH/temperature penetration pH meter (Testo 205, Testo, Inc., New Jersey, USA). The titratable acidity was determined according to the AOAC Official Method 920.124, (2002), procedure, by titration to a pH end-point of 8.9 using an automatic titrator with pH meter (Crison – Titromatic 1S with pH electrode 50 14, Barcelona, Spain). Measurements of water activity were performed using a Novasina LabSwift water activity ($a_w$) analyser (Lachen, Switzerland), by direct reading at room temperature after proper stabilization. Moisture content was determined by drying approximately 2 g of cheese to a constant weight (ca. 24 h) at 105 °C using a laboratory oven drying equipment (Venticell, MMM Medcenter Einrichtungen GmbH, Munic, Germany). All these physicochemical analyses were performed in triplicate per cheese sample. Fat content was determined, in duplicate, by the method of Gerber following the Portuguese Standard Protocol NP-2105 (Norma Portuguesa 2105, 1983) and ISO 3433:1975 (ISO 3433, 1975), using a butyrometer calibrated in a range from 0 to 40% fat. The total nitrogen (TN) content was determined by the micro-Kjeldahl procedure (AOAC 2001.14) using 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).

### 3.2.6. Colour

Colour parameters were measured using a Minolta Konica CM 2300d (Konica MinoltaCM 2300d, Osaka, Japan) at room temperature. The colour parameters were recorded in CIE Lab system and directly computed through the original SpectraMagic NX software (Konica Minolta, Osaka, Japan), according to the International Commission on Illumination regulations. Cheeses were kept 1 h at room temperature before measurements. The colour parameters $L^*$, $a^*$, and $b^*$ were measured in each cheese surface/rind and core/interior. The total colour difference ($\Delta E^*$) was calculated using the following Equation 3.1:

$$\Delta \boldsymbol{E}^* = [(\boldsymbol{L}^* - \boldsymbol{L}_0^*)^2 + (\boldsymbol{a}^* - \boldsymbol{a}_0^*)^2 + (\boldsymbol{b}^* - \boldsymbol{b}_0^*)^2]^{1/2} \quad \text{Equation 3.1}$$

where $\Delta E^*$ is the total colour difference between a sample and the control (initial values at 0 months of storage); $b^*$ and $L_0^*$ are the lightness of the sample and respective control; $a^*$ and $a_0^*$ are the redness of sample and control, respectively; and $b^*$ and $b_0^*$ are the yellowness of sample and control, respectively. In addition chroma (Equation 3.2) and hue angle (Equation 3.3) were also recorded:

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad \text{Equation 3.2}$$

$$^{\circ}h = arctg\left(\frac{b^*}{a^*}\right) \quad \text{Equation 3.3}$$

Measurements were performed selecting six random spots, read in triplicate, per cheese section (surface and core).

### 3.2.7. Statistical analyses

Analysis of variance (ANOVA) was performed to establish the effect of different processing conditions (three HPP treatments and control - $Ch_C$), the effect of storage and

the combined effect of processing conditions and storage. The significant difference Bonferroni test was applied to compare the mean values of parameters, with the significance assigned at $p < 0.05$. Mean values and standard error of mean are reported. SPSS software version 24.0 was used for the statistical analysis.

## 3.3. Results and discussion

### 3.3.1. Microbial composition of milk and fresh curd

Paramount to the safety of all raw milk cheeses is the microbiological quality of the raw milk. Ewes' milk samples revealed low microbial loads: *Enterobacteriaceae*, coliforms and enterococci viable cells numbers were found to be at a similar level, 2.40 ± 0.07, 2.25 ± 0.10 and 2.65 ± 0.04 log cfu/mL, respectively. *Escherichia coli* was detected at 1.44 ± 0.10 log cfu/mL. *Staphylococc*us spp. and *Pseudomonas* spp. were also quantified at 4.25 ± 0.91 and 3.30 ± 0.20 log cfu/mL. Counts of lactobacilli and lactococci were present at 2.33 ± 0.20 and 4.43 ± 0.34 log cfu/mL and total aerobic mesophilic bacteria counts at 4.60 ± 0.32 log cfu/mL. *Listeria* spp. and yeasts and moulds were not detected.

The coagulation of the milk followed by syneresis of the curd, concentrates the microorganisms in the curd. Salting of the curd also creates conditions that are not optimal for survival or growth of some microorganisms due to the decline of the water activity. The curd samples tested revealed viable cell numbers of 3.80 ± 0.04, 3.1 ± 0.9 and 3.30 ± 0.14 log cfu/g, for *Enterobacteriaceae*, coliforms and enterococci, respectively. *Escherichia coli* was detected at 3.10 ± 0.72, *Staphylococcus* spp. at 4.69 ± 0.08 and *Pseudomonas* spp. were measured at 4.71 ± 0.07, while lactobacilli and lactococci were counted at 8.89 ± 0.01 and 9.19 ± 0.10, respectively.

### 3.3.2. Changes in *Serra da Estrela* cheese microbial composition induced by HPP

#### 3.3.2.1. Lactic acid bacteria, enterococci, total aerobic mesophilic, anaerobic and psychotropic bacteria

Lactic acid bacteria (LAB), enterococci and total microbiota viabilities were significantly affected by HPP, the storage time and by the combination of both ($p < 0.05$). Figure 3.2 shows the effect of HPP on *Serra da Estrela* cheese microbiota, upon HPP and throughout the 15-month storage period. While treatment P1 was revealed to have the greatest impact on microbial load decrease (Figure 3.2 and Table 3.1), independently of the microbial group, no significant differences were visible among the treatments at 450 MPa (P2 vs P3) ($p > 0.05$).

Lactobacilli and lactococci were reported at similar orders of magnitude (Figure 3.2 A and B, respectively), independently of the treatment, although in general, lactococccci viable cell numbers were slightly higher, corroborating results reported in the literature for this type of cheese (Tavaria *et al.*, 2006; Tavaria and Malcata, 2000). In $Ch_C$ cheeses, the high lactobacilli ($9.55 \pm 0.04$ log cfu/g; Figure 3.2 A) and lactococci ($9.80 \pm 0.1$ log cfu/g; Figure 3.2 B) viable cell numbers reached at 45 days ripening were similar to those reported in a previous study (8 – 10 log cfu/g) during 100 days of storage at 4 ºC (Inácio *et al.*, 2014); Tavaria and Malcata (2000) also reported that these microbial groups counts were never below the 8 log cfu/g at 30 and 60 days of ripening. Despite these high viable cell numbers, upon HPP the lactobacilli counts were lowered 3.20-3.55 log cycle units in all treated cheeses, without significant differences among treatments P1, P2 and P3 ($p > 0.05$).

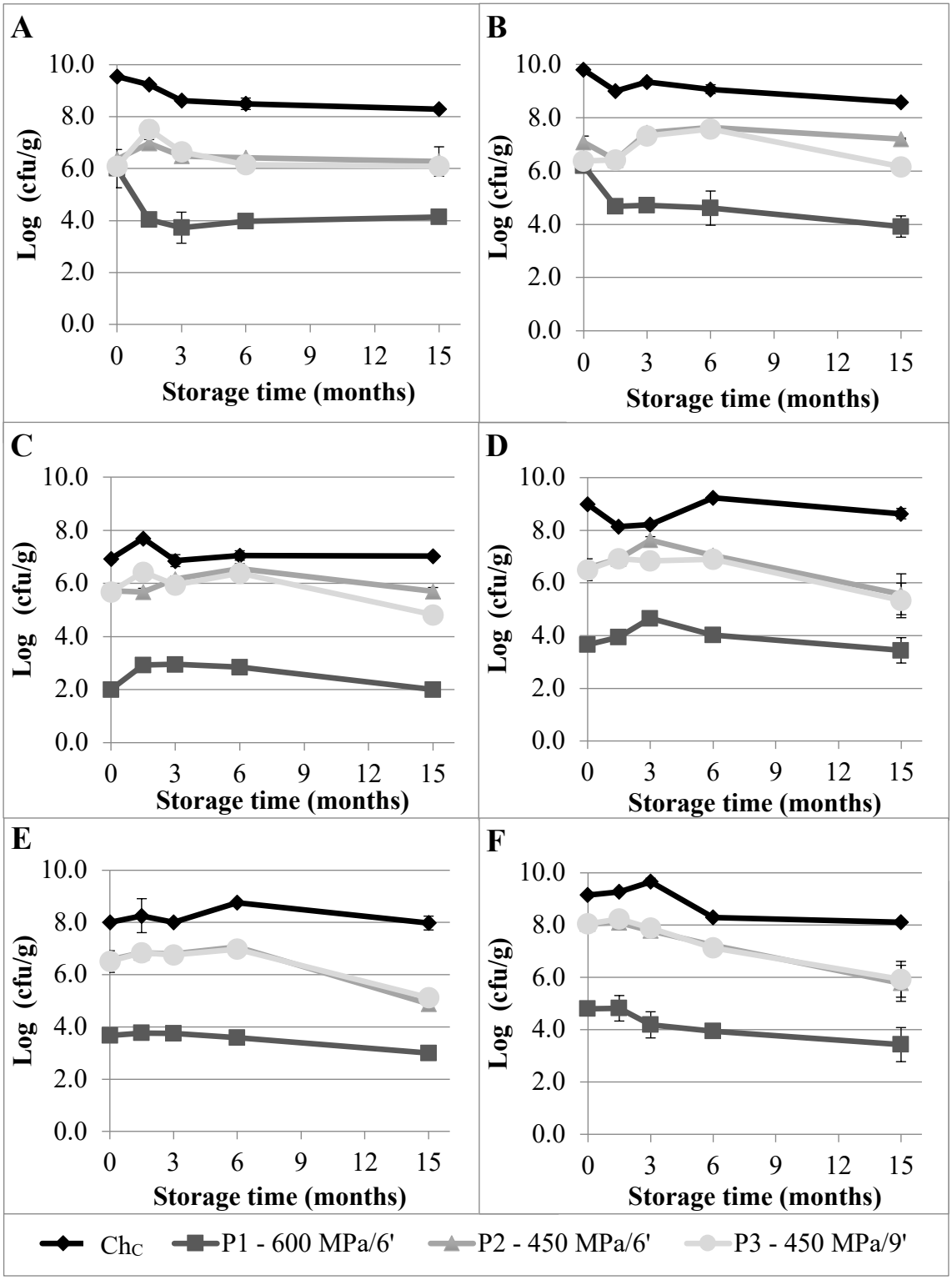


**Figure 3.2: (A) Lactobacilli, (B) lactococci, (C) enterococci, (D) total aerobic, (E) anaerobic and (F) psychrotrophic microorganisms viable cell numbers in *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage (4 ºC), of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3).**

Lactococci counts appeared to be more baroresistant than lactobacilli, in particular, at lower pressure intensity (P2 vs P1), reporting a 2.71 log cycle decrease upon HPP; higher pressure (P1) or longer time period (P3) led to a similar lowering effect as for lactobacilli, namely 3.60 and 3.41 log cycles decrease, respectively, without significant differences among P1 and P3 ($p > 0.05$). Indeed, the more intense pressure treatment (P1) caused a greater impact on LAB's viability ($p < 0.05$), than the increase in time under pressure (by comparing P2 with P3) ($p > 0.05$).

Enterococci are an important microbial group involved in the development of *Serra da Estrela* organoleptic quality given their complex enzyme make-up. Their counts in $Ch_C$ cheeses were found to be around 7 log cfu/g without significant losses along the 15 months storage period ($p > 0.05$) (Figure 3.2 C), which is in agreement with the results of Dahl *et al*., (2000). Immediately after HPP, the processing caused a similar 1.21 log cycle reduction in enterococci viable cell numbers in cheeses treated at 450 MPa (P2 and P3) ($p > 0.05$), while the decrease in cheeses treated at 600 MPa (P1) was greater than 4.93 log cycle units (to viable cell numbers below the detection limit). Similar results were reported by Calzada *et al*., (2013) in *Torta del Casar* ewe cheese (with 5 weeks of ripening) treated at 400 and 600 MPa/5 min ( $\approx 0.6$ and $\approx 4.6$ log cycle units).

In general, the viable cell numbers of total aerobic mesophilic, anaerobic and psychotropic microorganisms in $Ch_C$ cheeses remained stable over storage without significant differences ($p > 0.05$), as can be observed in Figure 3.2 D-F. Similar total aerobic counts and behaviour (8-10 log) were already reported by Macedo *et al*., (1996) at 35 days of ripening and by Inácio *et al*., (2014) for 100 days of storage. Immediately after HPP at 450 MPa (P2 and P3), about 2.4, 1.4 and 1.1 log cycle reductions were determined for mesophilic, anaerobic and psychotropic microbiota, respectively, without significant differences among P2 and P3 cheeses ($p > 0.05$); while 5.32, 4.34 and 4.35 log

cycle reductions were achieved at 600 MPa (P1) ($p < 0.001$), respectively. A previous study on *Serra da Estrela* cheese pieces revealed that HPP caused lower reductions (0.47-1.20 log cycles) independently of the pressure treatment applied (Inácio *et al.*, 2014). Likewise, 1.29 and 1.44 log cycle reductions were reported for total microbiota after similar HPP treatments in *Torta del Casar* cheese (Rodríguez-Pinilla *et al.*, 2015) and in *La Serena* cheeses (Arqués *et al.*, 2006). On the other hand, another study performed on *Torta del Casar* cheese reported 1.33 to 4.43 log cycle reductions in total viable cell numbers after HPP at 400 and 600 MPa/5 min at 35 days of ripening (Calzada *et al.*, 2013), which are aligned with those observed in the present work. Furthermore, Delgado *et al*., (2012) studied the effect of HPP (500 MPa/7 min) on *Ibores* cheeses (raw goat milk), and also verified a significant decrease in viable cell numbers of psychrotrophic microorganisms and at a similar order of magnitude (1.1 log cycle reductions) at 50 days of ripening.

#### 3.3.2.2. Contaminant microbial groups

The evolution of the studied microbial contaminants of *Serra da Estrela* cheese is depicted in Figure 3.3. At 0 months control *Serra da Estrela* cheeses ($Ch_C$) revealed viable cell numbers between 6-9 log cfu/g for all contaminant microbial groups tested except for *E. coli* (Figure 3.3 C). Over storage time viable cell numbers remained stable for coliforms (Figure 3.3 B) and *Bacillus* (Figure 3.3 F) ($p > 0.05$) but decreased to levels ≤ 4 log cfu/g for *Enterobacteriaceae* (Figure 3.3 A), *Pseudomonas* spp. (Figure 3.3 D) and *Staphylococcus* spp. (Figure 3.3 E) ($p < 0.001$). The loss in viability over time may possibly be due to competition between existing species (Tavaria and Malcata, 2000). Literature reports a large decrease in viable cell numbers of *Enterobacteriaceae* in this cheese over ripening (Dahl *et al.*, 2000; Inácio *et al.*, 2014).

Independently of the microbial group considered, and the associated microbial load present at 0 months, HPP treatment was able to effectively reduce viability either to levels below detection limit in the case of *Enterobacteriaceae* (Figure 3.3 A), *E. coli* (Figure 3.3 C) and *Pseudomonas* spp. (Figure 3.3 D) across all 3 treatments and remained stable throughout the storage period, or to 3-5 log cycles lower in the case of coliforms (Figure 3.3 B) or *Bacillus* spp. (Figure 3.3 F) in P1 treated cheese and *Staphylococcus* spp. (Figure 3.3 E) in all P1, P2 and P3 cheeses.

HPP treatment at 450 MPa, independently of application time (P2 and P3), promoted a milder effect than treatment at 600 MPa ($p < 0.01$), in the case of coliforms, staphylococci and *Bacillus* (Figure 3.3 B, E, F respectively). The significant decrease in viable cell numbers ($p < 0.01$) achieved by HPP at 450 MPa (about 0.3, 4.4 and 2.2 log cycles, respectively) was incremented further at 600 MPa (3, 6.4 and 4.7 log cycles, respectively).

Such overall behaviour is not always corroborated by similar studies with raw ewes' milk cheeses reported in literature. For example, minor reductions in *Enterobacteriaceae* (2.29 log cycles) or in *Pseudomonas* spp. (2.5 log cycles) were achieved in *Torta del Casar* cheese after HPP at 600 MPa/5 min at 60 days of ripening (Rodríguez-Pinilla *et al.*, 2015). In *La Serena* cheeses, while minor reductions in *Enterobacteriaceae* (1.98 log cycles) were also reported for cheeses after HPP at 400 MPa/10 min at 50 days of ripening, high log cycles reductions (> 5 log cycles) were reported for coliforms (Arqués *et al.*, 2006). Calzada *et al*., (2013) reported similar high log cycle reductions in total coliforms (> 3.5 log to counts below the detection limit) in *La Serena* cheese after HPP at 400 and 600 MPa/5 min at 35 days of ripening.

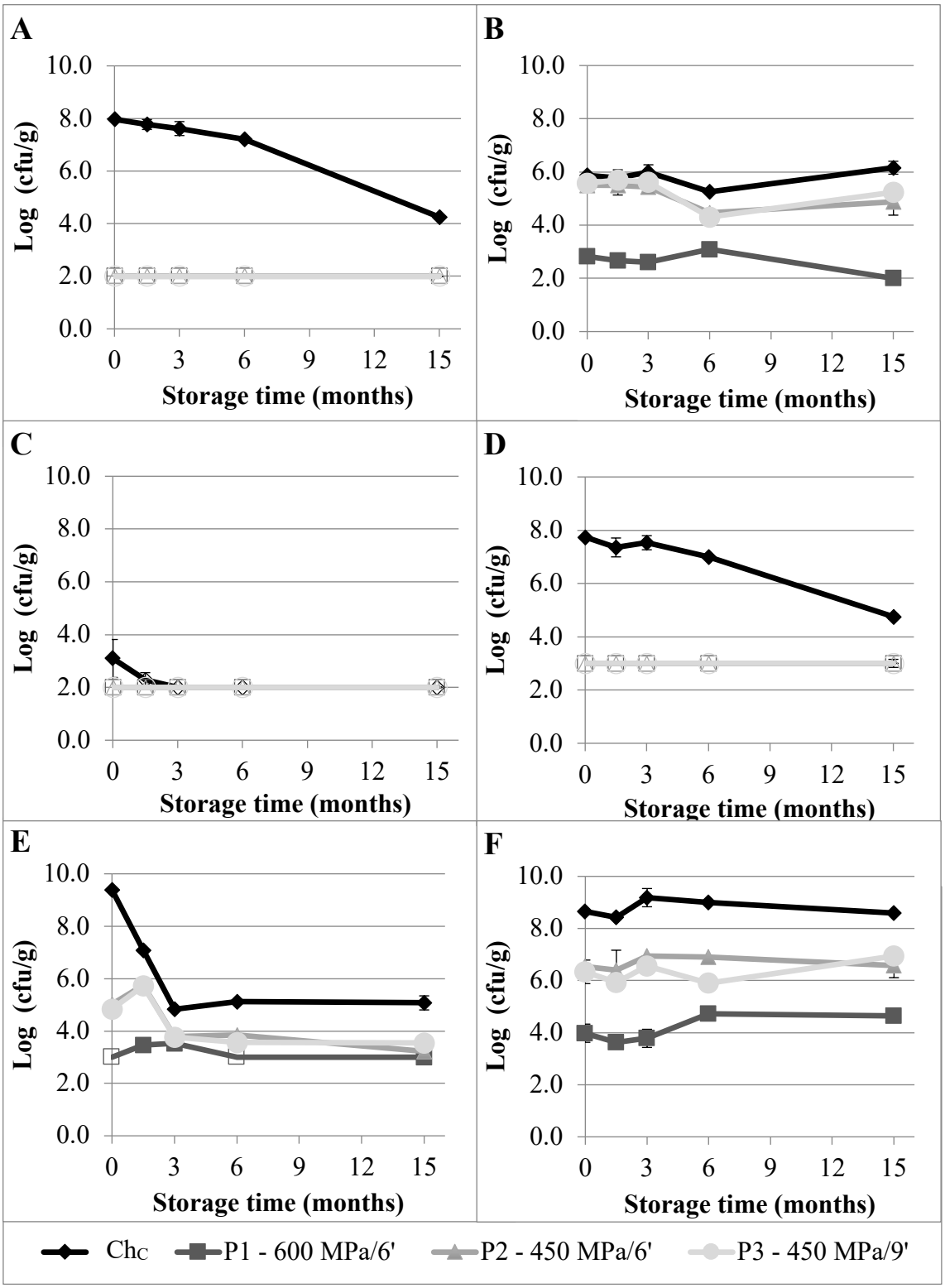


**Figure 3.3: (A) *Enterobacteriaceae*, (B) total coliforms, (C) *E. coli*, (D) *Pseudomonas* spp. (E) staphylococci and (F) *Bacillus* spp. viable cell numbers in *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage, of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3). Empty symbols represent microbial loads below the quantification limit.**

An important observation is related with viable cell numbers of staphylococci. The European Commission (European Commission, 2005) established staphylococci as a microbiology criterion to be analysed for cheeses made from raw milk. In $Ch_C$ cheeses staphylococci viable cell numbers decreased 4 log cycles to 4.83 ± 0.00 log cfu/g from 0 to 3 months of storage, remaining constant thereafter ($p > 0.05$) (Figure 3.3 E). The presence of staphylococci at 45 days of ripening in $Ch_C$ cheeses was above the established limit of $10^5$ cfu/g (European Commission, 2005); previous studies also reported levels above the required threshold at a similar ripening stage (6.60 log cfu/g at 42 days of ripening) of *Serra da Estrela* cheese (Macedo *et al.*, 2004). Despite the high viable cell numbers of staphylococci upon 45 days of ripening HPP treatment enabled a significant decrease ($p < 0.001$) of between 4-6 log cycles in cheeses treated at 450 MPa (P2, P3) or at 600 MPa (P1) (to below the detection limit). Cheeses treated at 600 MPa showed values below the detection limit or near the quantification limit (3 log cfu/g) during the 15 months of storage. Although, based on the European Commission criterion (2005), only the cheese submitted to HPP could be consumed at 0 months, from 3 months of storage onwards, all cheeses could be consumed including $Ch_C$ cheeses. Similar results were reported by Ávila *et al*., (2016) and Calzada *et al*., (2013), where HPP at 600MPa/5 min (at 35 days ripening and during storage of 240 days) staphylococci viable cell numbers were reduced to below the detection level. *Listeria* spp. were verified below detection limit in all cheeses during all storage period.

#### 3.3.2.3. Yeasts and moulds

Yeasts and moulds were present around 4 log cfu/g throughout the 15 months of storage (Figure A. 3.1) in $Ch_C$ cheeses ($p > 0.05$). Yeast loads reported in literature for *Serra da Estrela* cheese are variable, ranging in average between 3 and 6 log cfu/g

depending greatly on ripening and storage temperature and relatively humidity (Inácio *et al*., 2014; Macedo *et al*., 1996a, 1995; Tavaria and Malcata, 2000). HPP caused a reduction to below the quantification limit (< 3 log cfu/g equivalent to > 0.8 log reduction), in all treated cheeses and throughout the 6 months of storage. An identical effect was verified in a previous *Serra da Estrela* cheese study (using small cheese portions), although greater reductions were observed (≥ 3.6 log cycles, initial value of 5.58 log cfu/g) (Inácio *et al.*, 2014). Rodríguez-Pinilla *et al*., (2015) in *Torta del Casar* cheese reported 2.05 log cycle reductions of moulds after HPP at 600 MPa/5 min at 60 days of ripening. At 15 months of storage all cheeses revealed similar viable cell numbers of yeasts and moulds (3.6–4.2 log cfu/g), without significant differences in comparison to $Ch_C$ cheeses ($p > 0.05$).

#### 3.3.2.4. Overview of the effect of HPP on microbiota

As previously mentioned, the different microbial groups tested felt the impact of HPP differently, having expressed different reduction profiles at different pressure intensity, as can be observed in Table 3.1. The inactivation mechanisms induced by HPP have been correlated to cells damage in membranes, which are thought to be a primary target for HPP, in addition to enzyme denaturation and changes in cell morphology (Murchie *et al.*, 2005). HPP above 300 MPa induces enzyme and protein irreversible denaturation, alterations that have been shown to lead to ribosomes dissociation which may lead to limit the cell viability (Abe, 2007). In general, the changes in the different microbial groups induced by HPP are in agreement with those reported in literature. The prokaryotes microorganisms (bacteria) showed a higher resistance towards the pressure than eukaryotes microorganism (yeasts and moulds) (Georget *et al.*, 2015). Within bacteria, in general, Gram-positive bacteria (lactococci, lactobacilli, *Bacillus*,

staphylococci) were more resistant reflecting lower cycle reductions than Gram-negative bacteria (*E. coli* and *Pseudomonas*) (Smelt, 1998), which were reduced to below the detection limit. This different resistance can be correlated with the thicker peptidoglycan layer in Gram-positive microorganisms, which generally show to be more pressure resistance (Considine *et al.*, 2008; Murchie *et al.*, 2005; Smelt, 1998), but it also suggested that gram negative cell membrane complexity causes also more susceptibility to HPP (Shigehisa *et al.*, 1991). Among LAB bacteria, the coccoid shape were in general more resistance to HPP than rod-shape bacteria (Huang *et al.*, 2014), behaviour verified in general by the viable cell numbers inactivation of lactobacilli and lactococci.

**Table 3.1: Log reductions of HPP cheeses relatively to control *Serra da Estrela* cheese at 0 months of refrigerated storage.**

| | **P1 600 MPa/6'** | **P2 450 MPa/6'** | **P3 450 MPa/9'** |
|---|---|---|---|
| **Lactobacilli** | 3.55 | 3.20 | 3.47 |
| **Lactococci** | 3.60 | 2.71 | 3.41 |
| **Enterococci** | 4.93 | 1.21 | 1.24 |
| **Total aerobic microorganism** | 5.32 | 2.40 | 2.48 |
| **Total anaerobic microorganism** | 4.34 | 1.44 | 1.51 |
| **Total psychotropic microorganism** | 4.35 | 1.11 | 1.11 |
| ***Enterobacteriaceae*** | > 5.9 | > 5.9 | > 5.9 |
| **Coliforms** | 3.05 | 0.36 | 0.28 |
| ***Escherichia coli*** | > 1.1 | > 1.1 | > 1.1 |
| **Staphylococci** | 6.39 | 4.37 | 4.55 |
| ***Pseudomonas* spp.** | 4.74 | 4.74 | 4.74 |
| **Bacilli spp.** | 4.68 | 2.13 | 2.32 |
| **Yeasts and moulds** | > 1.1 | > 1.1 | > 1.1 |

### 3.3.3. Changes in physicochemical characteristics induced by HPP

#### 3.3.3.1. Moisture, fat and protein contents

As expected, the moisture content significantly decreased during 45 days of ripening from 59.7 ± 2.3 in curd to 47.8 ± 1.1 for $Ch_C$ cheeses ($p < 0.001$). The decrease in moisture content was caused by the natural and progressive water evaporation due to the relative humidity in the ripening chambers (Macedo *et al.*, 1997). Values inside the range 40.1-48.4 % were reported by Correia *et al*., (2016) and Macedo *et al*., (2004) at 42 days of ripening. At 0 months, no significant differences were found among the moisture content of $Ch_C$ and all HPP treated cheeses ($p > 0.05$). No significant differences were found between $Ch_C$ and P3 cheeses throughout 15 months of storage ($p > 0.05$). The results obtained in the present work are in agreement with those reported in literature, being HPP reported to cause no changes in the moisture content (Martínez-Rodríguez *et al.*, 2012). For example in *Torta del Casar* cheeses, Delgado *et al*., (2015) showed no significant differences in moisture content between control and HPP cheeses, despite the natural decrease during storage.

As far as the fat content is concerned, HPP had no significant ($p > 0.05$) effect on fat content values as listed in Table 3.2; values between 25 and 27 % were registered. Similar values have been reported for this type of cheese (Carocho *et al.*, 2016a, 2016b; Macedo and Malcata, 1997b). Delgado *et al*., (2012) also did not find significant differences in fat content of control and HPP *Ibores* cheeses.

$Ch_C$ cheeses revealed 22.0 ± 0.25 % (w/w) of protein content at 0 months of storage, which significantly increased to 23.7 ± 0.40 % ($p < 0.001$) up to 6 months, as shown in Table 3.2. A similar protein content (22.1 % (w/w) was determined by Macedo and Malcata (1997b) at 60 days of ripening, but lower values were recently reported by Correia *et al*., (2016) (14.69 – 19.35 % (w/w). HPP affected protein content in different

ways; at 0 months, treatment P1 revealed no significant difference ($p > 0.05$) whereas treatments P2 and P3 led to a higher or lower protein content ($p < 0.01$) in comparison to $Ch_C$ cheeses. Similarly, in *Ibores* cheeses, Delgado *et al*., (2012) quantified a high protein content in cheeses HPP treated at 400 MPa/7 min and significantly lower in HPP at 600 MPa/7 min treated cheeses at 50 days of ripening. In the present study, at 15 months of storage, no significant differences were found among HPP cheeses ($p > 0.05$), yet protein contents were a significantly higher compared to $Ch_C$ cheeses ($p < 0.05$).

**Table 3.2: Moisture, fat, protein content, pH values, titratable acidity and water activity measured in *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage of different HPP treatments and control cheeses ($Ch_C$).**

| | $Ch_C$ | | P1 - 600 MPa/6' | | P2 - 450 MPa/6' | | P3 - 450 MPa/9' | |
|---|---|---|---|---|---|---|---|---|
| **Water Content** | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | |
| 0 | 47.8 ± 1.10 | b,A | 49.4 ± 0.20 | a,A | 47.8 ± 0.89 | b,A | 48.0 ± 0.82 | b,A |
| 1.5 | 46.0 ± 0.31 | a,B | 45.8 ± 0.58 | a,B | 45.8 ± 1.02 | a,B | 45.4 ± 1.17 | a,B |
| 3 | 44.5 ± 0.86 | a,C | 44.1 ± 0.41 | a,D | 44.8 ± 0.18 | a,B,C | 44.4 ± 0.26 | a,B,C |
| 6 | 43.6 ± 1.18 | b,C | 44.5 ± 0.49 | a,b,C,D | 45.1 ± 0.37 | a,B,C | 43.9 ± 1.00 | a,b,C |
| 15 | 44.5 ± 0.21 | a,b,C | 44.9 ± 0.52 | a,C | 44.4 ± 0.56 | a,b,C | 43.9 ± 0.71 | b,C |
| **Fat Content** | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | |
| 0 | 26.1 ± 1.25 | a,B | 24.9 ± 1.97 | a,B | 25.4 ± 1.31 | a,B | 26.5 ± 1.47 | a,A |
| 3 | 27.4 ± 0.25 | a,A,B | 28.0 ± 0.71 | a,A | 27.9 ± 0.25 | a,A | 28.4 ± 1.11 | a,A |
| 6 | 28.3 ± 1.19 | a,A | 28.9 ± 1.60 | a,A | 26.5 ± 0.41 | a,A,B | 27.1 ± 1.49 | a,A |
| 15 | 28.1 ± 0.48 | a,A | 27.6 ± 0.48 | a,A,B | 27.9 ± 1.77 | a,A | 27.9 ± 0.75 | a,A |
| **Protein Content** | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | | **% (w/w) STD** | |
| 0 | 22.0 ± 0.25 | b,B | 22.6 ± 0.15 | a,b,B | 23.5 ± 0.29 | a,A | 20.4 ± 0.32 | c,C |
| 3 | 23.3 ± 0.19 | a,A | 22.4 ± 0.44 | b,c,B | 23.1 ± 0.19 | a,b,A | 21.8 ± 0.56 | c,B |
| 6 | 23.7 ± 0.40 | a,A | 23.1 ± 0.48 | a,A,B | 22.8 ± 0.41 | a,A | 23.5 ± 0.55 | a,A |
| 15 | 22.5 ± 0.29 | b,B | 23.7 ± 0.33 | a,A | 23.5 ± 0.34 | a,A | 23.8 ± 0.60 | a,A |
| **pH values** | **pH STD** | | **pH STD** | | **pH STD** | | **pH STD** | |
| 0 | 5.36 ± 0.17 | A,a | 5.27 ± 0.17 | B,a | 5.24 ± 0.01 | B,a | 5.23 ± 0.02 | B,a |
| 1.5 | 5.39 ± 0.07 | A,a | 5.34 ± 0.01 | A,a | 5.33 ± 0.02 | A,a | 5.36 ± 0.06 | A,a |
| 3 | 5.25 ± 0.04 | A,a | 5.22 ± 0.01 | C,a,b | 5.19 ± 0.02 | C,b | 5.21 ± 0.01 | B,C,b |
| 6 | 5.29 ± 0.02 | A,a | 5.17 ± 0.01 | D,b | 5.17 ± 0.01 | C,b | 5.15 ± 0.03 | C,b |
| 15 | 5.26 ± 0.02 | A,a | 5.22 ± 0.01 | C,b | 5.17 ± 0.01 | C,b | 5.18 ± 0.05 | B,C,b |

| **Titratable acidity** | **$g_{lactic\ acid}$/100 g** | **STD** | | **$g_{lactic\ acid}$/100 g** | **STD** | | **$g_{lactic\ acid}$/100 g** | **STD** | | **$g_{lactic\ acid}$/100 g** | **STD** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.694 ± | 0.008 | E,a | 0.825 ± | 0.085 | D,a | 0.814 ± | 0.177 | D,a | 0.801 ± | 0.086 | E,a |
| 1.5 | 1.09 ± | 0.059 | D,a | 1.00 ± | 0.097 | C,a | 1.03 ± | 0.026 | C,a | 1.02 ± | 0.086 | D,a |
| 3 | 1.27 ± | 0.073 | C,a | 1.17 ± | 0.048 | B,C,b | 1.18 ± | 0.058 | C,a,b | 1.21 ± | 0.051 | C,a,b |
| 6 | 1.50 ± | 0.101 | B,a | 1.20 ± | 0.086 | B,b | 1.63 ± | 0.063 | B,a | 1.47 ± | 0.173 | B,a |
| 15 | 1.94 ± | 0.056 | A,a | 1.61 ± | 0.158 | A,b | 1.84 ± | 0.097 | A,a | 1.88 ± | 0.088 | A,a |
| **Water activity** | **$a_w$** | **STD** | | **$a_w$** | **STD** | | **$a_w$** | **STD** | | **$a_w$** | **STD** | |
| 0 | 0.959 ± | 0.002 | a,A | 0.959 ± | 0.004 | a,A | 0.957 ± | 0.001 | a,A | 0.956 ± | 0.002 | a,A |
| 1.5 | 0.954 ± | 0.005 | a,A,B | 0.953 ± | 0.001 | a,B | 0.952 ± | 0.002 | a,B | 0.951 ± | 0.001 | a,B |
| 3 | 0.953 ± | 0.001 | a,b,B | 0.954 ± | 0.001 | a,A,B | 0.950 ± | 0.003 | c,B | 0.951 ± | 0.001 | b,c,B |
| 6 | 0.950 ± | 0.002 | b,B | 0.954 ± | 0.001 | a,A,B | 0.951 ± | 0.000 | b,B | 0.950 ± | 0.000 | b,B |
| 15 | 0.928 ± | 0.004 | b,C | 0.935 ± | 0.006 | a,C | 0.932 ± | 0.003 | a,b,C | 0.933 ± | 0.004 | a,b,C |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 3.3.3.2. pH values and titratable acidity

The milk average pH values were 6.69 ± 0.07, which decreased to 6.53 ± 0.06 in curd/fresh cheese. At 0 months of storage cheese pH values had decreased to 5.36 ± 0.17, remaining fairly constant over 15 months of storage, particularly from 3 months sampling point onwards ($p > 0.05$), as shown in Table 3.2. The pH variations observed are naturally related to the microbiota metabolism, mainly due to the production and consumption of lactic acid during cheese ripening (Macedo *et al.*, 1996a, 1995; Macedo and Malcata, 1997c). Similar pH values have been reported in literature (4.82-5.66) (Guiné *et al.*, 2016; Inácio *et al.*, 2014; Macedo *et al.*, 2004; Sousa and Malcata, 1997). HPP did not affect initial pH values; at 0 months of storage, no significant differences among $Ch_C$ and HPP treated cheeses were reported for pH and titratable acidity (TA) values ($p > 0.05$). The pH values of HPP cheese were maintained within limits reported for $Ch_C$ cheeses (5.23 and 5.39) throughout storage, except at 6 months where a slight decrease was observed in comparison to $Ch_C$ cheeses ($p < 0.001$). Martínez-Rodríguez *et al*., (2012) reviewed that pressure treatments modify the pH to an extension degree that differs according to treatment conditions and cheese age, where the pH differences between HPP and untreated samples fade out as the ripening process progresses. In the present study, although no significant differences were found in the first days of storage, significant differences were verified toward the end of the storage period (Table 3.2). Calzada *et al*., (2014a) reported a similar behaviour, having control cheeses revealed higher and significantly different pH values in relation to HPP cheese, only from day 120 onwards. Evolution of TA values were, in general, correlated with pH values; lower pH values corresponded to higher TA values. Over 15 months storage, TA values increased steadily and significantly ($p < 0.001$) to values between 1.61-1.94 g lactic acid/100g (fresh curd had 0.178 g lactic acid/100g). Lower TA values were determined for P1 cheeses, with

significant differences ($p < 0.05$) at 6 and 15 months relatively to other cheeses. *Serra da Estrela* cheese acidification depends on indigenous microbial viable cells numbers (Macedo *et al.*, 1993). Thus, this trend may be correlated with the number and type of microbiota present, which was higher and more diverse in $Ch_C$ cheeses than in HPP cheese samples (P1 cheeses underwent the highest reduction in bacterial viable numbers), as well as with the possible reduced ability to produce acid compounds induced by HPP (Martínez-Rodríguez *et al.*, 2012), resulting in very low TA values. A similar behaviour was reported in a previous study done with small portion samples of *Serra da Estrela* cheese (Inácio *et al.*, 2014)

#### 3.3.3.3. Water activity

As expected water activity ($a_w$) values depended strongly on storage time ($p < 0.001$) in both $Ch_C$ and HPP treated cheeses; $a_w$ decreased in all cheeses from 0.959, by 0 months to 0.928 at 15 months for $Ch_C$ cheeses, and from 0.956-0.959 to 0.932–0.935 at 15 months for HPP cheeses (Table 3.2). Ávila *et al*., (2016) also reported a decrease of $a_w$ for HPP and control raw ewe cheeses over 60 days of ripening. In the present study, during 3 months no significant $a_w$ differences were found among all cheeses, similarly to what had been already been reported in the previous study of 100 days of storage (Inácio *et al.*, 2014). During the whole storage period no significant $a_w$ differences ($p > 0.05$) were observed between $Ch_C$ and P3 cheeses.

#### 3.3.3.4. Colour

Colour analysis is a relevant parameter to understand the HPP and storage time impacts on visual appearance of cheese surface (Figure 3.4), but also in cheese core. *Serra da Estrela* cheeses are recognized by the slightly yellowish colour, which can be

instrumentally measured by the CIE *b** values. In this study, it was shown that HPP had an impact on both cheese surface and cheese core; HPP cheeses showed higher CIE *b** values, more yellowness, than the $Ch_C$ cheeses throughout the storage period. The cheese surface CIE *b** values decreased slightly until the end of storage (Table A. 3.1). The *b** values variations measured on $Ch_C$ cheeses (19.77 and 22.78) along 15 months were similar to those that Correia *et al*., (2016) reported (19.55 – 22.17). A similar behaviour was reported by Delgado *et al*., (2013) and Delgado *et al*., (2015) for goat and ewe cheeses, respectively.

Total colour difference ($\Delta E^*$) was calculated relatively to ChC cheeses at 0 months, with the results being expressed in Figure 3.5 A for cheese surface and B for cheese core. In cheese surface a total difference between 8.03 to 13.7 for ChC cheeses and 7.38 to 15.5 for HPP cheeses were verified. HPP P3 cheeses revealed lower total differences, but the HPP P1 cheeses showed higher values. Minor total differences were quantified in cheeses core.

In addition, determination of chroma (*C**) revealed that the HPP cheeses had higher values on associated surface and core, indicating that HPP led to a superior cheese colour intensity, which decreased along storage in cheese surface and increased in cheese core (Figure A. 3.2 A and B) corroborating CIE *b** values trends. The hue degree (ºh) values were higher in HPP cheeses (fluctuated between 90.8 – 94.2 for cheese surface and 94.2 – 97.4 for cheese core); values near to 90º correspond to a visual yellowish colour. $Ch_C$ cheeses showed similar values (ranged from 90.0 – 91.9 for cheese surface and 93. – 94.1 for core, (Figure A. 3.2 C and D) during storage.

According to the literature, the colour variations induced by HPP can be correlated with changes in moisture content and/or with modifications in the protein matrix and/or due to proteolytic alterations (Delgado *et al.*, 2015).

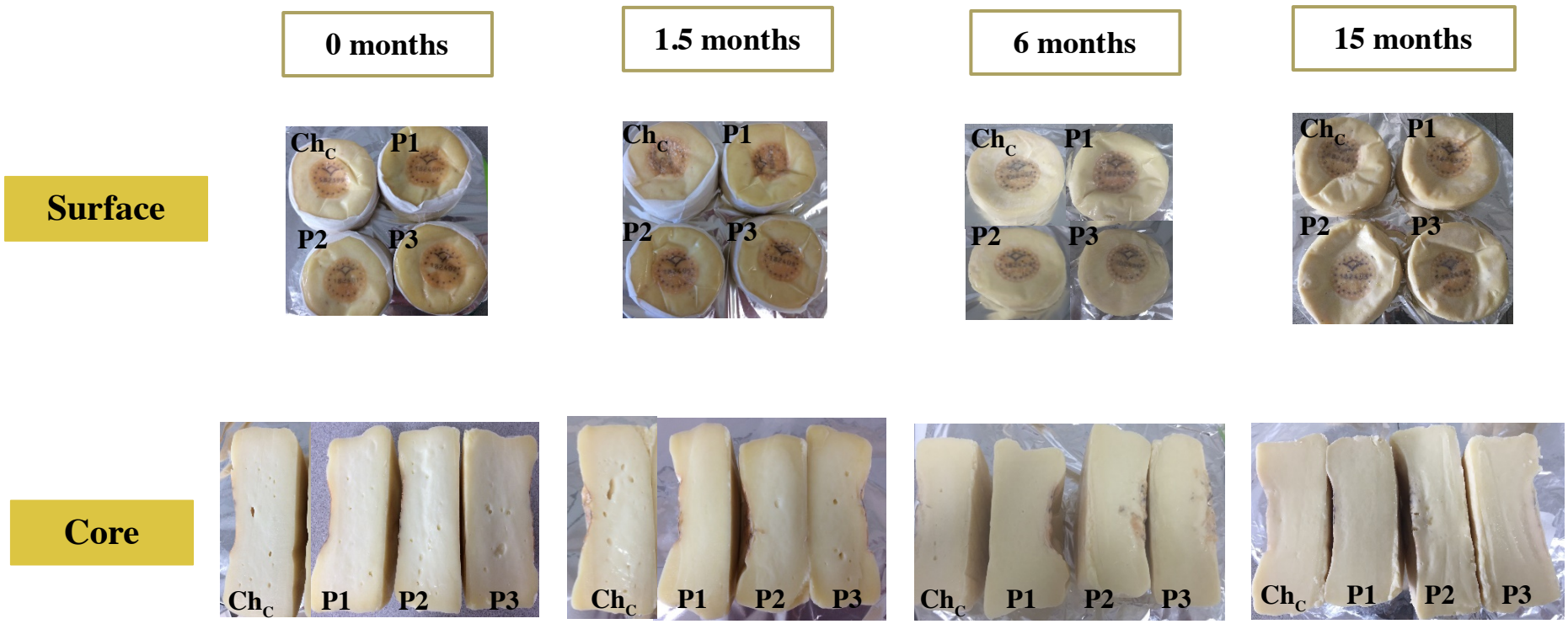


**Figure 3.4: Visual appearance of *Serra da Estrela* cheese at 0, 1.5, 6 and 15 months of refrigerated storage of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3).**

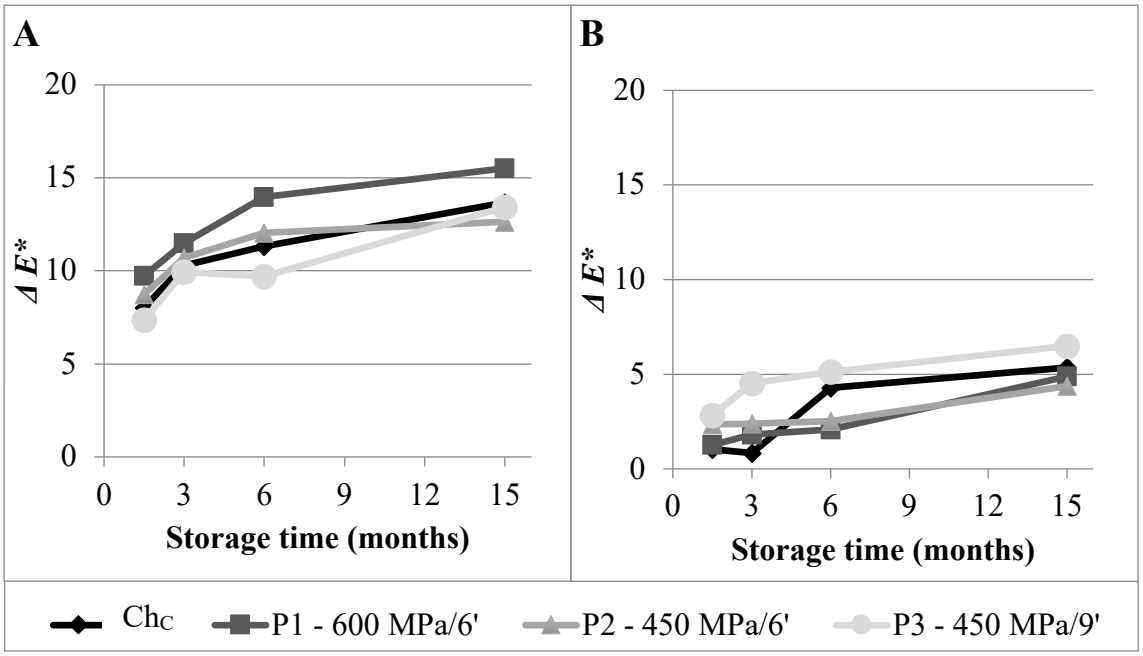


**Figure 3.5: Total colour differences in (A) cheese surface and (B) cheese core measured comparatively to control *Serra da Estrela* cheese at 1.5, 3, 6 and 15 months of refrigerated storage of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3).**

## 3.4. Conclusions

The results obtained in this work uphold a promising effect for the application of HPP to enhance microbial safety of *Serra da Estrela* cheese (a raw ewes' milk cheese) throughout extended storage (15 months shelf-life) without jeopardizing physicochemical quality. The nature of the HPP treatment applied was revealed to be significantly different: despite a few exceptions, in general, a longer period of time (6 min vs 9 min at 450 MPa) was not significant among all the parameters tested, whereas pressure intensity (450 MPa vs 600 MPa) was. In terms of microbial viable cell numbers, higher log cycle reductions were achieved in P1 cheeses treated at 600 MPa/6 min. If a minimal impact on microbial population with important metabolic activity for *Serra da Estrela* cheese (lactobacilli, lactococci, enterococci) is sought, while simultaneously inactivating pathogenic microorganisms, an intermediate pressure intensity can be the best treatment

to be applied. In what concerns cheese quality, interesting results were also obtained. HPP P2 cheeses showed similar moisture and fat content and higher protein content in comparison to $Ch_C$ cheeses. In cheese surface, smaller total colour differences were determined for HPP P3 (7.38–13.4), but a reverse effect was verified in cheese core (2.82-6.50), relativity to $Ch_C$ cheeses at 0 months. HPP P2 cheese surfaces showed similar total colour differences (8.73-12.7), in comparison to $Ch_C$ cheeses, along storage.

Such promising results open perspectives to probe further at the levels of proteolysis, lipid profiles, textural and sensory characteristics, to prove further the impact of HPP on biochemical and sensory quality, to further enhance effectiveness of such HPP treatment on preservation of *Serra da Estrela* quality over long term storage.

# CHAPTER 4 - Characterization of proteolysis, texture and sensorial attributes of HPP treated cheeses throughout storage

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*This chapter has been submitted for publication.*

## Abstract

One of the most appreciated traditional raw milk Portuguese cheeses, *Serra da Estrela* cheese with Protected Denomination of Origin (PDO), is well known for its unique flavour and texture, which are related with the use of raw ewe milk and production process. In this work, *Serra da Estrela* cheeses with 45 days of ripening were processed by high-pressure processing (HPP) at 600 MPa/6 minutes (P1), 450 MPa/6 minutes (P2) and 450 MPa/9 minutes (P3), to study the effect of HPP immediately and during 15 months storage at 4 ºC. The proteolysis indices were, in general, lower in HPP cheeses than in control chesses. HPP P1 cheeses kept the ripening extension index along 15 months of storage close to non-processed cheese at month 0. Progression of the ripening depth and free amino acids indices were also slowed down by HPP. HPP had no immediate effect on cheese texture parameters and minor changes were found up to 3 months of storage; moreover, HPP P2 cheeses maintained the hardness and consistency level during the 15 months of storage at values close to control cheeses at month 0. Sensory evaluation by trained panellists showed that HPP P2 cheeses were softer than control cheeses; furthermore, for HPP P3 cheese no treatment effects on the evaluated sensory attributes were uncovered at the end of storage. Overall, results uphold the potential of HPP processing in rendering *Serra da Estrela* cheese proteolysis levels similar to those of control cheese at 45 days of ripening with minor effects on texture.

## 4.1. Introduction

One of the seven wonders of Portuguese gastronomy is the raw ewe's milk *Serra da Estrela* cheese with Protected Designation of Origin (PDO) certification in the European Union. Its unique characteristics are mainly due to the milk's origin and characteristics and also to the traditional manufacture process; being the importance and relevance reported in CHAPTER 1- *Serra da Estrela* cheese: a review. These conditions result in a cheese with unique organoleptic characteristics, with a closed, moderately buttery, deformable when cutting, well connected, creamy and unctuous texture, with few or no eyes and smooth, clean and slightly acidic *bouquet* (Planning and Political Office, 2011). These organoleptic features are largely associated to proteolysis, the most important biochemical and complex process in cheese ripening. Proteolysis can be evaluated via proteolytic indices, mainly by the ripening extension index (ratio of water-soluble nitrogen to total nitrogen - WSN/TN ratio) that has been used to follow the aging of cheese. For *Serra da Estrela* cheese, this index was found to increase from 9.5–11 % at 1 day manufacture to 23-59 % at 35-180 days of ripening (Macedo *et al.*, 2004; Macedo and Malcata, 1997c; Reis and Malcata, 2011; Tavaria *et al.*, 2003). These high values were associated with the role the vegetable rennet plays in cheese proteolysis (Sousa and Malcata, 2002), causing a more extensive proteolysis which, in turn, is associated to a more homogeneous cheese structure, as well as increased creaminess and softness (Delgado *et al.*, 2015). Since *Serra da Estrela* cheese is a non-pasteurized product, manufactured from raw milk, microbial safety is a pertinent issue. From a safety point of view, previous results have demonstrated the usefulness of HPP to render *Serra da Estrela* cheese microbiologically safer during a more extended shelf-life of up to 100 (Inácio *et al.*, 2014). The observed effect of HPP on cheese properties is dependent on the pressure intensity, the holding time under pressure and the cheese ripening stage at which

it is applied. The effect of HPP on proteolysis (CHAPTER 2- High pressure processing on milk and raw milk cheese), in cheese manufactured with raw ewes' milk, has been studied during ripening of *La Serena* cheese (Garde *et al.*, 2007a); and during storage of *Torta del Casar* cheese (Calzada *et al.*, 2014a; Delgado *et al.*, 2015) and during storage of small portion pieces (~20 g) of *Serra da Estrela* cheese (Inácio *et al.*, 2014). *La Serena* cheese at 50 days of ripening was pressure treated at 300 and 400 MPa for 10 min at 10 °C and showed a similar proteolysis level compared to the control cheese after 10 days under conventional ripening conditions (Garde *et al.*, 2007a). During storage of *Torta del Casar* cheese, casein degradation was significantly retarded in HPP treated cheeses (600 MPa/5 min) (Calzada *et al.*, 2014a). Also, Delgado *et al.*, (2015) treated *Torta del Casar* cheese at 600 MPa/5 and 20 min at 60 days of ripening and verified a reduction in proteolysis of casein fractions (WSN/TN ratio decreased) compared with the control cheese after 240 days of storage. In a previous study with small portions of *Serra da Estrela* cheese, WSN content did not show significant differences between control and treated samples up to 100 days of refrigerated storage (Inácio *et al.*, 2014). According to these above mentioned studies, the HPP treatments, applied at the end of the ripening period, slowed down or maintained the casein hydrolysis state and nitrogen ratio comparable to that of the control cheese samples during storage. Thus, in this work the effects of HPP (450 for 6 or 9 min or 600 MPa for 6 min), applied to whole (~0.5 kg) *Serra da Estrela* cheeses after 45 days of ripening (Inácio *et al.*, 2014) on the evolution of cheese proteolysis, texture and sensorial properties over 15 months refrigerated storage period, were studied.

## 4.2. Materials and methods

### 4.2.1. Cheese manufacture, high pressure processing and sampling

As previously described in sections: 3.2.1 Cheese manufacture, High pressure processing, 3.2.2 High pressure processing and 3.2.3 Sampling.

### 4.2.2. Proteolytic indices

Proteolysis was monitored during storage by measuring the amount of nitrogen content of different cheese dispersions by the micro-Kjeldahl method (AOAC Official Method 2001.14, 2002; International IDF Standard 20D, 1993), using a Kjeltec system with a 2012 digester and a 1002 distilling unit (Tecator, Hoganas, Sweden). Cheese dispersions in water (WSN), in 12% (w/v) trichloroacetic acid (TCA) and in 5% (w/v) phosphotungstic acid (PTA) were prepared and the nitrogen content measured according to Macedo and Malcata (1997b). The analyses were run in duplicate per cheese. The quantity of nitrogen soluble in water, in 12% TCA and in 5% PTA was expressed as per unit mass of total nitrogen content (TN), and will be denoted hereafter as WSN/TN as ripening extension index, TCA/TN as ripening depth index and PTA/TN as free amino acid index.

### 4.2.3. Aminopeptidase activity

Aminopeptidase activity was measured, in triplicate, on an extract obtained by homogenizing 10 g of cheese with 20 mL of 10 mM sodium phosphate buffer, pH 7, at room temperature for 4 min in a Stomacher 80, followed by centrifugation (10000 x*g*, 15 min, 4 ± 1 ºC) and filtering through Filters Fiorinni 112A. Lysine *p*-nitroanilide (Lys-*p*-NA) and leucine *p*-nitroanilide (Leu-*p*-NA) were used as substrates at 1mM in 50 mmol

TRIS-HCl buffer, pH 7.0. The reaction mixture consisted of 275 µL substrate solution and 25 µL enzyme solution. The blank consisted in the same mixture without substrate in TRIS-HCl buffer. Assays were carried out at 30 ºC using a microplate spectrophotometer (Multiskan Go, Thermo Scientific, Thermo Fisher Scientific Inc., USA) and a Nunc UV plate of 96 wells. The absorbance of the *p*-nitroaniline released was read at 410 nm at 2 min intervals. Aminopeptidase activities were expressed in nmol of *p*-nitroaniline released per minute per g of cheese and presented as mean ± STD (n = 6) of triplicate determinations in two cheese-making experiments.

### 4.2.4. Instrumental texture profile analysis (TPA)

Each cheese, from each batch (batches A and B), was kept at room temperature (18 - 22 °C) for 2 h before analysis. Random cylinders of cheese (18 mm diameter) were taken with a cork borer inserted vertically through the cheeses from their top surface, crossing from side to side, and 3 mm were cut off from each side, corresponding to the cheese rind. For the analysis of texture it was used a texturometer TA-Hdi from Stable Micro system (England), connected to a 2 mm diameter probe. Each test was conducted as 2 sequential penetration events, of 10-mm penetration at a rate of 0.80 mm/s, separated by a rest period of 10 s. The tests generated a force-time curve, from which hardness (N), consistency (N/s), adhesiveness (N/s), cohesiveness and gumminess (N) were calculated (Bourne, 1978). All analyses were performed in sextuplicate per cheese.

### 4.2.5. Sensory evaluation

Sensory evaluation of $Ch_C$ and HPP cheeses was carried out by ten trained panellists from the Faculty of Biotechnology (CBQF, Porto, Portugal). Twelve sensory attributes

of the cheese paste, two appearance attributes, four odours (orthonasal olfaction) and two texture attributes were evaluated in the mouth along with three taste attributes and after-taste intensity. Sensory sessions took place at the ISO 8589:2007 compliant sensory evaluation laboratory of CBQF, equipped with white fluorescent lighting (6500 K). Analyses were carried out at room temperature (18-22 °C).

Attribute difference-from-control method was used to compare the magnitude of difference between each attribute of the HPP cheeses relatively to $Ch_c$ (control sample), using a bipolar anchored continuous scale (-10 to +10, 0=no difference). The attributes evaluated by panellists were: appearance (colour - from much lighter to much darker than the control; consistency - from much more fluid to much firmer than the control ) odour (lactic, acid, animal/stable and short-chain fatty acids (SCFA, vomit like odour), - from much less intense to much more intense than the control), texture (consistency - from much softer to much harder than the control, and friability - from much less friable to much more friable than the control), taste (salty, acid and bitter, - from much less intense to much more intense than the control) and after-taste (much less intense to much more intense than the control). Panellists were additionally asked to record defects or taints present in the samples.

In each testing day, the cheeses were removed from the refrigeration about 1 h prior to evaluation and kept at room temperature. The rind was removed and the cheeses were cut in slices with 0.7 cm of thickness of outermost cheese layer. Cheese slices were presented to panellists in labelled Petri dishes. A sample of $Ch_c$ identified as control was presented to each panellist, along with a second sample of this cheese coded with a three digit random number (blind control sample) and samples HPP also coded using three digit random numbers. The presentation order of coded samples was randomized across panellists. Mineral water and slices of granny smith apples were provided to the

panellists to cleanse their palates between samples. The panel sessions were held mid-morning. A Qualtrics (Qualtrics, LLC.) online questionnaire was used.

### 4.2.6. Statistical analysis

Experimental data were analysed by analysis of variance (ANOVA) to determine the main effects and interactions of different processing conditions (three HPP treatments and control - $Ch_C$) and of storage on all variables tested. The significant difference Bonferroni test was applied to compare the mean values of parameters, with the significance assigned at $p < 0.05$. Sensory data was analysed by paired Student t-test comparing the results for each HPP cheese with the $Ch_C$ (blind control sample) with the significance assigned at p-value < 0.05. When the distribution of the differences between the control and treated cheeses failed to follow a normal distribution, the non-parametric Wilcoxon test was applied. SPSS software version 24.0 was used for the statistical analysis.

## 4.3. Results and discussion

### 4.3.1. Effect of HPP on proteolytic indices

The assessment of the extent of proteolysis in cheese is of interest as an index of cheese maturity and quality. For each type of cheese the ideal ripening time is established based on achievement of desired texture, aroma and flavour properties. The evolution of the three proteolytic indices for *Serra da Estrela* cheeses, immediately upon HPP and throughout 15 months of refrigerated storage, are shown in Figure 4.1. The ripening extension index (WSN/TN ratio) is typically used to follow the aging of cheese, being proportional to proteolytic enzyme activity. It reflects the direct action of rennet, retained

in the curd after manufacture, on casein and consists of large to medium-sized peptides (Pereira *et al.*, 2008b).

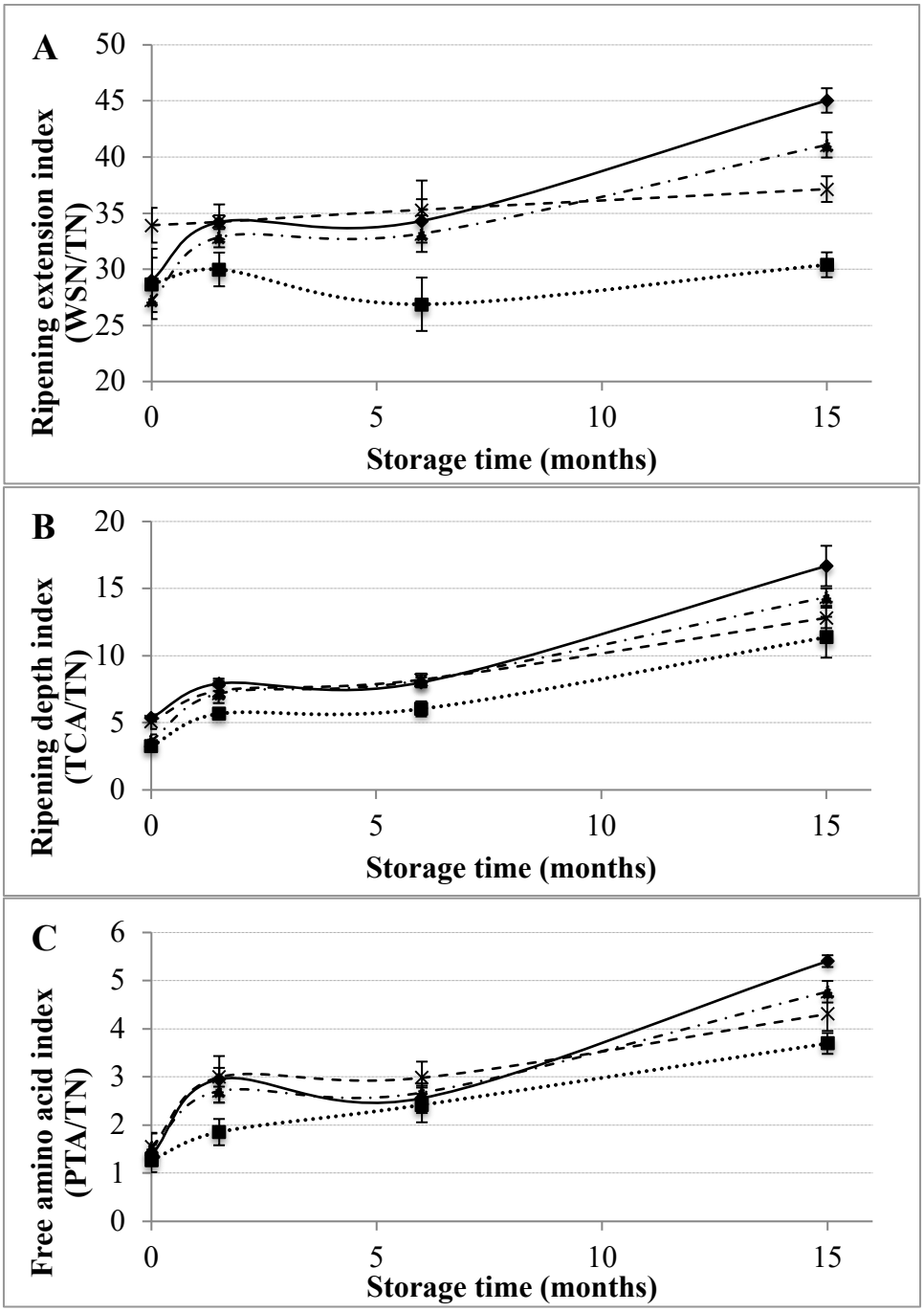


**Figure 4.1: (A) Ripening extension index (WSN/TN), (B) ripening depth index (TCA/TN) and (C) free amino acid index (PTA/TN) of *Serra da Estrela* cheese at 0, 1.5, 6 and 15 months of storage, for control cheeses (◆, $Ch_C$) and HPP cheeses (■ P1, ▲ P2 and X P3).**

At the beginning of storage (0 months), $Ch_C$ cheeses were characterised by WSN/TN of 29± 2.0 %. This index varies throughout ripening and all through the cheese-making season, having Macedo and Malcata, (1997a, 1997b) reported values between 27 and 36% of WSN/TN for *Serra da Estrela* cheese at 35 days of ripening. During the 15 months of refrigerated storage, this index increased steadily up to 1.5 months of storage, stabilized between 1.5 and 6 months of storage and then increased steadily again up to a final value of 45 ± 1.1 % WSN/TN at 15 months of storage. Similar values, between 23-59 % were reported for this type of cheeses within 35-180 days of ripening. In what concerns the HPP treated *Serra da Estrela* cheeses different behaviours were observed compared to the control $Ch_C$ cheese. At the beginning of storage (0 months), immediately after HPP treatments of 6 minutes of holding time, independently of the pressure intensity (P1 and P2), there were no significant changes to the WSN/TN index ($p > 0.05$) compared to the $Ch_C$ cheese. On the other hand, HPP treatment for a longer period of time (P3 – 450 MPa/9 minutes) caused a significant 17 % increase in the corresponding WSN/TN index ($p < 0.05$) immediately after HPP, yet no further changes were observed along the 15 months storage of these P3 cheeses ($p > 0.05$).

In general, a deceleration of the ripening extension index was verified for all HPP treated cheeses during 15 months of storage, especially for those treated under higher pressure (P1, 600 MPa) ($p > 0.05$). This behaviour was also verified by Delgado *et al.*, (2015) for HPP treated *Torta del Casar* cheeses (600 MPa/20 min) at 60 days of ripening and stored for 240 days. Application of HPP at the end of ripening (42 – 60 days) also lead to a lower proteolysis levels for *Ibores* and *La Serena* cheeses (Delgado *et al.*, 2013; Garde *et al.*, 2007a). Notably, P1 cheeses maintained their WSN/TN index stable during the whole storage period, with values ranging between 27 – 30 %, values similar to those of the $Ch_C$ cheese at 0 months (29 % obtained at 45 days of ripening considered to be the

best condition to generate ideal sensorial properties). Such results indicate that this HPP treatment (600 MPa/6 min) when applied to 45 days-ripened *Serra da Estrela* cheese (0 months storage), may halt proteolysis during 15 months of storage, keeping cheese properties at ideal levels, comparable to those of 45-day ripened $Ch_C$ cheeses. This observation is of considerable importance, since texture, aroma and flavour are influenced by proteolytic activity. The cheese matrix is in itself a network of casein particles which disintegrates as proteolytic enzymes take action. A lower proteolysis leads to a more consistent cheese whereas a higher proteolysis increases the softening and meltability of cheese (Delgado *et al.*, 2015). Given the lower ripening extension index by 15 months of storage, HPP P1 cheeses were in fact firmer and harder compared to $Ch_C$. This was confirmed by both instrumental (TPA profile) and sensorial analysis by the trained panel, (see details further in sections 3.3 and 3.4, respectively). A significant progressive increase in the value of TCA/TN, understood as a ripening depth index ($p < 0.001$), was observed for all cheeses during the 15 month refrigerated storage (Figure 4.1 B); an intermediate plateau similar to that previously observed for WSN/TN was also registered. This proteolysis index expresses the presence of medium and small-sized peptides (with a chain length between 2 and 20 amino acids residues) and free amino acids, which may result from the strong proteolytic action of *C. cardunculus* extract (Sousa and Malcata, 1996; Tavaria *et al.*, 2003), but mainly from peptidase activity from viable or lysed lactic acid bacteria (Macedo *et al.*, 2004; Macedo and Malcata, 1997c; Reis and Malcata, 2011) and/or from psychotropic bacteria (Macedo and Malcata, 1997e); in this study viable cell numbers of psychotropic bacteria were higher in $Ch_C$ and P2 and P3 cheeses compared to P1 cheeses (CHAPTER 3). The obtained TCA/TN values are in agreement with other reported results: Macedo and Malcata (1997b) reported 5.5–6.2 % TCA/TN at 35 days of ripening for *Serra da Estrela* cheeses, Tavaria *et al.*, (2003) 7–16 % at 35-180 days of

ripening, while Reis and Malcata (2011) reported 3.7 % at 60 days of ripening. In general, there were no significant differences in TCA/TN indices, between the $Ch_C$ cheeses and the HPP P2 and P3 cheeses (450 MPa/6 and 9 min, respectively). On the other hand, significantly lower TCA/TN indices were obtained for HPP P1 – 600 MPa/6 min cheeses than for $Ch_C$ cheeses ($p < 0.001$) over the whole storage period. This observation may be consequence of a reduced peptidase activity that can be linked with the reduction in microbial viable cell numbers caused by HPP as verified in a previous work (CHAPTER 3). This effect was also verified in HPP-treated *Torta del Casar* cheeses (600 MPa/20 min) at 60 days of ripening and stored for 240 days, having Delgado *et al.*, (2015) suggested that HPP at 600 MPa lead to a reduction in the production of medium-size to small peptides. On the other hand, Delgado *et al*., (2012) verified an increase in TCA/TN in *Ibores* Cheese (raw goat milk) HPP treated (400 and 600MPa/7 min) at 50 days ripening compared to control cheeses, possibly due to intracellular release of proteinases/peptidases.

The PTA/TN, as free amino acid (FAA) index, is related with the final product of proteolysis, the FAA and the very small peptides (containing less than 6 amino acid residues). Once again, the PTA/TN ratio significantly ($p < 0.05$) increased progressively during refrigerated storage for all non-treated and HPP treated cheeses (Figure 4.1 C). In the case of the $Ch_C$ cheeses, the values of PTA/TN almost quadruplicated, going from 1.4 ± 0.2 at 0 months storage to 5.4 ± 0.1 at 15 months of storage. These values are within the range previously reported by Tavaria *et al.*, (2003), i.e 3–12 % at 60-180 days of ripening, and also in line with the increase from 0.56 to 2.6 % during the first 60 days of ripening reported by Reis and Malcata, (2011) for the same type of cheese. This FAA index has been associated with the hypothesis that FAA are released by means of peptidases synthesized by adventitious microorganisms in *Serra da Estrela* cheese

(Tavaria *et al.*, 2003). In fact, the lowest value of PTA/TN determined for HPP P1 treated cheeses paralleled the higher reductions in viable cell numbers observed in these cheeses (CHAPTER 3). On the other hand, HPP P2 and P3 treated cheeses, which revealed a similar PTA/TN ratios compared to that of ChC cheeses ($p > 0.05$), suffered a minor effect on microbial composition. Delgado *et al.*, (2015) also observed a similar FAA content between *Torta del Casar* cheeses HPP (600 MPa/20 min) treated at 60 days and respective control.

### 4.3.2. Effect of HPP on aminopeptidase activity

HPP is capable of inactivating microorganisms, but can also affect enzyme activity due to its (in)activation or to changes in substrate's conformation (Martínez-Rodríguez *et al.*, 2012). Aminopeptidase activity contributes significantly to proteolysis in cheese (Macedo *et al.*, 2003b) and so its activity was quantified on both Lys-*p*-NA and Leu-*p*-NA with the results being listed in Table 4.1. The activity decreased along refrigerated storage for both substrates and for all cheeses, with exception for P1 cheeses that showed a significant increment in the activity on Leu-*p*-NA from 0 to 6 months of storage ($p < 0.001$). Aminopeptidase activity on Lys-*p*-NA was significantly higher ($p < 0.001$) in P1 (17 % higher) and P2 (+ 59%) cheeses at 0 month than in $Ch_C$ cheeses. A similar behaviour was observed throughout the 15 months of storage, although no significant activity variation was observed during the last 9 months of storage, for all cheeses. On the other hand, all cheeses registered a similar activity for Leu-*p*-NA at 0 months ($p > 0.05$); however, during the 15 months refrigerated storage associated aminopeptidase activity decreased significantly ($p < 0.05$) in $Ch_C$, P2 and P3 cheeses. (Trujillo *et al.*, 2000) suggested that HPP treatment could release intracellular enzymes due to incremented cell membrane permeability and microbial cells lysis, favouring the release

of intracellular material, including peptidases. In general, an inverse relationship was observed between proteolytic indices and aminopeptidase activity during storage; as values of proteolytic indices increased the aminopeptidase activity decreased. Indeed other proteolytic enzymes will have their activity increased along storage and/or HPP and storage may increase the proteolytic susceptibility of proteins. For instance, cheese casein conformation changes or casein aggregation was reported to occur by HPP (Garde *et al.*, 2007a). A higher aminopeptidase activity on HPP treated cheeses (400 MPa/10 min) was also verified for *La Serena* cheese treated at 50 days ripening and analysed 10 days later, even though a similar level of proteolysis was quantified (Garde *et al.*, 2007a). Furthermore, Juan *et al.,* (2007) also verified this tendency in HPP treated ewe cheese during ripening; authors tested two HPP treatments i.e 400 MPa and 500 MPa for 10 min each, and although some aminopeptidase inactivation was observed at the higher HPP treatment (500 MPa/10 min) and both HPP treated cheeses revealed similar WSN content compared to control.

**Table 4.1: Aminopeptidase activity of control ($Ch_C$) and HPP treated *Serra da Estrela* cheeses.**

| Property | Storage time (months) | $Ch_C$ | P1 600 MPa/6' | P2 450 MPa/6' | P3 450 MPa/9' |
|---|---|---|---|---|---|
| **Activity for Leu-*p*-Na*** | 0 | 11.0 ± 0.8 $^{a,A}$ | 11.2 ± 0.5 $^{a,B}$ | 10.4 ± 0.8 $^{a,A}$ | 10.4 ± 0.8 $^{a,A}$ |
| | 3 | 9.8 ± 0.6 $^{b,B}$ | 14.2 ± 0.6 $^{a,A}$ | 10.1 ± 0.8 $^{b,A}$ | 10.6 ± 0.2 $^{b,A}$ |
| | 6 | 7.9 ± 0.5 $^{c,C}$ | 13.3 ± 0.6 $^{a,A}$ | 8.2 ± 0.3 $^{c,B}$ | 9.8 ± 0.4 $^{b,A}$ |
| | 15 | 5.3 ± 0.8 $^{c,D}$ | 10.2 ± 0.9 $^{a,B}$ | 6.7 ± 0.4 $^{b,C}$ | 6.6 ± 0.6 $^{b,B}$ |
| **Activity for Lys-*p*-Na#** | 0 | 21.9 ± 2.5 $^{c,A}$ | 28.0 ± 4.1 $^{b,A}$ | 34.9 ± 3.7 $^{a,A}$ | 20.8 ± 2.6 $^{c,A}$ |
| | 3 | 7.2 ± 1.4 $^{c,B}$ | 20.6 ± 1.3 $^{a,B}$ | 13.7 ± 2.2 $^{b,B}$ | 17.6 ± 4.0 $^{a,A,B}$ |
| | 6 | 7.7 ± 0.3 $^{d,B}$ | 19.7 ± 2.1 $^{a,B,C}$ | 13.5 ± 0.7 $^{c,B}$ | 15.7 ± 1.2 $^{b,B,C}$ |
| | 15 | 9.0 ± 1.6 $^{b,B}$ | 16.2 ± 1.5 $^{a,C}$ | 15.8 ± 3.8 $^{a,B}$ | 13.0 ± 0.6 $^{a,C}$ |

* expressed in nmol Leu-p-NA/min.g cheese ± standard deviation
# expressed in nmol Lys-p-NA/min.g cheese ± standard deviation
P1 = 600 MPa, 6 min; P2 = 450 MPa, 6 min and P3= 450 MPa, 9 min.
Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 4.3.3. Effect of HPP on textural properties of cheese

Cheese texture is an important quality parameter that derives from the extensive chemical and biochemical changes that occur during ripening. Proteolysis is among the biochemical changes that may contribute to such textural properties.

Textural changes during refrigerated storage of control and HPP treated *Serra da Estrela* cheeses are shown in Table 4.2. Significant texture changes ($p < 0.05$) were verified throughout refrigerated storage for all cheeses under analysis. Albeit such trend HPP treatment did not significantly affect hardness, consistency, adhesiveness, cohesiveness and gumminess at 0 months in comparison to $Ch_C$ cheeses ($p > 0.05$). Similarly, Garde *et al*., (2007) and Delgado *et al*., (2012) noticed that HPP (400 MPa/10 min) applied to *La Serena* cheese at 50 days of ripening and HPP (400 or 600 MPa/7 min) applied on *Ibores* cheese (raw goats milk) at 30 days of ripening, had no significant effect on texture. However, a significant effect was observed after storage (10 days) of HPP treated and non-treated ripened (50 days) cheeses (Delgado *et al.*, 2013). As shown in Table 4.2, P1 cheeses became harder and more consistent along the storage period ($p < 0.001$), except for the samples measured after 15 months of storage. Such increase may be related, on the one hand, with the progressively lower moisture contents reported up to 6 months of refrigerated storage (Table 3.2). On the other hand, this variation in textural properties of P1 cheeses also revealed a positive correlation with the proteolysis levels previously discussed and with the sensorial analysis described below; as previously discussed a lower ripening extension index (less primary proteolysis) is related to a firmer cheese. Delgado *et al.,* (2013). reported an increase in hardness for ripened *Ibores* cheeses treated by HPP (600 MPa/7 min) and after storage for 90 days.

P2 cheeses maintained similar values of hardness and consistency throughout storage ($p > 0.05$), with values closer to those of $Ch_C$ cheeses at 0 months (upon 45 days of ripening), in line with what was observed and discussed for proteolytic indices. In general, the cohesiveness increased during the first 90 days of storage for all cheeses, but then showed no significant differences ($p > 0.05$), except for the $Ch_C$ cheeses at 15 months storage, which revealed significant lower values ($p < 0.001$) than HPP cheeses. This textural parameter expresses the energy needed to be applied during mastication to break down the product until it is ready to be swallowed. The adhesiveness decreased in all cheeses during storage, particularly for HPP P1 cheeses. A similar effect was verified by Delgado *et al*., (2015) after 120 days storage of *Torta del Casar* cheeses, which had been previously ripened for 60 days and HPP treated (600 MPa/5 and 20 min). HPP at 600 MPa (6 min) caused the most pronounced textural changes, increasing the gumminess when compared to $Ch_C$ cheeses. During the whole storage period, P3 cheeses showed similar gumminess to $Ch_C$ cheeses ($p > 0.05$), with exception at 15 months ($p < 0.001$). Similar results were obtained for *Ibores* cheese treated by HPP (400 MPa/7 min at 60 days), with no significant effects on gumminess after 30 days of storage (Delgado *et al.*, 2013).

Globally and according to the literature, HPP had no immediate effect on cheese texture parameters. However, along storage time, some textural parameters tended to show some changes between $Ch_C$ and HPP cheeses. Overall, lower differences in textural parameters were found for shorter storage times and for HPP treated cheeses at lower pressure intensity (P2).

**Table 4.2: Textural properties of control ($Ch_C$) and HPP *Serra da Estrela* cheeses.**

| Property | Storage Time (months) | $Ch_C$ | P1 600 MPa/6 min | P2 450 MPa/6 min | P3 450 MPa/9 min |
|---|---|---|---|---|---|
| **Hardness (N)** | 0 | 0.16 ± 0.03 $^{a,B,C}$ | 0.12 ± 0.03 $^{a,C}$ | 0.12 ± 0.05 $^{a,A}$ | 0.14 ± 0.04 $^{a,C}$ |
| | 1.5 | 0.19 ± 0.04 $^{a,b,B}$ | 0.22 ± 0.09 $^{a,A,B}$ | 0.14 ± 0.07 $^{b,A}$ | 0.20 ± 0.04 $^{a,b,B}$ |
| | 3 | 0.12 ± 0.01 $^{b,C,D}$ | 0.20 ± 0.02 $^{a,B}$ | 0.10 ± 0.04 $^{b,A}$ | 0.12 ± 0.04 $^{a,C}$ |
| | 6 | 0.25 ± 0.08 $^{a,A}$ | 0.28 ± 0.06 $^{a,A}$ | 0.14 ± 0.02 $^{b,A}$ | 0.26 ± 0.03 $^{a,A}$ |
| | 15 | 0.11 ± 0.01 $^{c,D}$ | 0.22 ± 0.04 $^{a,A,B}$ | 0.12 ± 0.01 $^{c,A}$ | 0.18 ± 0.03 $^{b,B}$ |
| **Consistency (N/s)** | 0 | 0.96 ± 0.25 $^{a,B,C}$ | 0.92 ± 0.33 $^{a,C}$ | 1.1 ± 0.57 $^{a,A}$ | 1.2 ± 0.47 $^{a,C,D}$ |
| | 1.5 | 1.4 ± 0.37 $^{b,c,B}$ | 1.8 ± 0.88 $^{b,A,B}$ | 1.0 ± 0.65 $^{c,A}$ | 2.7 ± 0.64 $^{a,B}$ |
| | 3 | 0.72 ± 0.26 $^{b,C,D}$ | 1.5 ± 0.19 $^{a,B}$ | 0.68 ± 0.31 $^{b,A}$ | 0.89 ± 0.34 $^{b,D}$ |
| | 6 | 1.9 ± 0.76 $^{a,A}$ | 2.1 ± 0.54 $^{a,A}$ | 1.1 ± 0.14 $^{b,A}$ | 1.9 ± 0.32 $^{a,A}$ |
| | 15 | 0.67 ± 0.10 $^{c,D}$ | 1.6 ± 0.28 $^{a,A,B}$ | 0.78 ± 0.17 $^{c,A}$ | 1.3 ± 0.25 $^{b,B,C}$ |
| **Adhesiveness (N/s)** | 0 | 0.16 ± 0.08 $^{a,D}$ | 0.16 ± 0.09 $^{a,D}$ | 0.25 ± 0.18 $^{a,B,C}$ | 0.15 ± 0.05 $^{a,C}$ |
| | 1.5 | 0.36 ± 0.13 $^{a,b,B}$ | 0.53 ± 0.27 $^{b,B,C}$ | 0.26 ± 0.17 $^{a,B,C}$ | 0.38 ± 0.12 $^{a,b,C}$ |
| | 3 | 0.18 ± 0.05 $^{a,C,D}$ | 0.48 ± 0.09 $^{c,C}$ | 0.18 ± 0.10 $^{a,C}$ | 0.30 ± 0.05 $^{b,C}$ |
| | 6 | 0.82 ± 0.26 $^{b,A}$ | 0.97 ± 0.26 $^{a,A}$ | 0.47 ± 0.09 $^{b,A}$ | 0.89 ± 0.19 $^{b,A}$ |
| | 15 | 0.33 ± 0.06 $^{a,B,C}$ | 0.72 ± 0.16 $^{b,B}$ | 0.39 ± 0.06 $^{a,A,B}$ | 0.68 ± 0.16 $^{b,B}$ |
| **Cohesiveness (dimensionless)** | 0 | 0.56 ± 0.12 $^{a,B}$ | 0.47 ± 0.06 $^{a,B}$ | 0.56 ± 0.11 $^{a,B}$ | 0.54 ± 0.19 $^{a,B}$ |
| | 1.5 | 0.72 ± 0.14 $^{a,A}$ | 0.73 ± 0.15 $^{a,A}$ | 0.61 ± 0.24 $^{a,B}$ | 0.61 ± 0.11 $^{a,B}$ |
| | 3 | 0.68 ± 0.11 $^{a,A,B}$ | 0.71 ± 0.12 $^{a,A}$ | 0.73 ± 0.08 $^{a,A,B}$ | 0.57 ± 0.08 $^{a,B}$ |
| | 6 | 0.76 ± 0.09 $^{a,b,A,B}$ | 0.73 ± 0.06 $^{b,A}$ | 0.73 ± 0.09 $^{b,A,B}$ | 0.82 ± 0.08 $^{a,A}$ |
| | 15 | 0.012 ± 0.003 $^{b,C}$ | 0.78 ± 0.07 $^{a,A}$ | 0.87 ± 0.14 $^{a,A}$ | 0.80 ± 0.09 $^{a,A}$ |
| **Gumminess (N)** | 0 | 0.09 ± 0.03 $^{a,B,C}$ | 0.06 ± 0.02 $^{a,C}$ | 0.07 ± 0.04 $^{a,A}$ | 0.07 ± 0.04 $^{a,C}$ |
| | 1.5 | 0.14 ± 0.04 $^{a,A,B}$ | 0.17 ± 0.09 $^{b,A,B}$ | 0.10 ± 0.07 $^{b,A}$ | 0.11 ± 0.04 $^{a,b,B}$ |
| | 3 | 0.08 ± 0.01 $^{b,C}$ | 0.14 ± 0.03 $^{a,B}$ | 0.07 ± 0.04 $^{b,A}$ | 0.07 ± 0.03 $^{b,C}$ |
| | 6 | 0.16 ± 0.09 $^{a,A}$ | 0.20 ± 0.04 $^{a,A}$ | 0.10 ± 0.01 $^{b,A}$ | 0.22 ± 0.03 $^{a,A}$ |
| | 15 | 0.001 ± 0.000 $^{d,D}$ | 0.17 ± 0.03 $^{a,A,B}$ | 0.10 ± 0.03 $^{c,A}$ | 0.14 ± 0.03 $^{b,B}$ |

P1 = 600 MPa, 6 min; P2 = 450 MPa, 6 min and P3= 450 MPa, 9 min. Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 4.3.4. Effect of HPP on sensory attributes

The results for the sensory evaluation of *Serra da Estrela* cheese are presented in Table 4.3. Some significant differences ($p < 0.05$) between $Ch_C$ and HPP were found for the appearance, odour, texture and taste attributes. Instrumental measurement of colour showed that the colour of the paste of $Ch_C$ cheeses becomes darker than the paste of HPP cheeses (less luminous and less yellowness) (CHAPTER 3) along storage, this much was observed for sensory evaluation of colour at 15 months of storage, for which significant differences ($p < 0.05$) were found between all HPP and $Ch_C$ cheeses. Delgado *et al.*, (2013) also found that HPP *Ibores* cheeses (raw goat milk at 50 days of ripening at 400 and 600 MPa/7 min) were yellower than control cheeses. At 15 months of storage, the lower consistency (in paste appearance and texture) attributed to $Ch_C$ relativity to HPP P1 is in accordance to the textural measurement, with the results showing that HPP P1 cheeses became harder and more consistent. The lactic, acid, animal and short-chain fatty odours did not significantly change for the P1 cheeses during all storage period ($p > 0.05$). Also, no significant differences in odour intensity between HPP (400 or 600 MPa/5 min) and control raw ewe (Arqués *et al.*, 2007; Calzada *et al.*, 2014b) or cow cheeses (Calzada *et al.*, 2015), during 10, 60 and 240 days of storage, were reported. Significant odour differences between $Ch_C$ and HPP cheeses were found for P2 (acid and short-chain fatty acids) and P3 (acid) at 90 days storage, these results may derived from some particular heterogeneity of the samples.

No significant differences between $Ch_c$ and P3 cheeses ($p > 0.05$) were found for texture attributes, showing that consistency and friability were not affected by HPP up to 15 months. Similarly a HPP treatment (400 MPa/10 min at 50 days of ripening) on *La Serena* cheeses also revealed no significant effect on texture preference (Garde *et al.*, 2007a). On the other hand P1 cheeses were perceived to be harder than $Ch_C$ cheeses at 15

months of storage ($p < 0.05$). Conversely, a hardness and friability decrease was verified for HPP *Ibores* cheeses (600 MPa/7 min) in comparison to control cheeses (Delgado *et al.*, 2013). Instrumental textural analysis revealed minor differences in the hardness and consistency of P2 cheeses, however, these minor differences were perceived by the panel at 15 months storage ($p < 0.05$), which considered the P2 cheeses softer compared to $Ch_C$ cheeses.

The different evaluation, instrumental versus sensory, observed in some of the cheeses, is probably related to the specific particularities of each method and to sampling heterogeneity analysis. Cheese sensory analyses were performed using cheese slices, as cheese is usually consumed, which allowed to evaluate all cheeses zones (near to rind and cheese interior). On the other hand, texture analysis was evaluated using cylindrical samples (crossed rind to rind), thus representing different cheese zones and leading to a great cheese texture parameters variation.

Relatively to the flavour attributes, in general, the HPP cheeses revealed a similar salty and acid taste ($p > 0.05$) than $Ch_c$ cheeses, being only significantly less intense for P1 and P2 cheeses at 15 months ($p < 0.05$) for acid taste and for P1 for salty taste. Also no significant differences in sour, (sweet) and salty taste were denoted after 10 days of HPP (400 MPa/10 min at 50 days of ripening) of *La Serena* cheeses (Garde *et al.*, 2007a). In our study, the bitter taste was not affected by HPP. However, according to the lower TCA/TN index observed in HPP cheeses, a less bitter flavour was expected for these cheeses (Macedo *et al.*, 2004). The after-taste attribute evidenced no effect of HPP ($p > 0.05$).

Globally, few sensorial attributes were affected by HPP, being the P3 treatment (450 MPa/9 min) that which revealed minor differences in comparison to $Ch_C$ cheeses.

**Table 4.3: Sensory ratings of attribute difference-from-control test: paired comparisons between blind control cheese ($Ch_C$) and HPP *Serra da Estrela* cheeses at 0, 1.5, 6 and 15 months.**

| Appearance | Months | P1 vs $Ch_C$ | | P2 vs $Ch_C$ | | P3 vs $Ch_C$ | |
|---|---|---|---|---|---|---|---|
| Colour | **0** | -0.38 ± 1.19 | | -0.88 ± 1.36 | | -0.25 ± 0.89 | |
| | **1.5** | -1.22 ± 2.28 | | -0.56 ± 1.74 | | -1.11 ± 2.15 | |
| | **6** | -0.90 ± 1.29 | | -0.60 ± 1.58 | | -1.00 ± 2.00 | |
| | **15** | -1.00 ± 0.82 | * | -1.00 ± 1.05 | * | -1.00 ± 0.94 | * |
| Consistency | **0** | -1.43 ± 2.70 | | -1.29 ± 3.50 | | -0.43 ± 2.23 | |
| | **1.5** | 0.89 ± 2.21 | | 1.00 ± 2.18 | | 0.67 ± 2.06 | |
| | **6** | 1.50 ± 2.72 | | 0.20 ± 2.74 | | -0.20 ± 1.87 | |
| | **15** | 0.30 ± 1.06 | | -1.40 ± 1.27 | * | -0.70 ± 1.06 | |
| **Odour** | | | | | | | |
| Lactic | **0** | -0.57 ± 1.13 | | 0.00 ± 1.29 | | -0.29 ± 1.98 | |
| | **1.5** | -0.10 ± 1.52 | | -0.10 ± 2.42 | | 0.50 ± 2.22 | |
| | **6** | -0.90 ± 2.89 | | -0.40 ± 1.35 | | -0.50 ± 1.43 | |
| | **15** | -0.30 ± 1.25 | | -0.20 ± 2.15 | | -0.60 ± 1.84 | |
| Acid | **0** | -0.75 ± 1.91 | | -0.13 ± 1.96 | | 0.13 ± 1.73 | |
| | **1.5** | -0.30 ± 1.16 | | -2.10 ± 1.52 | * | -1.80 ± 1.93 | * |
| | **6** | 0.50 ± 2.46 | | -0.20 ± 1.55 | | 0.50 ± 1.43 | |
| | **15** | 0.00 ± 1.00 | | 1.00 ± 2.18 | | 0.33 ± 1.23 | |
| Animal | **0** | 0.43 ± 1.72 | | -0.71 ± 0.95 | | -0.29 ± 1.11 | |
| | **1.5** | -0.20 ± 1.62 | | -1.10 ± 2.08 | | -0.70 ± 2.00 | |
| | **6** | -0.60 ± 1.78 | | -0.10 ± 1.73 | | -0.20 ± 1.48 | |
| | **15** | -0.44 ± 0.73 | | -0.78 ± 1.09 | | -0.44 ± 1.01 | |
| SCFA[#] | **0** | 0.13 ± 1.73 | | 0.13 ± 2.53 | | -0.38 ± 1.77 | |
| | **1.5** | -0.80 ± 1.40 | | -1.90 ± 1.52 | * | -0.80 ± 1.87 | |
| | **6** | -0.78 ± 2.17 | | -0.44 ± 0.88 | | -0.22 ± 1.72 | |
| | **15** | 0.60 ± 2.12 | | 0.30 ± 3.65 | | -0.10 ± 2.23 | |
| **Texture** | | | | | | | |
| Consistency | **0** | -0.71 ± 1.25 | | -0.86 ± 0.90 | | 0.00 ± 1.29 | |
| | **1.5** | 0.11 ± 1.36 | | 0.33 ± 1.80 | | 0.67 ± 1.23 | |
| | **6** | 1.30 ± 1.57 | | -1.10 ± 1.52 | | -0.40 ± 1.51 | |
| | **15** | 1.00 ± 0.94 | * | -2.00 ± 0.67 | * | -0.20 ± 0.79 | |
| Friability | **0** | 0.38 ± 1.06 | | 0.13 ± 0.84 | | 0.50 ± 1.07 | |
| | **1.5** | 0.40 ± 1.17 | | 0.40 ± 0.97 | | 0.30 ± 0.68 | |
| | **6** | 0.50 ± 0.85 | | -0.30 ± 0.68 | | -0.30 ± 0.68 | |
| | **15** | 0.50 ± 1.08 | | -1.00 ± 1.70 | | -0.40 ± 0.70 | |
| **Taste** | | | | | | | |
| Salty | **0** | -0.71 ± 1.80 | | -1.00 ± 2.24 | | -1.14 ± 1.77 | |
| | **1.5** | 0.00 ± 1.58 | | -0.22 ± 1.72 | | -0.89 ± 1.69 | |
| | **6** | -1.00 ± 1.66 | | -0.11 ± 0.93 | | -0.56 ± 1.74 | |
| | **15** | -1.89 ± 2.03 | * | -0.56 ± 1.94 | | -0.11 ± 2.09 | |
| Acid | **0** | 2.33 ± 0.56 | | 1.19 ± 0.18 | | 1.25 ± 0.79 | |
| | **1.5** | 1.16 ± 0.09 | | 0.92 ± 0.51 | | 1.25 ± 0.47 | |
| | **6** | 1.67 ± 0.45 | | 1.87 ± 0.61 | | 1.05 ± 0.76 | |
| | **15** | 1.64 ± 0.58 | * | 1.99 ± 0.76 | * | 1.16 ± 1.00 | |
| Bitter | **0** | 0.50 ± 1.60 | | -0.63 ± 1.41 | | -0.13 ± 1.36 | |
| | **1.5** | 0.70 ± 0.71 | | 0.20 ± 1.55 | | 0.30 ± 1.77 | |
| | **6** | -0.44 ± 2.50 | | -0.33 ± 1.39 | | -0.11 ± 1.00 | |
| | **15** | -0.30 ± 2.44 | | 0.20 ± 2.00 | | 0.00 ± 1.49 | |
| **After-taste** | **0** | -0.50 ± 1.60 | | -0.38 ± 1.41 | | 1.13 ± 1.36 | |
| | **1.5** | 0.25 ± 0.71 | | 0.13 ± 1.55 | | 0.63 ± 1.77 | |
| | **6** | -0.67 ± 2.50 | | -0.22 ± 1.39 | | 0.00 ± 1.00 | |
| | **15** | 1.75 ± 2.44 | | 0.00 ± 2.00 | | 0.75 ± 1.49 | |

Data expressed as mean (n =10); * means significant difference ($p < 0.05$). [#] SCFA means short-chain fatty acids, vomit like odour. P1 = 600 MPa, 6 min; P2 = 450 MPa, 6 min and P3= 450 MPa, 9 min.

## 4.4. Conclusion

HPP had a significant effect on cheese ripening indices, with the greater impact being verified for HPP at 600 MPa for 6 min compared to 450 MPa (6 or 9 min). This effect can be related with the reduction in microorganisms' viable cell numbers caused by HPP (higher for the first condition) but also to the effect that pressure has on proteolytic enzymes, such as aminopeptidase. Albeit this observation, no straightforward correlation could be drawn between ripening indices, microbial inactivation and aminopeptidase activity, probably due to the influence of other factors on ripening indices.

Higher ripening indices (associated with more intense proteolysis) resulted in softer cheeses (HPP at 450 MPa (6 or 9 min), while proteolysis deceleration along storage occurred for 600 MPa for 6 min, allowing maintaining the characteristic texture of the cheeses, confirmed at sensorial level as being firmer and harder.

Considering all the sensorial attributes evaluated, the cheeses treated at 450 MPa for 9 min suffered less significant influence from HPP treatment.

Overall, the more intense HPP treatment P1 (600 MPa/6 min) studied in this work led to proteolysis deacceleration, while the less intense HPP (450MPa) P2 (for 6 min) and P3 (for 9 min) treatments underwent fewer changes on textural parameters and sensorial attributes, respectively.

# CHAPTER 5 - Evolution of qualitative and quantitative lipid profiles of HPP treated cheeses throughout storage

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*This chapter has been submitted for publication.*

## Abstract

*Serra da Estrela* cheese is a highly recognized and referenced raw ewes' milk cheese that is mandatorily ripened for a minimum period of 30 days, before commercialization under refrigerated storage. As all raw milk cheeses, *Serra da Estrela* cheese poses microbial safety issues, that can be overcome by high pressure processing (HPP) cold pasteurization. However, it is important that such treatment does not impact negatively on the overall biochemical quality, in particular, the lipid composition responsible for part of *Serra da Estrela* cheese unique sensorial and textural attributes. Hence, the major aim of this work was to assess the effect of HPP (600 MPa/6 min and 450 MPa/6 and 9 min) on the qualitative and quantitative lipid profiles of *Serra da Estrela* cheese along a 15-month refrigerated storage period. A triglycerides total content of about 65-66g TG/100 g just after processing was similarly determined for HPP treated cheeses at 450 MPa/6 min and control cheeses. About 60 fatty acids were identified and quantified, the major fatty acids being palmitic, stearic, capric, lauric and butyric; similar total contents of saturated, monounsaturated and polyunsaturated fatty acids for all cheeses along storage were reported. A high total conjugated linoleic acid content quantified in all cheeses along storage (1.29-1.65 g FA/100g fat), relatively to commercial dairy products, is highlighted and all cheeses revealed similar atherogenicity and thrombogenicity indices (~2.3 and ~2.6, respectively). The results clearly indicate that HPP can be used to process *Serra da Estrela* cheese with no changes in the lipid profiles at conditions that assure the cheese microbial safety.

## 5.1. Introduction

One of the most famous raw milk traditional Portuguese cheese is *Serra da Estrela* cheese, with Protected Designation of Origin (PDO) certification in the European Union. This cheese is manufactured using only raw ewes' milk, *Cynara cardunculus* L. (as coagulant), and salt. The milk used must be from specific ewe breeds (Bordaleira and/or Churra Mondegueira da *Serra da Estrela*), which live in particular geoclimatic conditions of the PDO region rich in natural pastures. The raw milk is manipulated daily according to strict PDO indications for cheese production, followed by ripening, resulting in a cheese with unique organoleptic characteristics (Macedo *et al.*, 1993). During ripening several and complex biochemical events occur as a result of milk microbiota and enzymes' action on lactose, lipids and proteins, with these having a great impact on cheese characteristics.

Lipolysis is an important biochemical event in cheese, with the lipid fraction and the primary products of its degradation, i.e., free volatile fatty acids, playing an important role in the development of particular aroma characteristics of *Serra da Estrela* cheese (Partidário *et al.*, 1998). There are several studies on lipolysis in *Serra da Estrela* cheese during ripening (Macedo *et al.*, 2003a, 1996b; Macedo and Malcata, 1997e; Partidário *et al.*, 1998), yet the lipolysis phenomenon continues through the storage period (i.e. during transportation and commercialization), particularly in the case of extended storage.

High pressure processing (HPP) is being increasingly applied and studied as a non-thermal alternative process to thermal pasteurization, with minimal deleterious effects on quality and a shelf-life extension up to 2-3 fold higher for dairy foods was reported compared to the non-pasteurized counterparts (Dhineshkumar *et al.*, 2016). However, the effects of HPP on cheese are dependent of the pressure intensity applied, the holding time used and the ripening stage (Garde *et al.*, 2007b). In a previous study (CHAPTER 3) HPP (450 or 600 MPa for 6 or 9 min) was shown to have beneficial effects on *Serra da Estrela* cheese microbiota (pejorative

microbial groups were significantly reduced by HPP, yet beneficial lactobacilli and lactococci were not significantly hampered and retained their positive metabolic activity), on proteolytic indices (HPP deaccelerated proteolysis and kept the ripening extension index closer to that of control cheeses at month 0 of storage), and on texture parameters and sensorial attributes (few attributes were affected) throughout prolonged 15 months refrigerated storage (CHAPTER 4). Since some of these changes (and particularly sensorial attributes) may, at least, be related to the lipids state and associated lipolysis extension in cheese, it is important and relevant to study the effect of HPP on the qualitative and quantitative lipid profiles of cheese, immediately after processing and throughout subsequent storage. To the best of the authors' awareness, this effect has been assessed only by quantification of the free fatty acids (FFA) and mainly during cheese ripening of HPP processed cheeses (Ávila *et al.*, 2007; Juan *et al.*, 2008, 2007b; Rynne *et al.*, 2008; Saldo *et al.*, 2003). Only three studies focus on the effect of HPP on lipolytic features during storage of raw cows and ewes milk cheeses (Calzada *et al.*, 2015, 2014b; Rodríguez-Pinilla *et al.*, 2015). Two studies were performed with *Torta del Casar* cheese (raw ewes milk), having the authors reported results for the sum of short- (SC-FFA), medium- (MC-FFA) and long-chain fatty acids (LC-FFA) (Calzada *et al.*, 2014b; Rodríguez-Pinilla *et al.*, 2015). When cheeses were HPP treated (200 and 600 MPa for 5 and 20 min) at 60 days of ripening, the FFA levels tended to increase during 240 days of refrigerated storage, but without significant differences among HPP and control chesses (Rodríguez-Pinilla *et al.*, 2015). On the other hand, HPP treatment (400 and 600 MPa for 5 and 20 min) at 14 and 21 days of ripening in cows milk cheeses had significant lower influence on SC FFA, MC FFA and LC FFA levels (Calzada *et al.*, 2015). To the best of our knowledge no previous studies have addressed such an in depth study of the lipid fraction and also no data are available on the free/non-esterified fatty acids profile –(NEFA) of raw ewes milk cheeses.

The aim of the present work was, therefore, to study the possible effects of HPP on triglycerides, free fatty acids and conjugated linoleic acid (CLA) composition of raw ewes milk PDO *Serra da Estrela* cheese, immediately after processing and during subsequent storage under refrigeration during 15 months.

## 5.2. Materials and methods

### 5.2.1. Cheese manufacture and high pressure processing

As previously described in sections: 3.2.1 Cheese manufacture, High pressure processing, 3.2.2 High pressure processing and 3.2.3 Sampling.

### 5.2.2. Chemicals

All solvents used in the present work (hexane, methanol, dichloromethane (DCM), dimethylformamide (DMF), methyl tert-butyl ether (MTBE) were HPLC grade and purchased to LABSCAN (Dublin, Ireland). Sulphuric acid was analytical grade (VWR Scientific, Carnaxide, Portugal). Supelco 37 fatty acid methyl esters (FAME) mix, methyl tricosanoate (99%; FAME-C23), undecanoic acid (99.9%; FFA C11), tritridecanoin (99%; C13), trinonanoin (99%; TG C27), and sodium methoxide (MetNa; 95%) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA); GLC-Nestle´36 FAME mix, and tritridecanoin (99%) and CLA standards from Nu-Chek Prep were from Nu-Chek Prep, Inc. (Elysian, MN, USA). Undecanoic acid (99%; C11; ALFA AESAR, Karlsruhe, Germany) was obtained from VWR (Carnaxide, Portugal). All the experiments were performed using a 14 mL borosilicate glass tubes (16mm x 125mm) with acid/heat resistant cap (VWR international, Carnaxide, Portugal).

### 5.2.3. Lipid extraction

The lipid fraction extraction was performed with MTBE, according to Matyash *et al.,* (2008) with some modifications. Methanol (1.5 mL) and MTBE (5.0 mL) were added to 200 mg of lyophilized cheese, into a glass tube with Teflon-lined cap and the tube was vortexed (1 min). Then, the mixture was incubated for 1 h at room-temperature (18-22 ºC) in a roller stirrer. Ultra-pure water (1.25 mL) was added, and the mixture left 10 min at room temperature for phase separation to occur, followed by centrifugation at 1 000 xg for 10 min. The upper phase was then collected, and the lower phase was re-extracted alike with 2 mL of the solvent mixture (obtained by mixing MTBE/methanol/water 10:3:2.5, v/v/v). The combined organic phases were dried in a multi-channel nitrogen system and the dried extracted lipids were weighted and dissolved in 1 mL of dichloromethane for storage. The lipid fraction extractions were done in duplicate.

### 5.2.4. Triglycerides determination

Sample (25 mg) was weighted into a vial with 200 µl TG-C9 (1.52 mg/mL) and 800 µL DCM. Upon preparation samples were analyzed using a CLARUS 500 gas chromatograph (PerkinElmer, Massachusetts, USA) equipped with FID detector and a Rtx-65TG column (30 m × 0.25 mm × 0.10 µm; Restek Corporation, Bellefonte, Pennsylvania, USA) Analysis conditions were as described in Castro-Gómez *et al.*, (2015). The oven program was 120 °C held for 30 s, 10 °C/min to 220 °C held for 30s, and 6 °C/min to 350 °C held for 30 min. Injector and FID temperatures were 355 and 370 °C, respectively. Helium was used as the carrier gas at 25 psi, and the injection volume was 0.5 µL.

### 5.2.5. Methyl esters fatty acids quantification

The preparation of methyl esters was performed according to Pimentel *et al.*, (*2*015), obtaining two fractions: the esterified fatty acids (EFA) and the non-esterified fatty acids (NEFA) fractions. For quantitative purposes, three internal standards were used: a triacylglycerol (tritridecanoin - C13; 1.36 mg/mL to calculate the concentration of EFA), a free fatty acid (undecanoic acid - C11; 0.68 mg/mL to calculate NEFA) and a FAME (methyl tricosanoate – FAME-C23; 0.66 mg/mL to control isolation of FAME and cross-contamination). Briefly, 20 mg of sample were weighted into a glass tube and standards were added - 400 µl TG C13 and 200 µl FFA C11. Then 100 µL of MetAc 3.4 mL of hexane and 2.46 mL of methanol were added and mixed. Subsequently, 240 µL of MetNa was added. The tubes were shaken for 10 seconds using a vortex and placed in a heating block at 40 °C for 10 minutes. After this, 500 µL of $H_2O$ were added to each of the tubes and they were once again vortexed for 10 seconds. The samples were centrifuged at 1250 xg for 5 minutes. The upper layer was collected and transferred into a new tube where 400 µL of FAME C23 had been previously added. Two mL of hexane were added to what remained in the glass tube and the procedure repeated akin. The collected phases contained the esterified fatty acids. In order to obtain the non-esterified fatty acids a second reaction was carried out by adding 100 µL of FAME C23, 1.25 mL of DMF and then 1.25 mL of $H_2SO_4$ /MetOH (3M). This reaction mixture was then placed in a heating block at 60°C for 30 minutes. After cooling down, 700 µL of hexane were added. The sample was vortexed for 1 minute and centrifuged at 1250 g for 5 minutes and the upper phase was collected into a vial.

### 5.2.6. Fatty acids identification and quantification

For the analysis of esterified fatty acids methyl esthers (EFAME) and non-esterified fatty acids methyl esthers (NEFAME) fractions of cheese samples, a 120 m x 0.25 mm x 0.25 mm

BPX70 column (SGE Europe Ltd, Courtaboeuf, France) was installed in a HP6890 gas chromatograph. Conditions were, split 10:1, injection volume 1 μL, injector temperature 250 ºC, detector (FID) temperature 290 ºC, carrier pressure (Hydrogen) 30 psi while the oven programme was as follows: 70 ºC hold 1 min, 7 ºC/min to 170 ºC (hold 41 min), 5 ºC/min to 23 ºC (hold 17 min). Fatty acids were identified by comparing their retention times with the fatty acid methyl standards Supelco 37. GLC-Nestlé36 was assayed for calculation of response factors. All the preparations and analysis were performed in triplicate.

### 5.2.7. Nutritional quality indices of lipids

The atherogenicity index and thrombogenicity indices were calculated according to Ulbricht *et al.*, (1991). In what concerns the atherogenicity index, which indicates the relationship between the sum of the main saturated and that of the main classes of unsaturated fatty acids - the former being considered pro-atherogenic (favour the adhesion of lipids to cells the immunological and circulatory systems cells), and the latter anti-atherogenic (inhibit the aggregation of plaque and diminish the levels of esterified fatty acids, cholesterol, and phospholipids, thus preventing the appearance of micro- and macrocoronary diseases) - C14:0 is considered to be 4 times more atherogenic than other FA (hence, it is assigned the coefficient '4'). In this regard, the following equation was applied:

$$IA = \frac{C12{:}0 + (4 \times C14{:}0) + C16{:}0}{\sum MUFA + PUFA_{n-6} + PUFA_{n-3}} \qquad \textbf{Equation 5.1}$$

The ratio of C14, C16, and C18 (pro-thrombogenetic) to USFAs (anti-thrombogenetic) is described as the index of thrombogenicity (IT); n−6 and remaining monounsaturated FA are assigned coefficients of 0.5 because they are less anti-atherogenic than the n−3 FA, which are

assigned a coefficient of 3. This index refers to the tendency for clot formation in the blood vessels. The IT value was measured by the following equation:

$$IT = \frac{C14{:}0 + C16{:}0 + C18{:}0}{(0.5 \times \sum MUFA + 0.5 \times PUFA_{n-6} + 3 \times PUFA_{n-3}) + \frac{PUFA_{n-3}}{PUFA_{n-6}}} \quad \textbf{Equation 5.2}$$

### 5.2.8. Conjugated linoleic acid determination

Separation of CLA as FAME was performed as previously described by Rodríguez-Alcalá *et al.*, (2014). HPLC (Waters Alliance, Series 600, Mildford MA, USA) equipped with UV detector (Waters 996 PDA) at 233 nm and a Chrom-Spher 5 Lipid analytical column (4.6 mm i.d. × 250 mm stainless steel, 5 μm particle size; Varian). The mobile phase was 0.1% acetonitrile in hexane, operated isocratically at a flow rate of 1.0 mL/min. The injection volume was 10 μL. For identification, a standard mixture of pure CLA FAME isomers was used.

### 5.2.9. Statistical analysis

Data obtained from each chromatographic method were statistically processed according to the Kruskal-Wallis test to establish the effect of different processing conditions (three HPP treatment and control-$Ch_C$) on the lipid profiles, with the significance assigned at $p < 0.05$. SPSS software version 24.0 was used for the statistical analysis.

## 5.3. Results and discussion

### 5.3.1. Triglycerides composition of *Serra da Estrela* cheese

Mean values of the triglycerides (TG) present in *Serra da Estrela* cheeses subject to different HPP treatment and control ($Ch_C$) are shown in Table 5.1. In $Ch_C$ *Serra da Estrela* cheeses at 0 months of storage, among the TG identified, the most abundant, in descending order of magnitude, were the C42 (8.35 g/100g fat), C40 (7.91 g/100g fat), C36 (7.14 g/100g fat), C44 (6.38 g/100g fat), and C34 (5.98 g/100g fat). These results are in agreement with the TG composition of *Serra da Estrela* cheeses reported by Partidário *et al.*, (1998), with exception that C38 was the highest quantified TG in the latter study. Along the 15 months of storage, the amount of each TG remained basically constant for the $Ch_C$ cheeses with total TG content of 66.8 TG/100 g fat at 0 months, to 57.4 and 67.5 TG/100 g fat at 6 and 15 months of storage, respectively.

#### 5.3.1.1. Effect of HPP on triglycerides composition of *Serra da Estrela* cheese

The total TG content was influenced by HPP treatment upon ripening (0 months storage). In particular, P1 and P3 cheeses revealed a lower total amount of TG content after HPP (60.0 and 57.8 g TG/100 g fat, respectively), while a similar total TG content was determined for P2 cheeses (65.1 g TG/100 g fat), in comparison to $Ch_C$ cheeses (66.8 g TG/100 g fat). Concerning the effect of storage, no defined trend was observed between HPP cheeses and between these and $Ch_C$ cheeses in terms of total TG content, with values ranging between 69.2 (P1) and 72.5 (P3) TG/100 g fat at 6 months storage and 53.7 (P2) and 72.3 (P1) TG/100 g fat at 15 months. Interestingly, total TG content in P1cheeses rose mildly but steadily (+20% from 0 to 15 months storage) throughout storage, whereas in P2 and P3 cheeses increases were observed between 0 and 6 months storage (+ 10 and + 25%, respectively) but then original

values (P3) or less (P2) were recovered by 15 months storage. Such oscillations were mainly due to changes, over storage, in the contents of the six most abundant TG, these being generally similar to those of ChC cheeses (i.e. C42, C40, C36, C44, C34 and C46 in descending order). In terms of HPP, pressure intensity (P1 vs P2, P3) seems to influence TG quantitative profile over storage more than holding time at the same pressure (P2 vs P3). To the best of the authors knowledge, there are no works, reporting on the effect of HPP on the TG profile of cheese and so comparative discussion is not possible. Nevertheless, a study performed in raw cows milk did not reveal variations in triglycerides composition among raw and HPP (900 MPa/5 min) treated milk (Rodríguez-Alcalá *et al.*, 2015).

**Table 5.1: Mean composition of triglycerides (TG) (g TG/ 100 g of fat) in control ($Ch_C$) and HPP treated (600 MPa/6 min - P1, 450 MPa/6 min – P2 and 450 MPa/9 min) *Serra da Estrela* cheeses.**

| Storage time (Months) | $Ch_C$ | | | P1 - 600 MPa/6 min | | | P2 - 450 MPa/6 min | | | P3 - 450 MPa/9 min | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 15 | 0 | 6 | 15 | 0 | 6 | 15 | 0 | 6 | 15 |
| **C24** | 0.367±0.054 | 0.217±0.038 | 0.354±0.167 | 0.308±0.147 | 0.293±0.131 | 0.680±0.01 | 0.454±0.056 | 0.438±0.246 | 0.543±0.008 | 0.299±0.084 | 0.434±0.017 | 0.58±0.013 |
| **Ch** | 0.285±0.097 | 0.210±0.134 | 0.269±0.119 | 0.314±0.014 | 0.345±0.008 | 0.379±0.049 | 0.338±0.003 | 0.367±0.022 | 0.279±0.021 | 0.232±0.077 | 0.246±0.193 | 0.293±0.032 |
| **C26** | 0.500±0.301 | 0.287±0.02 | 0.700±0.154 | 0.188±0.104 | 0.672±0.074 | 0.831±0.045 | 0.691±0.155 | 0.658±0.033 | 0.510±0.016 | 0.537±0.035 | 0.580±0.346 | 0.577±0.003 |
| **IS** | 0.894±0.205 | 1.13±0.141 | 1.03±0.122 | 1.12±0.18 | 0.893±0.042 | 1.04±0.011 | 0.891±0.023 | 1.08±0.391 | 0.856±0.159 | 0.874±0.063 | 0.982±0.101 | 0.839±0.025 |
| **C28** | 1.70±0.25 | 1.50±0.11 | 1.43±0.24 | 1.54±0.19 | 1.45±0.30 | 1.64±0.81 | 1.15±0.11 | 1.90±0.03 | 1.38±0.14 | 1.29±0.07 | 1.77±0.01 | 1.52±0.02 |
| **C30** | 2.68±0.42 | 2.35±0.11 | 2.63±0.06 | 2.49±0.28 | 2.95±0.32 | 2.59±1.06 | 2.56±0.08 | 2.90±0.03 | 2.21±0.02 | 2.39±0.23 | 2.78±0.01 | 2.26±0.19 |
| **C32** | 4.10±0.57 | 3.60±0.09 | 4.17±0.21 | 3.75±0.35 | 4.51±0.41 | 4.59±1.10 | 4.06±0.16 | 4.47±0.08 | 3.3±0.11 | 3.78±0.22 | 4.23±0.13 | 3.49±0.24 |
| **C34** | **5.98**±0.77 | 5.25±0.11 | 6.10±0.24 | **5.40**±0.54 | **6.33**±0.42 | 6.56±1.42 | **5.81**±0.21 | **6.53**±0.13 | **4.93**±0.01 | **5.30**±0.24 | **6.15**±0.36 | **5.11**±0.47 |
| **C36** | **7.14**±1.47 | 6.59±0.06 | 7.83±0.37 | **6.86**±0.58 | **7.84**±0.32 | 8.16±1.46 | **8.33**±1.56 | **8.28**±0.35 | **6.19**±0.14 | **6.55**±0.15 | **7.92**±0.43 | **6.54**±0.71 |
| **C38** | 3.73±2.23 | 1.72±0.05 | 2.03±0.14 | 1.77±0.20 | 2.01±0.09 | 2.15±0.47 | 1.89±0.08 | 2.13±0.03 | 1.61±0.02 | 1.62±0.09 | 2.02±0.06 | 1.69±0.13 |
| **C40** | **7.91**±0.67 | 6.21±0.14 | 7.18±0.03 | **6.40**±0.39 | **8.09**±1.35 | 7.61±1.24 | **6.83**±0.10 | **7.68**±0.59 | **5.74**±0.34 | **7.64**±0.09 | **8.32**±0.76 | **6.07**±0.91 |
| **C42** | **8.35**±0.69 | 7.37±0.14 | 8.57±0.09 | **7.59**±0.54 | **8.55**±0.07 | 9.04±1.42 | **8.07**±0.08 | **9.05**±0.73 | **6.82**±0.24 | **6.97**±0.05 | **8.66**±0.83 | **7.18**±0.93 |
| **C44** | **6.38**±0.58 | 5.71±0.13 | 6.67±0.01 | **5.93**±0.54 | **6.64**±0.19 | 7.05±1.15 | **6.23**±0.03 | **7.03**±0.51 | **5.23**±0.30 | **5.42**±0.07 | **6.75**±0.61 | **5.62**±0.69 |
| **C46** | **4.79**±1.63 | 5.08±0.16 | 5.91±0.02 | **5.27**±0.52 | **5.81**±0.21 | 6.22±1.00 | **5.55**±0.07 | **6.19**±0.38 | **3.**92±0.84 | **4.76**±0.03 | **8.47**±3.04 | **4.95**±0.61 |
| **C48** | 4.55±0.31 | 4.07±0.22 | 4.74±0.08 | 4.37±0.22 | 4.81±0.13 | 5.15±0.88 | 4.47±0.08 | 5.05±0.45 | 3.83±0.18 | 3.79±0.13 | 5.07±0.1 | 4.08±0.56 |
| **C50** | 3.37±0.19 | 3.03±0.16 | 3.54±0.03 | 3.16±0.06 | 3.49±0.08 | 3.97±0.66 | 3.4±0.02 | 3.78±0.37 | 2.84±0.24 | 2.88±0.02 | 3.62±0.43 | 3.05±0.4 |
| **C52** | 2.94±0.17 | 2.44±0.46 | 3.09±0.09 | 2.73±0.04 | 3.03±0.11 | 3.28±0.40 | 2.91±0.10 | 3.24±0.30 | 2.52±0.23 | 2.53±0.09 | 3.20±0.56 | 2.69±0.53 |
| **C54** | 2.06±0.38 | 1.74±0.29 | 2.26±0.02 | 1.92±0.18 | 2.39±0.38 | 2.44±0.44 | 2.37±0.18 | 2.27±0.34 | 1.83±0.04 | 1.77±0.18 | 2.28±0.19 | 2.00±0.28 |
| **Total** | 66.8 | 57.4 | 67.5 | 60.0 | 69.2 | 72.3 | 65.1 | 72.0 | 53.7 | 57.8 | 72.5 | 57.7 |

Bold value represent the six most abundant TG in each cheese.

### 5.3.2. Fatty acid composition of *Serra da Estrela* cheese

The evolution of the fatty acid (FA) profile for EFAME and NEFAME, during the 15 months of storage, is presented in Table 5.2 and Table 5.3, respectively. A total of 60 FA were identified, a number higher than the 39 and 26 FA previously identified in *Serra da Estrela* cheese by Partidário *et al*., (1998) and Carocho *et al*., (2015), respectively, which improvement can be due to use a specific high polarity column for FAME analysis with extensive length, which increase the column efficiency, required for separation of complex samples and able to provide the isomeric FAME-separations. EFAME included a total content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of 84.9 g FA/100g fat. Partidário *et al*., (1998) reported a lower total FFA content of 0.374 g/100g fat, for *Serra da Estrela* cheese. During 6 months the total FA content of EFAME fraction remained fairly constant for $Ch_C$ cheeses. For *Torta del Casar* raw ewes milk cheese an increase in FA of almost 2-fold during storage was reported (from 5.60 to 11.6 g/kg cheese) from 0 to 6 months of storage (Rodríguez-Pinilla *et al.*, 2015).

Focusing on the composition of FA of $Ch_C$ cheeses at 0 months of storage, in the EFAME fraction, SFA were the main FA present (58.9 g FA/100g fat) corresponding to 69.4 percentage of EFAME FA composition, with palmitic (C16; 18.4 g FA/100g fat), myristic (C14; 9.65 g FA/100g fat), stearic (C18; 8.40 g FA/100g fat), capric (C10; 7.15 g FA/100g fat), lauric (C12; 4.02 g FA/100g fat), and butyric acids (C4; 3.28 g FA/100g fat), being those present in higher amounts. A similar composition of FA for the EFAME fraction was reported before for *Serra da Estrela* cheese (Carocho *et al.*, 2015; Partidário *et al.*, 1998). Among the SFA, palmitic, myristic and lauric acids have been associated with hypercholesterolemia and with the increase of the levels of low-density lipoproteins (LDL) (Naydenova *et al.*, 2014). On the other hand, those risks can be reduced by increasing high-density lipoproteins (HDL), through dietary

MUFA and PUFA (Naydenova *et al.*, 2014). In EFAME fractions, $Ch_C$ *Serra da Estrela* cheeses at 0 months revealed 20.5 g MUFA/100g fat (24.1 %) and 5.49 g PUFA/100g fat (6.5 %). In the MUFA fraction, oleic (C18:1 9*c* 13.45 g FA/100g fat) and *trans* vaccenic acids (C18:1 11*t*; 2.09 g FA/100g fat) were the most abundant. High MUFA diets were reported to lower cholesterol and triacylglycerol concentration, with favourable effects on the cardiovascular disease (CVC) risk (Kris-Etherton *et al.*, 1999). Among the PUFA fraction, linoleic (C18:2 9*c*,12*c* 1.30 g FA/100g fat) and rumenic acids (C18: 2 9*c*,11*t* CLA 1.09 g FA/100g fat), were the most abundant found. It is worthwhile mentioning that the total CLA content quantified in the $Ch_C$ cheeses was 1.38 g FA/100g fat, higher that those previously reported for other ewes' milk cheeses (0.986 g/100g of fat) (Prandini *et al.*, 2011) and CLA fortified dairy products (0.48 g/100 g of fat) (Rodríguez-Alcalá and Fontecha, 2007). Such a high content of CLA compounds (rumenic acid and the other CLA isomers) may reflect a high amount of CLA precursors in the milk and its presence can arise from dietary PUFA biohydrogenation in the ewe's rumen and the Δ9-desaturase action on *trans* vaccenic acid in the mammary gland (Rodríguez-Alcalá *et al.*, 2014), a FA present at high amounts in these $Ch_C$ cheeses. During storage the CLA content was kept (1.47 g FA/100g fat at 15 months). Clinical studies have associated CLA consumption with positive human health effects, such as anti-obesity (reduced body fat mass), reduced incidence of cardiovascular diseases (improvement of the blood lipid profile and reduction of total cholesterol) and reduced immune disorders (enhanced the levels of protective antibodies) (Abdelhamid *et al.*, 2018).

Due to the high presence of CLA verified in the cheeses, an Ag+-HPLC separation of CLA isomers of $Ch_C$ and HPP P1 (the most intense pressure treatment studied) cheeses at 0 months of storage was performed (Figure 5.1). As observed from Figure 5.1, similar qualitative and quantitative CLA isomers profiles were registered, demonstrating that HPP had no direct effect on CLA isomers profile. Studies performed on *Pecorino* ewes milk cheese (made with

pasteurized milk) showed a similar relative FA profile with higher percentages of saturated (SFA = 68.0 % of total FA) and monounsaturated fatty acids (MUFA = 22.5 % of total FA), and consequently, lower percentages of polyunsaturated fatty acids (PUFA = 4.43 % of total FA) (Prandini *et al.*, 2011). Notable, in the present work a higher relative amount of PUFA in the EFAME fat fraction of *Serra da Estrela* cheese was quantified relatively to other ewes milk cheeses reported in the literature, such as the previously mentioned *Pecorino*, *Roquefort* (Prandini *et al.*, 2011) and *Serra da Estrela* cheeses with incorporation of dried plants (Carocho *et al.*, 2015). As previously discussed, the ingestion of PUFA from animal sources such as cheese can reduce blood cholesterol and reduce the risk of cardiovascular diseases (Abdelhamid *et al.*, 2018). It is well known that the animal's diet plays a major role in modulating the fatty acid composition of ruminant milk(Addis *et al.*, 2005). *Serra da Estrela* ewe's diet is mainly from pasture, fresh forages in extensive or semi-extensive grazing, but also based on preserved forage according to lands availability and climatic conditions.

In the $Ch_C$ cheese NEFAME fractions, the total content of FA was 4001 mg FA/100g fat, which increased to 5793 mg FA/100g fat over 15 months storage. The main FA corresponded, once again, to SFA (3783 mg FA/100g fat) with pelargonic (C9; 3007 mg FA/100g fat), butyric (C4; 153.9 mg FA/100g fat), capric (C10; 148.5 mg FA/100g fat), and palmitic acids (C16; 118.6 mg FA/100g fat) as the main NEFAME present. In terms of the MU NEFA, the NEFAME fractions revealed a composition similar to that of EFAME fractions, being oleic (C18:1 9*c*, 108.7 mg FA/100g fat) and *trans* vaccenic acids (C18:1 11*t*; 14.7 g FA/100g fat) quantified as the main MU NEFA present. Among the PU NEFA fraction, linoleic (C18:2 9*c*,12*c*, 16.25 mg FA/100g fat) and ∝-linolenic acids (∝-C18:3 9c,12c,15c, 14.5 mg FA/100g fat) were the main PU NEFA present; these FA are known as essential FA since they are not able to be synthesized by humans or animals. In general this qualitative profile was maintained throughout storage.

**Table 5.2: Means fatty acid (FA) composition (g FA/ 100 g fat) of EFAME fractions of control ($Ch_C$) and HPP treated (600 MPa/6 min - P1, 450 MPa/6 min – P2 and 450 MPa/9 min) *Serra da Estrela* cheeses.**

| | $Ch_C$ | | | P1 - 600 MPa/6 min | | | P2 - 450 MPa/6 min | | | P3 - 450 MPa/9 min | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Storage time (months)** | **0** | **6** | **15** | **0** | **6** | **15** | **0** | **6** | **15** | **0** | **6** | **15** |
| **SFA** | | | | | | | | | | | | |
| C4 | 3.28±0.34 | 2.71±0.54 | 3.09±0.09 | 2.93±0.43 | 2.81±0.24 | 3.23±0.72 | 3.52±0.17 | 2.86±0.72 | 2.96±0.28 | 2.80±0.69 | 2.95±0.88 | 2.68±0.11 |
| C6 | 2.16±0.02 | 2.02±0.44 | 2.27±0.01 | 2.19±0.38 | 2.11±0.19 | 2.34±0.57 | 2.55±0.02 | 2.15±0.61 | 2.14±0.25 | 2.04±0.48 | 2.18±0.59 | 1.95±0.01 |
| C8 | 2.32±0.04 | 2.18±0.52 | 2.42±0.06 | 2.31±0.48 | 2.26±0.21 | 2.51±0.67 | 2.78±0.01 | 2.31±0.66 | 2.27±0.25 | 2.19±0.49 | 2.31±0.54 | 2.09±0.02 |
| C9 | 0.061±0.004 | 0.057±0.015 | 0.065±0.000 | 0.062±0.012 | 0.061±0.003 | 0.067±0.019 | 0.073±0.001 | 0.062±0.018 | 0.061±0.012 | 0.056±0.013 | 0.062±0.013 | 0.056±0.002 |
| C10 | 7.15±0.18 | 6.74±1.66 | 7.40±0.11 | 7.18±1.43 | 6.94±0.60 | 7.81±2.14 | 8.56±0.00 | 7.09±2.07 | 7.01±0.80 | 6.70±1.51 | 7.09±1.64 | 6.47±0.04 |
| C12 | 4.02±0.02 | 3.79±0.85 | 4.18±0.13 | 3.97±0.78 | 3.92±0.40 | 4.41±1.13 | 4.8±0.08 | 3.98±1.09 | 3.95±0.39 | 3.77±0.94 | 4.01±0.99 | 3.66±0.07 |
| C13i | 0.078±0.003 | 0.061±0.012 | 0.064±0.005 | 0.079±0.012 | 0.069±0.016 | 0.062±0.016 | 0.069±0.004 | 0.059±0.009 | 0.057±0.002 | 0.055±0.017 | 0.056±0.016 | 0.054±0.004 |
| C13ai | 0.033±0.003 | 0.033±0.005 | 0.037±0.004 | 0.034±0.001 | 0.035±0.002 | 0.034±0.006 | 0.039±0.003 | 0.034±0.006 | 0.033±0.001 | 0.033±0.01 | 0.035±0.010 | 0.032±0.002 |
| C14 | 9.65±0.03 | 9.08±2.00 | 10.1±0.24 | 9.53±1.85 | 9.43±0.95 | 10.68±2.7 | 11.51±0.26 | 9.58±2.64 | 9.57±0.98 | 9.03±2.27 | 9.65±2.41 | 8.84±0.11 |
| C14i | 0.25±0.01 | 0.24±0.04 | 0.26±0.02 | 0.25±0.03 | 0.25±0.04 | 0.27±0.05 | 0.30±0.02 | 0.25±0.06 | 0.25±0.02 | 0.24±0.07 | 0.25±0.08 | 0.23±0.01 |
| C14ai | 0.23±0.01 | 0.22±0.04 | 0.24±0.02 | 0.22±0.03 | 0.22±0.03 | 0.25±0.05 | 0.27±0.02 | 0.23±0.05 | 0.23±0.01 | 0.22±0.06 | 0.23±0.07 | 0.21±0.02 |
| C15 | 0.86±0.02 | 0.81±0.16 | 0.9±0.05 | 0.85±0.14 | 0.84±0.10 | 0.94±0.21 | 1.02±0.05 | 0.86±0.21 | 0.85±0.07 | 0.81±0.22 | 0.86±0.24 | 0.79±0.03 |
| C16 | 18.42±0.04 | 17.36±3.79 | 19.26±0.45 | 18.17±3.47 | 17.99±1.82 | 20.41±5.13 | 21.95±0.49 | 18.32±5.01 | 18.27±1.86 | 17.26±4.39 | 18.42±4.63 | 16.87±0.17 |
| C16i | 0.12±0.00 | 0.09±0.02 | 0.1±0.00 | 0.13±0.02 | 0.13±0.01 | 0.10±0.02 | 0.11±0.00 | 0.1±0.02 | 0.09±0.01 | 0.09±0.02 | 0.09±0.02 | 0.09±0.00 |
| C17 | 0.47±0.02 | 0.45±0.08 | 0.5±0.03 | 0.47±0.07 | 0.47±0.07 | 0.52±0.11 | 0.56±0.02 | 0.47±0.11 | 0.47±0.03 | 0.45±0.12 | 0.47±0.13 | 0.43±0.02 |
| C17i | 0.33±0.02 | 0.32±0.05 | 0.36±0.02 | 0.33±0.05 | 0.33±0.05 | 0.38±0.08 | 0.40±0.02 | 0.34±0.07 | 0.33±0.01 | 0.32±0.09 | 0.34±0.10 | 0.31±0.02 |
| C17ai | 0.52±0.02 | 0.49±0.09 | 0.56±0.04 | 0.52±0.08 | 0.52±0.08 | 0.58±0.12 | 0.63±0.03 | 0.52±0.12 | 0.52±0.03 | 0.50±0.14 | 0.54±0.16 | 0.48±0.03 |
| C18 | 8.40±0.46 | 7.87±1.23 | 8.79±0.71 | 8.23±1.09 | 8.26±1.29 | 9.25±1.76 | 10.01±0.80 | 8.3±1.79 | 8.32±0.37 | 7.93±2.47 | 8.47±2.63 | 7.7±0.51 |
| C18i | 0.054±0.006 | 0.051±0.002 | 0.063±0.007 | 0.053±0.01 | 0.055±0.009 | 0.065±0.014 | 0.06±0.008 | 0.055±0.008 | 0.063±0.005 | 0.053±0.019 | 0.056±0.013 | 0.057±0.00 |
| C20 | 0.23±0.01 | 0.22±0.03 | 0.24±0.01 | 0.22±0.03 | 0.23±0.04 | 0.25±0.04 | 0.27±0.02 | 0.23±0.05 | 0.23±0.01 | 0.22±0.07 | 0.24±0.08 | 0.21±0.01 |
| C21 | 0.074±0.004 | 0.075±0.003 | 0.079±0.01 | 0.073±0.008 | 0.076±0.012 | 0.081±0.011 | 0.081±0.005 | 0.082±0.018 | 0.077±0.00 | 0.07±0.018 | 0.071±0.021 | 0.07±0.004 |
| C22 | 0.13±0.00 | 0.13±0.02 | 0.15±0.02 | 0.14±0.02 | 0.13±0.02 | 0.15±0.03 | 0.16±0.01 | 0.13±0.03 | 0.14±0.01 | 0.13±0.04 | 0.14±0.04 | 0.13±0.01 |
| C24 | 0.075±0.005 | 0.079±0.017 | 0.077±0.008 | 0.07±0.009 | 0.078±0.014 | 0.087±0.008 | 0.072±0.004 | 0.075±0.013 | 0.072±0.011 | 0.071±0.021 | 0.084±0.026 | 0.068±0.012 |
| **MUFA** | | | | | | | | | | | | |
| C10:1 | 0.26±0.01 | 0.25±0.04 | 0.27±0.02 | 0.26±0.04 | 0.26±0.03 | 0.28±0.06 | 0.31±0.01 | 0.26±0.06 | 0.25±0.01 | 0.25±0.07 | 0.26±0.07 | 0.24±0.01 |
| C12:1 | 0.10±0.01 | 0.09±0.01 | 0.09±0.01 | 0.09±0.01 | 0.10±0.02 | 0.10±0.02 | 0.11±0.01 | 0.09±0.02 | 0.09±0.00 | 0.08±0.03 | 0.09±0.03 | 0.08±0.01 |
| C14:1 | 0.55±0.03 | 0.52±0.07 | 0.59±0.05 | 0.54±0.07 | 0.55±0.08 | 0.61±0.12 | 0.65±0.05 | 0.55±0.12 | 0.56±0.03 | 0.52±0.16 | 0.56±0.16 | 0.51±0.03 |
| C15:1 | 0.25±0.01 | 0.24±0.04 | 0.27±0.01 | 0.25±0.04 | 0.25±0.04 | 0.27±0.06 | 0.30±0.02 | 0.25±0.06 | 0.25±0.01 | 0.24±0.07 | 0.25±0.07 | 0.23±0.01 |
| C16:1 9t | 0.17±0.01 | 0.16±0.03 | 0.18±0.01 | 0.17±0.03 | 0.17±0.02 | 0.19±0.03 | 0.21±0.01 | 0.17±0.04 | 0.17±0.01 | 0.16±0.05 | 0.18±0.05 | 0.16±0.01 |
| C16:1 9t | 0.36±0.01 | 0.35±0.08 | 0.39±0.02 | 0.36±0.07 | 0.37±0.05 | 0.41±0.09 | 0.44±0.02 | 0.37±0.09 | 0.36±0.03 | 0.35±0.09 | 0.38±0.11 | 0.34±0.01 |
| C16:1 7c | 0.71±0.03 | 0.66±0.12 | 0.74±0.05 | 0.7±0.11 | 0.69±0.10 | 0.77±0.16 | 0.85±0.05 | 0.70±0.16 | 0.69±0.03 | 0.63±0.13 | 0.72±0.21 | 0.64±0.04 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C16:1 9c | 0.08±0.006 | 0.076±0.008 | 0.094±0.008 | 0.079±0.009 | 0.085±0.017 | 0.092±0.015 | 0.095±0.006 | 0.081±0.016 | 0.079±0.001 | 0.076±0.024 | 0.088±0.028 | 0.076±0.005 |
| C17:1 9c | 0.051±0.004 | 0.049±0.006 | 0.057±0.007 | 0.048±0.008 | 0.051±0.007 | 0.057±0.013 | 0.055±0.002 | 0.052±0.009 | 0.051±0.002 | 0.051±0.015 | 0.055±0.012 | 0.044±0.001 |
| C17:1 10c | 0.17±0.01 | 0.16±0.02 | 0.18±0.02 | 0.17±0.02 | 0.17±0.03 | 0.19±0.04 | 0.21±0.01 | 0.17±0.03 | 0.17±0.01 | 0.16±0.05 | 0.18±0.05 | 0.16±0.01 |
| C18:1 4t+5t | 0.026±0.003 | 0.025±0.004 | 0.03±0.004 | 0.027±0.002 | 0.032±0.004 | 0.033±0.005 | 0.03±0.001 | 0.03±0.008 | 0.032±0.000 | 0.027±0.006 | 0.03±0.007 | 0.027±0.002 |
| C18:1 6-9t | 0.39±0.01 | 0.35±0.04 | 0.38±0.05 | 0.39±0.05 | 0.37±0.07 | 0.41±0.08 | 0.43±0.06 | 0.39±0.08 | 0.38±0.02 | 0.35±0.07 | 0.39±0.12 | 0.05±0.00 |
| C18:1 10t | 0.23±0.02 | 0.22±0.03 | 0.22±0.01 | 0.19±0.04 | 0.23±0.03 | 0.25±0.08 | 0.26±0.02 | 0.24±0.07 | 0.19±0.01 | 0.23±0.07 | 0.22±0.05 | 0.31±0.00 |
| C18:1 11t | 2.09±0.10 | 2.01±0.37 | 2.17±0.19 | 2.08±0.26 | 2.06±0.28 | 2.27±0.42 | 2.50±0.19 | 2.03±0.42 | 2.05±0.04 | 1.97±0.59 | 2.11±0.65 | 1.99±0.01 |
| C18:1 12t | 0.32±0.02 | 0.31±0.05 | 0.34±0.03 | 0.33±0.05 | 0.33±0.05 | 0.36±0.07 | 0.38±0.03 | 0.33±0.07 | 0.33±0.02 | 0.31±0.10 | 0.33±0.10 | 0.29±0.01 |
| C18:1 9c | 13.45±0.91 | 12.55±1.96 | 13.92±1.47 | 13.18±1.68 | 13.15±2.25 | 14.49±2.65 | 16.09±1.38 | 13.15±2.64 | 13.08±0.27 | 12.73±4.03 | 13.52±4.3 | 12.21±1.14 |
| C18:1 15t | 0.21±0.01 | 0.2±0.01 | 0.25±0.04 | 0.21±0.01 | 0.23±0.03 | 0.27±0.04 | 0.27±0.01 | 0.24±0.04 | 0.24±0.01 | 0.22±0.06 | 0.24±0.05 | 0.20±0.00 |
| C18:1 11c | 0.27±0.01 | 0.25±0.04 | 0.28±0.03 | 0.27±0.04 | 0.27±0.05 | 0.30±0.06 | 0.32±0.03 | 0.27±0.06 | 0.27±0.00 | 0.26±0.05 | 0.28±0.08 | 0.25±0.02 |
| C18:1 12c | 0.13±0.00 | 0.12±0.02 | 0.14±0.02 | 0.13±0.02 | 0.13±0.02 | 0.15±0.04 | 0.15±0.01 | 0.13±0.04 | 0.13±0.00 | 0.13±0.03 | 0.14±0.03 | 0.12±0.00 |
| C18:1 13c | 0.072±0.004 | 0.07±0.007 | 0.076±0.009 | 0.072±0.012 | 0.081±0.011 | 0.077±0.019 | 0.085±0.008 | 0.075±0.014 | 0.077±0.003 | 0.076±0.021 | 0.079±0.02 | 0.068±0.002 |
| C18:1 14c+16t | 0.59±0.04 | 0.56±0.08 | 0.61±0.08 | 0.59±0.09 | 0.59±0.10 | 0.64±0.12 | 0.68±0.06 | 0.58±0.14 | 0.59±0.02 | 0.57±0.16 | 0.60±0.19 | 0.54±0.04 |
| C20:1 c9 | 0.046±0.006 | 0.049±0.002 | 0.057±0.002 | 0.046±0.006 | 0.055±0.012 | 0.062±0.012 | 0.056±0.001 | 0.067±0.018 | 0.066±0.006 | 0.053±0.014 | 0.061±0.016 | 0.051±0.004 |
| **PUFA** | | | | | | | | | | | | |
| C18:2 9t,12t | 0.54±0.02 | 0.53±0.08 | 0.58±0.06 | 0.56±0.09 | 0.55±0.08 | 0.60±0.11 | 0.60±0.13 | 0.55±0.12 | 0.56±0.02 | 0.54±0.17 | 0.57±0.17 | 0.51±0.03 |
| C18:2 9c,12t | 0.22±0.02 | 0.21±0.03 | 0.23±0.03 | 0.22±0.03 | 0.23±0.04 | 0.24±0.05 | 0.25±0.03 | 0.21±0.03 | 0.22±0.00 | 0.22±0.07 | 0.23±0.07 | 0.20±0.02 |
| C18:2 9t,12c | 0.47±0.03 | 0.44±0.08 | 0.48±0.03 | 0.45±0.07 | 0.46±0.06 | 0.50±0.12 | 0.53±0.04 | 0.45±0.11 | 0.45±0.02 | 0.43±0.13 | 0.47±0.13 | 0.41±0.02 |
| C18:2 9c,15c | 0.14±0.01 | 0.15±0.03 | 0.16±0.01 | 0.14±0.03 | 0.16±0.02 | 0.16±0.03 | 0.16±0.02 | 0.16±0.03 | 0.15±0.01 | 0.15±0.05 | 0.15±0.04 | 0.14±0.00 |
| C18:2 9c,12c | 1.30±0.03 | 1.23±0.25 | 1.35±0.09 | 1.29±0.22 | 1.29±0.17 | 1.40±0.31 | 1.55±0.07 | 1.29±0.31 | 1.27±0.07 | 1.24±0.34 | 1.31±0.35 | 1.18±0.05 |
| C18:2 9c,11t CLA | 1.09±0.08 | 1.01±0.15 | 1.14±0.12 | 1.06±0.14 | 1.07±0.19 | 1.18±0.22 | 1.30±0.11 | 1.07±0.21 | 1.06±0.03 | 1.03±0.33 | 1.09±0.35 | 0.99±0.09 |
| CLA c,c | 0.10±0.01 | 0.12±0.01 | 0.13±0.01 | 0.12±0.02 | 0.13±0.02 | 0.14±0.03 | 0.13±0.00 | 0.13±0.03 | 0.15±0.00 | 0.12±0.03 | 0.14±0.03 | 0.12±0.00 |
| CLA t,t | 0.19±0.00 | 0.20±0.01 | 0.21±0.02 | 0.19±0.03 | 0.21±0.04 | 0.21±0.04 | 0.22±0.02 | 0.19±0.04 | 0.22±0.01 | 0.21±0.09 | 0.20±0.04 | 0.18±0.00 |
| ∂ C18:3 6c,9c,13c | 0.028±0.001 | 0.069±0.01 | 0.039±0.002 | 0.039±0.00 | 0.035±0.003 | 0.036±0.006 | 0.033±0.003 | 0.034±0.011 | 0.037±0.001 | 0.028±0.007 | 0.036±0.007 | 0.03±0.00 |
| ∝C18:3 9c,12c,15c | 0.92±0.01 | 0.87±0.18 | 0.94±0.06 | 0.91±0.17 | 0.91±0.11 | 0.98±0.22 | 1.11±0.03 | 0.91±0.22 | 0.90±0.05 | 0.88±0.24 | 0.92±0.23 | 0.83±0.05 |
| C18:4 n6 | 0.12±0.01 | 0.11±0.02 | 0.12±0.01 | 0.12±0.03 | 0.12±0.00 | 0.13±0.04 | 0.13±0.00 | 0.13±0.04 | 0.12±0.02 | 0.11±0.02 | 0.11±0.02 | 0.1±0.00 |
| C20:4 n6 | 0.088±0.003 | 0.089±0.014 | 0.099±0.003 | 0.089±0.019 | 0.092±0.012 | 0.103±0.029 | 0.101±0.002 | 0.098±0.028 | 0.094±0.009 | 0.098±0.033 | 0.091±0.02 | 0.085±0.008 |
| EPA 20:5 n3 | 0.064±0.004 | 0.065±0.014 | 0.072±0.002 | 0.073±0.005 | 0.071±0.003 | 0.069±0.016 | 0.071±0.003 | 0.069±0.021 | 0.068±0.000 | 0.066±0.025 | 0.07±0.015 | 0.064±0.003 |
| C22:5 n3 DPA | 0.14±0.01 | 0.15±0.04 | 0.15±0.01 | 0.15±0.02 | 0.14±0.02 | 0.16±0.04 | 0.15±0.00 | 0.15±0.04 | 0.14±0.01 | 0.14±0.04 | 0.15±0.03 | 0.14±0.00 |
| C22:6 n3 DHA | 0.078±0.003 | 0.074±0.006 | 0.087±0.007 | 0.093±0.006 | 0.077±0.003 | 0.09±0.016 | 0.093±0.003 | 0.086±0.02 | 0.083±0.01 | 0.084±0.03 | 0.074±0.015 | 0.073±0.005 |
| **Total** | | | | | | | | | | | | |
| SFA | 58.92±0.05 | 55.07±11.61 | 61.19±2.04 | 58.0±10.43 | 57.21±6.2 | 64.45±15.61 | 69.77±2.06 | 58.08±15.38 | 57.98±5.41 | 55.02±14.17 | 58.61±15.32 | 53.45±1.2 |
| MUFA | 20.51±1.25 | 19.28±3.04 | 21.33±2.14 | 20.17±2.67 | 20.21±3.3 | 22.26±4.21 | 24.47±1.98 | 20.23±4.21 | 20.1±0.54 | 19.45±5.91 | 20.76±6.39 | 18.57±1.35 |
| PUFA | 5.49±0.11 | 5.32±0.92 | 5.77±0.45 | 5.50±0.87 | 5.54±0.77 | 6.00±1.29 | 6.42±0.44 | 5.53±1.26 | 5.52±0.26 | 5.35±1.61 | 5.62±1.55 | 5.07±0.27 |
| CLA | 1.38±0.07 | 1.33±0.12 | 1.47±0.18 | 1.37±0.05 | 1.41±0.49 | 1.54±0.02 | 1.65±0.18 | 1.39±0.45 | 1.43±0.25 | 1.36±0.23 | 1.43±0.15 | 1.29±0.01 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lipid quality indices** | | | | | | | | | | | | |
| IA | 2.36 | 2.35 | 2.37 | 2.36 | 2.33 | 2.40 | 2.37 | 2.37 | 2.38 | 2.32 | 2.33 | 2.38 |
| IT | 2.60 | 2.55 | 2.60 | 2.58 | 2.56 | 2.64 | 2.62 | 2.59 | 2.61 | 2.55 | 2.56 | 2.61 |
| Omega 6/Omega 3 | 12.47 | 10.59 | 11.51 | 11.21 | 11.97 | 11.78 | 12.89 | 11.29 | 11.68 | 11.57 | 11.96 | 11.57 |

Data expressed as mean (n =2). ai: anteiso; i: iso; c/t: cis/trans double bond; CLA: conjugated linoleic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid, SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; IA index of atherogenicity; IT index of thrombogenicity.

**Table 5.3: Mean fatty acid (FA) composition (mg FA/ 100 g fat) of NEFAME fractions of non-processed ($Ch_C$) and HPP treated (600 MPa/6 min - P1, 450 MPa/6 min – P2 and 450 MPa/9 min) *Serra da Estrela* cheeses**

| Storage time (months) | $Ch_C$ | | | P1 - 600 MPa/6 min | | | P2 - 450 MPa/6 min | | | P3 - 450 MPa/9 min | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 15 | 0 | 6 | 15 | 0 | 6 | 15 | 0 | 6 | 15 |
| **SFA** | | | | | | | | | | | | |
| C4 | 153.9±13.74 | 148.34±16.75 | 148.12±2.26 | 136.76±11.87 | 137.97±1.22 | 117.45±14.84 | 136.02±9.52 | 145.51±7.37 | 116.34±9.37 | 132.3±3.94 | 166.96±3.62 | 112.61±5.5 |
| C6 | 88.83±3.25 | 91.09±12.55 | 90.7±1.18 | 80.65±8.25 | 80.18±1.7 | 77.18±12.39 | 80.61±9.4 | 85.23±4.4 | 73.71±2.82 | 77.29±5.65 | 101.99±1.59 | 71.02±0.79 |
| C8 | 67.7±3.04 | 76.87±13.31 | 80.97±4.46 | 64.07±8.7 | 69.21±2.74 | 72.97±14.04 | 65.78±8.91 | 70.21±3.32 | 65.15±0.78 | 63.11±5.67 | 88.82±3.08 | 61.82±3.33 |
| C9 | 3007±1090 | 3724±489.6 | 4263±63.62 | 3077±386.2 | 3647±69.94 | 2659±385.7 | 3005±74.43 | 3861±861.2 | 3524±419.0 | 4075±500.8 | 4353±322.1 | 3476±570.2 |
| C10 | 148.47±10.42 | 172.42±32.73 | 211.65±45.42 | 136.93±22.04 | 161.35±11.52 | 193.02±46.04 | 143.93±20.07 | 170.04±20.41 | 163.18±6.37 | 140.86±20.3 | 203±13.93 | 151.04±13.41 |
| C12 | 53.8±3.53 | 77.72±1.06 | 84.57±21.4 | 51±7.2 | 61.26±7.07 | 82.7±20.35 | 52.97±7.34 | 67.84±12.01 | 75.84±9.22 | 48.71±3.93 | 73.42±6.01 | 64.66±9.57 |
| C13i | 1.15±0.06 | 1.33±0.17 | 1.88±0.08 | 0.93±0.16 | 1.63±0.09 | 2.1±0.16 | 1.19±0.02 | 1.19±0 | 2.17±0.24 | 1.18±0.21 | 1.69±0.19 | 1.4±0.2 |
| C14 | 79.6±6.08 | 103.07±15.07 | 126.79±28.48 | 75.63±8.11 | 91.64±8.2 | 125.3±29.17 | 77.82±9.13 | 104.25±18.63 | 126.47±15.27 | 68.84±3.39 | 107.05±11.27 | 101.98±16.1 |
| C14i | 2.06±0.37 | 2.17±0.1 | 3.04±0.55 | 2.16±0.33 | 2.15±0.07 | 2.81±0.31 | 1.58±0.06 | 2.48±0.37 | 3.3±0.07 | 1.34±0.02 | 2.55±0.37 | 2.14±0.04 |
| C14ai | 3.16±0.11 | 3.94±0.58 | 6.03±0.84 | 2.97±0.04 | 3.62±0.36 | 5.82±0.68 | 2.75±0.28 | 4.45±0.95 | 5.09±0.32 | 2.7±0.25 | 4.51±0.66 | 4.18±0.52 |
| C15 | 6.9±0.29 | 8.26±0.78 | 10.2±1.78 | 6.41±0.18 | 7.49±0.26 | 10.35±1.87 | 6.26±0.41 | 9.01±1.3 | 10.85±0.51 | 5.62±0.29 | 8.83±0.77 | 8.35±1.1 |
| C16 | 118.62±6.8 | 145.94±20.35 | 192.88±26.82 | 113.73±8.52 | 133.55±9.24 | 179.23±44.02 | 114.26±11.66 | 154.14±31.91 | 192.57±20.35 | 98±7.55 | 151.39±16.66 | 148.07±20.63 |
| C16i | 2.11±0.09 | 2.3±0.34 | 2.97±0.44 | 2.22±0.04 | 2.24±0.04 | 2.84±0.59 | 1.91±0.14 | 2.31±0.14 | 2.8±0.46 | 1.66±0.13 | 2.55±0.38 | 2.21±0.22 |
| C17 | 2.91±0.18 | 3.79±0.43 | 4.8±0.65 | 2.97±0.29 | 3.46±0.51 | 5.26±0.31 | 2.85±0.07 | 4±0.24 | 5.31±0.32 | 2.23±0.03 | 4.75±0.23 | 3.8±0.5 |
| C17i | 2.11±0.05 | 2.29±0.27 | 2.99±0.33 | 1.99±0.1 | 2.36±0.2 | 3.73±0.19 | 1.74±0.06 | 2.84±0.35 | 3.77±0.14 | 1.38±0.13 | 2.68±0.3 | 2.7±0.44 |
| C17ai | 3.72±0.14 | 4.44±0.47 | 5.85±0.86 | 3.58±0.18 | 4.45±0.18 | 6.58±0.97 | 3.19±0.16 | 5.14±0.66 | 6.94±0.14 | 2.98±0.05 | 5.27±0.83 | 4.9±0.46 |
| C18i | 41.03±0.07 | 49.23±5.78 | 63.7±12.62 | 39.47±0.32 | 46.9±1.54 | 63.07±13.4 | 38.68±1.96 | 53.86±9.51 | 68.22±5.4 | 33.15±2.38 | 51.86±4.35 | 51.88±5.23 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MUFA** | | | | | | | | | | | | |
| C10:1 | 7.25±0.09 | 8.33±0.5 | 9.46±1.08 | 6.63±0.5 | 7.65±0.03 | 8.7±1.49 | 6.78±0.48 | 7.98±0.4 | 8±0.91 | 6.8±0.87 | 9.79±0.11 | 7.27±0.25 |
| C12:1 | 1.96±0.39 | 2.54±0.33 | 2.88±0.33 | 2.21±0.16 | 1.94±0.24 | 2.78±0.57 | 1.83±0 | 2.35±0.16 | 2.38±0.35 | 1.7±0.02 | 2.79±0.18 | 2.11±0.17 |
| C14:1 | 4.45±0.53 | 5.29±0.46 | 6.94±1.08 | 4.15±0.26 | 5±0.02 | 7.25±0.95 | 3.84±0.01 | 5.7±0.68 | 6.78±0.48 | 3.23±0.05 | 9.59±0.37 | 8.66±0.64 |
| C15:1 | 1.75±0.08 | 1.98±0.03 | 2.87±0.59 | 1.61±0.19 | 1.69±0.07 | 2.77±0.45 | 1.63±0.07 | 2±0.22 | 3.29±0.02 | 1.38±0.01 | 2±0.17 | 2.37±0.38 |
| C16:1 7c | 3.84±0.08 | 5.54±0.05 | 7.52±0.06 | 3.09±0.06 | 4.41±0.64 | 7.39±1.48 | 2.69±0.39 | 5.87±0.55 | 6.93±0.27 | 1.96±0.15 | 5.02±0.33 | 5.15±0.8 |
| C16:1 9c | 2.91±0.18 | 3.79±0.43 | 4.8±0.65 | 2.97±0.29 | 3.46±0.51 | 5.26±0.31 | 2.85±0.07 | 4±0.24 | 5.31±0.32 | 2.23±0.03 | 4.75±0.23 | 3.8±0.5 |
| C16:1 9t | 2.04±0.1 | 2.82±0.24 | 3.73±0.43 | 1.59±0.11 | 2.25±0.19 | 4.17±0.69 | 1.58±0.13 | 3.13±0.59 | 3.97±0.77 | 1.31±0.03 | 3.13±0.57 | 2.97±0.79 |
| C17:1 10c | 2.13±0.22 | 2.9±0.16 | 4.62±1.31 | 2.24±0.19 | 3.08±0.11 | 5.52±0.75 | 1.96±0 | 4.39±0.14 | 5.56±0.43 | 1.69±0.16 | 2.81±0.38 | 3.53±1.04 |
| C18:1 6-9t | 3.15±0.81 | 3.24±0.39 | 5.39±1.01 | 2.65±0.1 | 3.99±0.06 | 5.86±0.56 | 2.32±0.05 | 4.17±0.26 | 5.57±0.66 | 2.74±0.35 | 4±0.2 | 4.83±1.1 |
| C18:1 10t | 1.77±0.37 | 1.22±0.09 | 3.26±0.97 | 1.2±0.26 | 1.73±0.02 | 2.37±0.2 | 0.67±0.05 | 1.4±0.2 | 3.4±0.09 | 1.13±0.27 | 1.59±0.24 | 2.05±0.49 |
| C18:1 11t | 14.72±0.06 | 20.67±2.38 | 30.15±10.12 | 13.88±1.96 | 19.03±1.46 | 32.12±8.45 | 14.21±1.62 | 24.56±8.27 | 32.18±5.38 | 11.97±2.51 | 22.51±4.4 | 25.96±6.39 |
| C18:1 12t | 2.39±0.02 | 3.05±0.14 | 4.82±1.51 | 2.33±0.23 | 3.19±0.12 | 4.3±1.14 | 2.32±0.18 | 3.12±0.34 | 4.43±0.32 | 2.03±0.38 | 3.34±0.57 | 3.92±1.15 |
| C18:1 15t | 1.93±0.12 | 2.23±0.06 | 3.47±1.3 | 1.52±0.23 | 1.93±0.11 | 3.1±0.42 | 1.68±0.18 | 2.46±0.19 | 2.64±0.14 | 1.36±0.05 | 1.9±0.04 | 2.25±0.09 |
| C18:1 9c | 108.7±2.93 | 152.24±26.73 | 261.11±103.16 | 100.54±12.43 | 153.28±34.25 | 278.02±75.48 | 94.35±17.2 | 190.46±80.23 | 256.64±61.84 | 84.69±19.2 | 183.08±28.68 | 217.35±73.59 |
| C18:1 11c | 2.94±0.23 | 3.31±0.47 | 6.18±1.58 | 2.85±0.23 | 3.8±0.6 | 6.28±1.85 | 2.82±0.2 | 4.39±1.51 | 6.22±0.59 | 2.06±0.15 | 3.63±0.59 | 5.07±1.62 |
| C18:1 12c | 3.58±0.23 | 4.7±0.46 | 8.33±2.46 | 3.67±0.44 | 4.67±0.61 | 8.77±1.33 | 3.62±0.33 | 6.04±1.3 | 8.58±0.73 | 3.2±0.16 | 5.83±1.18 | 6.44±2.17 |
| C18:1 14c+16t | 5.15±0.48 | 7.94±0.65 | 11.66±3.35 | 5.51±0.01 | 7.7±1.81 | 12.77±2.51 | 4.77±0.63 | 8.75±1.7 | 12.44±0.94 | 4.37±0.43 | 8.76±1.06 | 9.22±2.6 |
| **PUFA** | | | | | | | | | | | | |
| C18:2 9t,12t | 2.37±0.22 | 4.28±0.42 | 6.1±0.78 | 2.38±0.54 | 4.61±0.05 | 5.75±1.28 | 2.77±0.39 | 4.42±0.86 | 6.14±0.69 | 2.03±0.16 | 4.09±0.22 | 3.89±0.12 |
| C18:2 9c,12t | 16.25±0.06 | 23.74±5.43 | 39.19±17.69 | 14.82±1.86 | 22.67±6.55 | 40.58±14.03 | 13.18±2.66 | 28.87±13.67 | 37.55±11.08 | 12.8±3.73 | 25.93±9.41 | 34.72±16.47 |
| C18:2 9c,11t CLA | 8.58±0.31 | 13.06±1.51 | 19.77±5.77 | 8.65±0.63 | 12.15±1.1 | 21.08±4.71 | 8.2±0.89 | 14.26±5.27 | 19.08±3.27 | 7.45±1.16 | 13.31±2.9 | 16.2±4.05 |
| ∝C18:3 9c,12c,15c | 14.45±2.22 | 24.14±6.33 | 38.85±18.14 | 14.37±2.15 | 22.04±7.27 | 38.79±14.06 | 13.74±2.3 | 28.47±14.66 | 34.57±11.72 | 14.35±1.88 | 25.62±8.03 | 32.26±12.99 |
| **Total** | | | | | | | | | | | | |
| **SFA** | 3783.45±1085.5 | 4617.74±371.95 | 5301±83.52 | 3798.89±692.56 | 4457.02±28.6 | 3609.95±186.44 | 3736.68±153.51 | 4743.97±749.97 | 4445.76±374.73 | 4757.19±554.45 | 5330.89±377.53 | 4268.92±636.89 |
| **MUFA** | 175.95±0.56 | 238.82±34.05 | 388.8±2.38 | 163.02±22.46 | 235.94±41.61 | 410.16±103.08 | 154.49±21.85 | 289.69±101.09 | 385.2±73.32 | 137.87±24.86 | 282.29±40.85 | 323.01±97.1 |
| **PUFA** | 41.65±2.38 | 65.22±13.69 | 103.9±42.39 | 40.22±7.45 | 61.47±14.96 | 106.20±34.08 | 37.88±6.24 | 76.02±34.46 | 97.35±26.77 | 36.64±6.61 | 68.96±20.56 | 87.06±33.4 |

Data expressed as mean (n =2); CLA: conjugated linoleic acid; SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids.

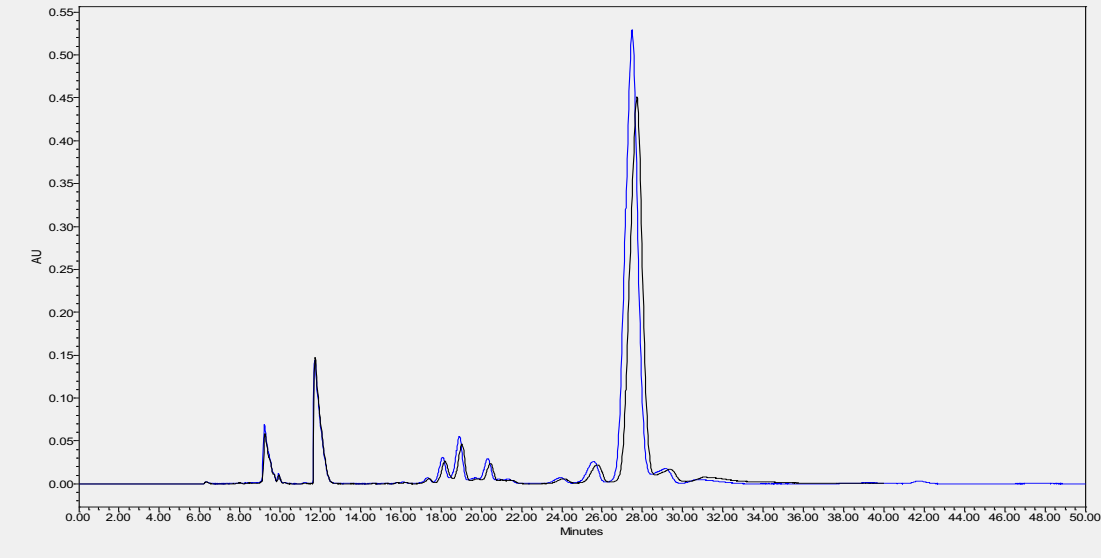



**Figure 5.1: Example of HPLC separation of CLA isomers of $Ch_C$ (blue line) and HPP P1 – 600 MPa/6 min (black line) of *Serra da Estrela* cheese at month 0 of storage.**

#### 5.3.2.1. Effect of HPP on fatty acid composition of *Serra da Estrela* cheese

The total content of SFA, MUFA and PUFA in EFAME fractions of HPP cheeses were similar to those of $Ch_C$ cheeses, exception for HPP P2 cheeses at month 0, which revealed an increment of about 18% in relation to both $Ch_C$ and other HPP (P1 and P3) cheeses. At 15 months of storage, the SFA content was numerically, although not significantly, slightly lower in HPP P2 and P3 cheeses (57.9 and 53.5 g FA/100 g fat) than in $Ch_C$ cheese (61.2 g FA/100 g fat), while HPP P1 cheeses revealed a higher content (64.5 g FA/100 g fat). Analysing the main SFA at 15 months of storage, somewhat lower values were observed for HPP P3 cheeses in comparison to $Ch_C$ cheeses, in what concerns palmitic (C16:0, 16.87 g FA/100 g fat vs. 19.26 g FA/100 g fat, respectively), myristic (C14:0, 8.84 g FA/100 g fat vs. 10.10 g FA/100 g fat), lauric (C12:0, 3.66 g FA/100 g fat vs. 4.18 g FA/100 g fat) and butyric acids (C4:0 2.68 g FA/100 g fat vs. 3.09 g FA/100 g fat). To also be highlighted the lower content of butyric acid in HPP NEFAME P3 fraction, since butyric acid has been associated to rancid-like cheese odour

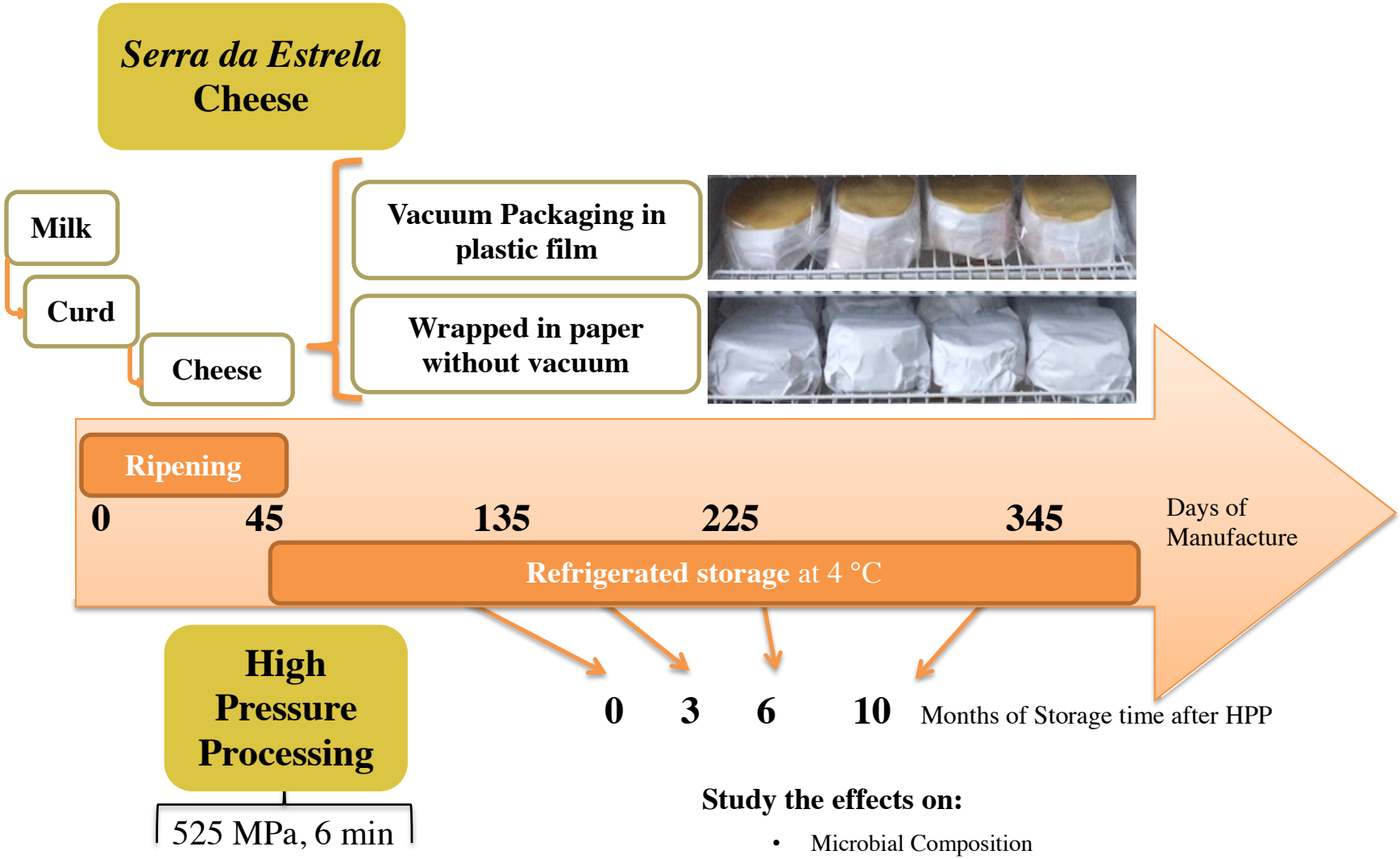
(Delgado *et al.*, 2009). Similar lower amounts of butyric acid were reported in HPP (400 and 600 MPa for 5 and 20 min) processed *Torta del Casar* cheeses (Rodríguez-Pinilla *et al.*, 2015). Nevertheless, sensory evaluation of those *Serra da Estrela* cheeses did not reveal significant odour differences between $Ch_C$ and HPP cheeses, and the characteristic lactic, acid, animal/stable and short-chain fatty acids (vomit like odour) attributes were detected (CHAPTER 4).

Nutritional indices are presented in Table 5.2. The PUFA/SFA ratio is measured to indicate the risk of incidence of CVD. It has also been discussed, although with some uncertainties, that single fatty acids might play negative roles on human health, in particular on the probability of increasing atheroma (C12:0, C14:0 and C16:0) and thrombus formation (C14:0, C16:0 and C18:0) (Ulbricht and Southgate, 1991), hence, in many studies, the index of atherogenicity (IA) and the index of thrombogenicity (IT) are calculated (based on experimental values) to assess diet nutritional quality and the associated propensity to influence the incidence of CVD (Ulbricht and Southgate, 1991). According to the results presented in Table 5.2, the EFAME fractions of all cheeses revealed similar atherogenic (between 2.32 and 2.40) and thrombogenic (between 2.55 and 2.64) indices, although the HPP P3 cheese revealed slightly lower (less 2%) values compared with the $Ch_C$ cheeses. Dairy products with lower atherogenic and thrombogenic indices are potentially healthier for humans (Naydenova *et al.*, 2014). Mature buffalo cheeses revealed an IA index of 2.16 and an IT index of 1.20 (Naydenova *et al.*, 2014).

The balance of PUFA omega n-6/omega n-3 is relevant, since PUFA depress HDL, which has a protective action against CVD (Ulbricht and Southgate, 1991). EFAME extracts of HPP and $Ch_C$ cheeses revealed similar omega n-6/omega n-3 ratios, where the EFAME fat fractions of HPP P3 cheeses were also those that revealed lower values.

## 5.4. Conclusions

HPP did not affect *Serra da Estrela* cheese characteristics in terms of lipid composition: triglycerides, esterified and non-esterified fatty acids methyl esters and conjugated fatty acids, and nutritional features: atherogenicity and thrombogenicity indices were similar among all cheeses. Furthermore, this research work presents for the first time, the effect of HPP on the mean triglycerides composition of a raw ewe's milk cheese (no major effects were observed) as well as a comprehensive identification of esterified and non-esterified fatty acids methyl esters fractions of *Serra da Estrela* cheese. In terms of free fatty acids identification, the procedure used in this study was able to identify 60 FFA (including, SFA, MUFA and PUFA and associated conjugated isomers) in comparison to the 39 FFA previously identified in research studies on *Serra da Estrela* cheese. The high CLA content could be ascribed to the presence of a large number of precursors in milk and effective enzyme activity, which were not affected by HPP. Overall, having no major impact on *Serra da Estrela* cheese qualitative and quantitative lipid profiles, HPP may be considered a potential cold pasteurization process to assure the microbial safety and quality of *Serra da Estrela* cheese.




**$Ch_C$+V** Control raw cheese under vacuum in plastic film
**$Ch_P$+V** HPP cheese under vacuum in plastic film
**$Ch_C$ +P** Control raw cheese wrapped in paper without vacuum
**$Ch_P$+P** HPP cheese wrapped in paper without vacuum

**Study the effects on:**

- Microbial Composition
- Physicochemical parameters
- Colour
- Textural parameters
- Proteolysis
- Visual appearance
- Sensorial

General schematic flow diagram of the work carried in Part II – CHAPTER 6.

# CHAPTER 6 - Comparison of packaging methods and materials: vacuum vs paper wrapping, on quality stability of HPP treated cheeses during storage

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*This chapter has been submitted for publication.*

## Abstract

Raw ewes' milk cheeses such as the Portuguese *Serra da Estrela* cheese with Protected Designation of Origin (PDO) are traditional food products, quite common in several countries, which require careful handling given the fact that they are produced with raw milk. High pressure processing (HPP) can be used for cold pasteurization of foods such as raw milk cheese, overcoming safety issues, but with minimal changes in quality. An adequate packaging system is important to preserve *Serra da Estrela* cheese physicochemical and microbial quality throughout refrigerated storage and commercialization enabling an extended shelf-life. Such cheese is generally wrapped in paper and kept without vacuum, as for other raw milk cheeses, but in the case of HPP treatment cheeses must be vacuum-packed in impermeable packages (usually using plastic films) beforehand. Hence, two packaging systems with potential for maintaining microbiological quality and extending shelf-life of HPP treated *Serra da Estrela* cheese were studied: (i) wrapping in paper without vacuum and packaging in plastic film under vacuum. Unpasteurized cheeses were used as control cheeses. The results revealed that lactococci, lactobacilli, enterococci and total mesophiles microbial groups achieved viable cell numbers close to 8 log cfu/g in control cheeses, while in HPP treated cheeses these bacterial groups reached lower viable cell numbers, between 4 and 6 log cfu/g with no significant differences between both packaging systems: non-vacuum wrapped in paper and vacuum-packed in polyamide-polyethylene plastic film being observed. Spoilage microorganisms such as *Enterobacteriaceae*, coliforms, *E. coli* and staphylococci counts were reduced to below the quantification limit in HPP treated cheeses (<3 log cfu/g), independently of the packaging system. However, yeasts and moulds grew in non-vacuum paper wrapped cheeses (>5 log cfu/g) and the colour of these cheeses' rind was more yellowish – brown. Overall, for a short storage period of less than 3 months the conventional non-vacuum paper wrapping could be an interesting methodology to package cheese, but for long periods the vacuum packaging in plastic film method is preferable.

## 6.1. Introduction

Traditional cheeses made from raw milk have been manufactured for centuries and are well known for their unique organoleptic characteristics. A common example is the Portuguese *Serra da Estrela* Cheese with Protected Designation of Origin (PDO) certification in the European Union. For the production of this cheese, only three ingredients are used: raw ewes' milk, crude extract of *Cynara cardunculus* L. as coagulant, and salt (Macedo *et al.*, 1993), as reported in CHAPTER 1- *Serra da Estrela* cheese: a review. Since milk is not thermally pre-treated to produce this cheese, both spoilage and pathogenic microorganisms may be found in this type of cheese if not properly handled (Macedo *et al.*, 1995), thus raising safety issues. Current efforts are constantly directed in meeting the needs of consumers by ensuring cheese quality with prolonged shelf-life during storage and commercialization. Two possible ways are related with milk non-thermal pasteurization and with protective packaging systems. As a non-thermal technology, high pressure processing (HPP) has been studied and applied as a non-thermal alternative process to the conventional thermal pasteurization, with a 2-3 fold shelf-life extension for dairy foods compared to the raw counterparts (Dhineshkumar *et al.*, 2016), and with low impact on quality. According to CHAPTER 2 - High pressure processing on milk and raw milk cheese, several studies have revealed the advantage of HPP application at optimum organoleptic ripening time of cheeses, ensuring effective inactivation of microorganisms and thus improving safety (Delgado *et al.*, 2015; Rodríguez-Pinilla *et al.*, 2015). For example studies performed with *Torta del Casar* cheese (Delgado *et al.*, 2015; Rodríguez-Pinilla *et al.*, 2015) demonstrated that HPP treatment of 600 MPa/5 min at 60 days of ripening caused 2.3 and 2.5 log cycle reductions in *Enterobacteriaceae* and in Pseudomonas spp. viable cells numbers, respectively (Rodriguez-Pinilla *et al.* 2015). In what concerns *Serra da Estrela* cheese HPP treatment

of 450 and 600 MPa for 6 minutes enabled lactic acid bacteria (LAB) viable cells numbers to be kept at similar levels to control cheeses and only minor changes were verified among physicochemical parameters (CHAPTER 3). Both studies also showed interesting results in terms of proteolytic indices; HPP deaccelerated proteolysis keeping the ripening extension index closer to that of control cheeses, and therefore closer to the optimum organoleptic attributes (CHAPTER 4) and delaying the over-ripening during refrigerated storage up to commercialization (Rodríguez-Pinilla *et al.*, 2015).

HPP treatment requires products to be packed in water impermeable packages (usually polyamide-polyethylene plastic films for solid foods such as cheese) previously to processing. However, consumer preference for these type of traditional cheeses (raw milk cheeses) is for wrapping paper package system without vacuum. Nevertheless, foods cannot be processed by HPP if wrapped only in paper.

In order to study the feasibility of HPP as a non-thermal treatment for raw milk cheese pasteurization, once processed the cheeses were stored as they were processed, i.e., under vacuum (Delgado *et al.*, 2015; Rodríguez-Pinilla *et al.*, 2015). However, in these studies it was verified that the cheeses' rind became whiter, a negative aspect that could be avoided allowing access to oxygen by using oxygen permeable packages (Rodríguez-Pinilla *et al.*, 2015).

Based on this, two packaging systems for storage of *Serra da Estrela* raw milk cheeses after HPP application at optimum organoleptic time were assessed: (i) vacuum packaging in polyamide-polyethylene plastic bag and (ii) non-vacuum packed in greaseproof wrapping paper (cheeses were removed from the plastic bag after HPP and kept wrapped in paper for the study). To the best of our knowledge no other study is available in the literature on this specific research topic.

## 6.2. Materials and methods

### 6.2.1. Milk supply and cheese manufacture

One hundred and fifty litres of raw ewe milk (from two farms in *Serra da Estrela* cheese production region, Portugal) were collected and kept in a refrigerated reservoir until further use. Prior to sampling, milk was well mixed to ensure a homogeneous sampling. Two batches of *Serra da Estrela* cheese were produced at the dairy, one in the morning and the other early afternoon, according to the PDO procedures (Macedo *et al.*, 1993). The resulting 56 cheeses (of about 500 g each) were ripened during 45 days according to the PDO practices (first 15 days at 8 ± 2 ºC and 95% RH and then at 10 ± 2º C and 85% RH) (Macedo *et al.*, 1993) in order to reach the optimum organoleptic level. Upon 45 days of ripening at the dairy, half of the cheeses (28) were wrapped in paper (50 g/m$^2$ white Kraft paper plus 10 g/m$^2$ low density polyethylene (LDPE) from Seilimp, Oliveira do Hospital, Portugal) and all 56 cheeses were then vacuum packaged (vacuum packaging machine HenkoVac E-193, Albipack, Aveiro, Portugal) in heat sealed polyamide-polyethylene film (PA-PE, Plásticos Macar – Indústria de Plásticos Lda, Santo Tirso, Portugal).

### 6.2.2. High pressure processing

Twenty-eight cheeses were treated by HPP treatments in a 55-liter capacity industrial scale high pressure equipment (model 55, Hiperbaric, Burgos, Spain) at 525 MPa for 6 min (this condition was selected based on previous results obtained for *Serra da Estrela* cheese pasteurization (CHAPTER 3-5). The initial temperature of the water used as transmitting fluid was 8 °C and the two cheese batches were processed in two different high-pressure processing cycles.

### 6.2.3. Samples identification and sampling

Milk used for cheese manufacture and the resulting curd from each batch: the morning and the early afternoon batches, were collected from the refrigerated reservoir mixer and after squeezing the curd (1.5 h after starting milk coagulation), respectively, for their chemical characterization.

The fifty-six cheeses were divided equally into two groups: HPP processed (28) and unprocessed cheeses (control cheeses, 28). In order to study the effect of the packaging system, half of the unprocessed cheeses ($Ch_C$) were kept under vacuum in polyamide-polyethylene plastic film ($Ch_C$+V, 14 cheeses) and the other 14 cheeses were kept wrapped in greaseproof paper without vacuum ($Ch_C$+P); similarly the HPP treated cheeses ($Ch_P$) were kept half (14 cheeses) under vacuum in polyamide-polyethylene plastic film ($Ch_P$+V) and the other half (14 cheeses) were removed from the plastic bag and kept wrapped in greaseproof paper without vacuum ($Ch_P$+P). All 56 cheeses were kept refrigerated at 4 ºC for 10 months, having the non-vacuum paper wrapped cheeses been washed due to visible mold development, after 3 and 6 months storage. The washing step was carried out according to PDO mandatory procedures (water and a wash brush) except that sterilized water was used and the washing took place under aseptic conditions in a laminar flow cabinet to avoid microbial contamination; it was important to keep the non-vacuum paper wrapped cheeses under conditions as similar as possible to those of the vacuum packed cheeses, in what concerns avoidance of microbial contamination. At each storage time (0, 3, 6, and 10 months), aliquots of each cheese (≈35 g per sample) were stored at −80 °C until physicochemical analyses were carried out.

### 6.2.4. Microbiological analyses

As previously described in section 3.2.4 Microbiological analyses 6.2.4 Microbiological analyses, with some modifications. The Miles and Misra technique (Miles *et al.*, 1938) was used for enumeration of: *Enterococcus* spp. on kanamycin aesculin azide agar base (KAAA, Oxoid, United Kingdom) and incubated at 37 ºC for 24 h; *Lactobacillus* spp. on Man, Rogosa and Sharpe (MRS, Merck) and incubated at 30 ºC for 3 d; *Lactococcus* spp. on M17 (Liofilchem, Italy) and incubated at 30 ºC for 3 d; and *Bacillus* spp. on HiChrome (Fluka, India) and incubated at 30 ºC for 2 d; total aerobic mesophilic microorganisms on plate count agar (PCA, Merck) and incubated at 30 ºC for 3 d; total anaerobic microorganisms on PCA and incubated at 37 ºC for 2 d in anaerobic jars (Merck) with Merck Anaerocult A (Merck); total psychotropic microorganisms on PCA and incubated at 20 ºC for 5 d; yeasts and molds on rose-bengal chloramphenicol agar (RBCA, Merck) and incubated at 25 ºC for 5 d; *Staphylococcus* spp. on Baird-Parker agar (BPA, Merck) with egg yolk tellurite emulsion (Liofilchem) and incubated at 37 ºC for 2 d; *Listeria* spp. on PALCAM agar selective agar base (Liofilchem), with selective supplement for PALCAM (Liofilchem) and incubated at 37 ºC for 2 d; and *Pseudomonas* spp. on pseudomonas agar base (PAB from Liofilchem) with glycerol and pseudomonas CFC supplement (CFC, Liofilchem) and incubated at 30 ºC for 2 d. Petri dishes containing 30 - 300 and 10 – 100 colony forming units (cfu) were selected for counting for pour plate and Miles and Misra, respectively. The results were converted into logarithmic decimals of the number of cfu *per* g of cheese sample, and the values were considered below the limit of quantification of 2.0 log cfu/g for pour plate technique and 3.0 log cfu/g for Miles and Misra technique. Less than 1 log cfu/g was considered for milk samples due to direct liquid sample plating.

### 6.2.5. Physicochemical analyses

As previously described in section 3.2.5 Physicochemical analyses.

### 6.2.6. Colour

As previously described in section 3.2.6 Colour.

### 6.2.7. Proteolysis

As previously described in section 4.2.2 Proteolytic indices.

### 6.2.8. Instrumental texture profile analysis (TPA)

As previously described in section 4.2.4 Instrumental texture profile analysis (TPA).

### 6.2.9. Sensory evaluation

Sensory sessions were carried out in the conditions previously described in section 4.2.5 Sensory evaluation. Three sensory evaluation sessions were carried out at each sampling date: 0 months, 3 months and 6 months, and only one session at 10 months. Firstly, two paired comparison tests were carried out in two sessions: in the first session panellists compared $Ch_P$+V with $Ch_C$+V to ascertain possible differences caused by the effect of HPP on cheeses stored under vacuum packaging in plastic film; in the second session, $Ch_P$+P was compared with $Ch_C$+P to assess possible differences caused by the effect of HPP on cheeses stored wrapped in paper without vacuum. Attribute difference-

from-control method was used to compare the magnitude of difference between the intensity of each evaluated attribute of the HPP cheeses relatively to control cheeses, using a bipolar anchored continuous scale (-10=much less intense.., 0=no difference, 10=much more intense…). The attributes evaluated by panellists were: rind appearance (tonality from much lighter to much darker, homogeneity and defects), paste appearance (colour from much lighter to much darker and consistency from much more fluid to much firmer), odour (lactic, acid, animal/stable and short-chain fatty acids (SCFA)/vomit from much less intense to much more intense), texture (consistency from much softer to much harder, unctuosity and friability from much less to much more), taste (salty, acid and bitter from much less intense to much more intense) and after-taste (much less intense to much more intense). For rind evaluation one half cheese was presented to panellists, for the remaining attributes cheese slices were provided to panellists in Petri dishes. In each of the two sessions one sample of the control cheese was presented to panellists identified as such; a second sample of the control cheese (blind control sample) plus a sample of the HPP cheese were presented to panellists coded with three digit random numbers. In the third session, a rating test was used to evaluate the intensity of the above mentioned attributes on the four cheeses. A continuous anchored scale was used (0=absent, 10=strong) and samples were coded with three digit random numbers. This third evaluation session allowed evaluating the effect of HPP and type of storage packaging system.

### 6.2.10. Statistical analyses

Analysis of variance (ANOVA) was performed to establish the effect of different processing/packaging systems conditions, the effect of storage and both. Significant differences were investigated using a post-hoc test – Bonferroni procedure, with the

significance assigned at $p < 0.05$. Data without normal distribution were analyzed by Kruskal Wallis test. Attribute difference-from-control test sensory data was analyzed by paired *t*-student comparison between $Ch_P$+V - $Ch_C$+V and $Ch_P$+P – $Ch_C$+P, with the significance assigned at $p < 0.05$; when the distribution of the differences between the control and treated cheeses did not show a normal distribution, non-parametric test Wilcoxon was applied. Sensory rating test data were analysed by one-way ANOVA and a Tukey's post-hoc test was applied to compare the mean values of attributes for each storage time. SPSS software version 24.0 was used for the statistical analysis.

## 6.3. Results and discussion

### 6.3.1. Microbial composition of milk and fresh curd

In milk samples, lactobacilli and lactococci revealed a microbial load of 4.23 and 5.72 log cfu/mL, respectively, and a total aerobic mesophilic bacteria viable cell numbers of 5.73 log cfu/mL. *Enterobacteriaceae*, coliforms and enterococci viable cell numbers were found at similar levels, namely, 2.98, 2.60 and 2.42 log cfu/mL, respectively. Staphylococci and *Pseudomonas* spp. were detected at 4.21 and 3.36 log cfu/mL, respectively. In the curd viable cell numbers of 5.21, 5.70, 4.13 and 6.36 log cfu/g were measured for lactobacilli, lactococci, enterococci and total aerobic mesophiles, respectively, while *Enterobacteriaceae* and coliforms were quantified at 4.96 and 4.28 log cfu/g (ca. 2 log cycle increase in comparison to milk microbial load), respectively. Viable cell numbers of staphylococci remained fairly stable at 4.13 log cfu/g and *Pseudomonas* spp. increased ca. 1 log cycle to viable cell numbers of 4.73 log cfu/g.

### 6.3.2. Changes in microbial composition induced by HPP and type of packaging

Figure 6.1 shows the viable cell numbers of the different microbial groups found in HPP treated and non-treated *Serra da Estrela* cheeses stored for 10 months at 4 ºC under vacuum in plastic film or wrapped in paper without vacuum. In general, viable cell numbers were not significantly affected ($p > 0.05$) by the packaging system type but they were affected by HPP treatment ($p < 0.05$).

Lactobacilli and lactococci (Figure 6.1 A and B) were found at a similar order of magnitude independently of the cheese treatment and packaging system; in $Ch_C$+V cheeses viable cell numbers were found at 8.37 and 8.29 log cfu/g, respectively, at the beginning of storage (0 months), and these remained relatively constant ($p > 0.05$) throughout the 10 months of storage (exception for lactococci viable cell numbers that increased to 9.30 log cfu/g at 3 months of storage ($p < 0.05$). Similar values were reported in literature, never below the 8 log cfu/g at 30-60 days of ripening (Inácio *et al.*, 2014; Tavaria and Malcata, 2000). The HPP treatment caused a significant reduction in lactobacilli and lactococci viable cell numbers ($p < 0.001$) of about 3.8 and 2.5 log cycles, respectively, values close to those previously obtained for *Serra da Estrela* cheese (450 and 600 MPa/6 min) (CHAPTER 3) and in *Torta del Casar* cheese (400 and 600 MPa/5 min) (Calzada *et al.*, 2013). At each sampling point, no significant differences in viable cell numbers between cheeses stored under vacuum in plastic film and non-vacuum wrapped in paper were observed ($p > 0.05$). Enterococci counts revealed a behaviour similar to that of LAB (Figure 6.1 C) having the $Ch_P$+V and $Ch_P$+P cheeses showed a significant decrease of 1.00 and 1.12 log cycle reduction in enterococci viable cell numbers in comparison to $Ch_C$+V and $Ch_C$+P cheeses ($p < 0.001$), respectively. A higher reduction of about 2-3 log cfu/g was verified for HPP (400 or 600 MPa/5 min) *Torta del*

*Casar* cheeses (Calzada *et al.*, 2013). Total aerobic mesophiles revealed viable cell numbers of 8.61 log cfu/g in $Ch_C$+V cheeses at 0 months of storage, with no further variations along storage time ($p > 0.05$), as can be observed in Figure 6.1 D. The packaging system type also had no significant effect on total aerobic mesophiles ($p > 0.05$), while HPP led to a significant decrease of about 2.6 log cycles of this group of microorganisms ($p < 0.01$). In the literature, lower reductions (0.88-1.33 log units) were achieved for both lower and higher pressure intensity (400 or 600 MPa/5 min) of *Torta del Casar* cheeses treated at 35 days of ripening (Calzada *et al.*, 2013). Nevertheless, the results in the present work are in agreement with values previously reported for total aerobic mesophiles in HPP *Serra da Estrela* cheese (2.4-5.3 log cycle reductions) (CHAPTER 3). For anaerobic and psychotropic microorganisms (Figure 6.1 E and F), a behavior similar to that of total aerobic mesophilics was found.

In agreement with the 5-6 log cfu/g reported at 35 days of ripening by Macedo *et al.*, (1996, 1995) were the total coliforms viable cell numbers in $Ch_C$+V cheeses at 0 months, which were kept stable throughout storage ($p > 0.05$) (Figure 6.1 G). HPP caused 1 log cycle reduction at 0 months storage, which intensified along storage, making viable cell numbers drop to values closer to 3 log cfu/g; a higher reduction (> 3.5 log cycle units) were reported for similar HPP treatments by Arqués *et al*., (2006) and Calzada *et al*., (2013).

*Enterobacteriaceae*, *Pseudomonas* spp. and *E. coli* viable cell numbers were reduced to below the quantification limit by HPP treatment (Figure 6.1 H). On the other hand, in the literature, *Torta del Casar* cheeses HPP (200 or 600 MPa/5 or 20 min) revealed *Pseudomonas* counts on cheese rind, having the cheeses been kept under vacuum for 180 days (Rodríguez-Pinilla *et al.*, 2015). The HPP treatment caused staphylococci viable cell numbers to reduce to viable cell numbers of ~3.8 log cfu/g, below the

established limit of $10^5$ cfu/g (European Commission, 2005) (Figure 6.1 I). *Listeria* spp. was below the detection limit in all cases.

Yeasts and moulds viable cell numbers in $Ch_C$+V cheeses reached between 4.07 and 6.48 log cfu/g over the 10 months of storage, as can been seen in (Figure 6.1 J). HPP caused significant reductions of > 3.4 log cycles to below the quantification limit, however, in this case the packaging system played an important role; in the case of $Ch_P$+P cheeses, this effect vanished with time, since yeasts and moulds proliferated in the rind, leading to the need to wash the cheeses.

As far as the authors are aware, there are no reports in the literature concerning the impact of storage on the microbial quality of non-vacuum paper wrapped cheeses. On the other hand, in the case of vacuum packed ewe cheese storage, HPP treatments (600 MPa/5 min), caused > 2.1 log units reduction of moulds, to below the quantification limit at 180 days (Rodríguez-Pinilla *et al.*, 2015).

Overall, $Ch_C$+V cheeses revealed viable cell numbers closer to those already reported for *Serra da Estrela* cheese. HPP caused microbial reductions in the range reported in the literature for vacuum packaged and stored cheeses. In general, the packaging system type caused no significant differences in viable cell numbers, exception for yeasts and moulds, which showed growth in non-vacuum paper wrapped cheeses, but not in vacuum-packed in plastic film cheeses, independently of non-treated or HPP treatment.

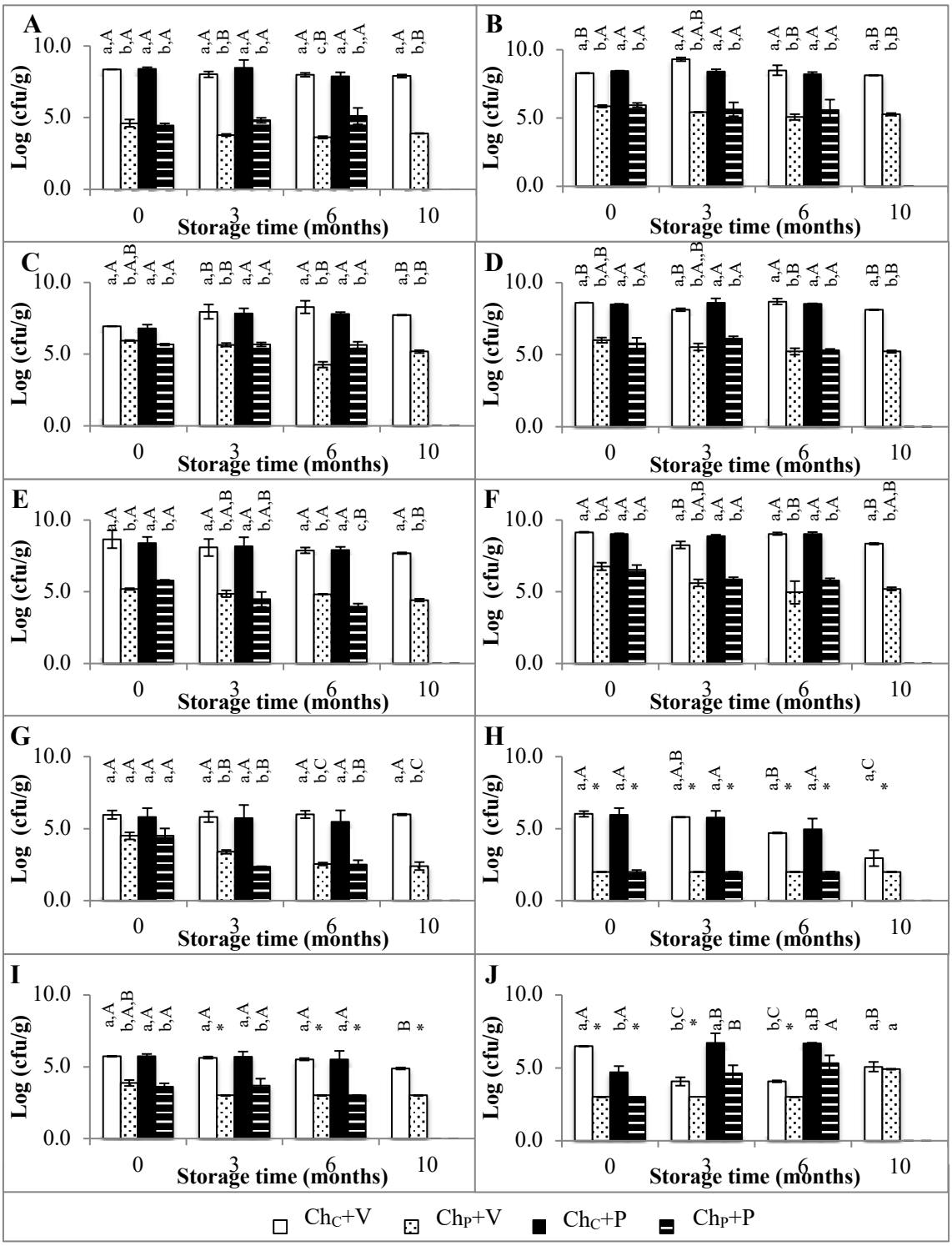



**Figure 6.1: (A) Lactobacilli, (B) lactococci, (C) enterococci, (D) total aerobic, (E) anaerobic, (F) psychrotrophic, (G) total coliforms, (H) *Enterobacteriaceae*, (I) staphylococci, (J) yeasts and moulds counts in *Serra da Estrela* cheese at 0, 3, 6 and 10 months of refrigerated storage (control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P)). * means below the quantification limit. Different non-capital letters (a, b, c) indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) indicate statistically significant differences among the same condition ($p < 0.05$).**

### 6.3.3. Changes in physicochemical characteristics

Despite the natural decrease of the moisture content during storage, neither the HPP treatment nor the packaging system (vacuum vs non-vacuum/paper vs plastic film) influenced moisture and protein contents significantly (Table 6.1). The measured values were found within the ranges reported in literature for this cheese, i.e. 40-48 and 14–26 %, respectively (Correia *et al.*, 2016; Macedo *et al.*, 2004). Similarly, Delgado *et al.*, (2015) demonstrated that HPP treatments (200 or 600 MPa/5 or 20 min) did not have an impact on the moisture content of ripened ewes' cheese.

At the beginning of storage (0 months) the $Ch_C$+V cheeses had pH values of 5.24, similarly to the other three types ($Ch_C$+P, $Ch_P$+V and $Ch_P$+P) of cheeses ($p > 0.05$). As storage time increased so did pH values of $Ch_C$+P cheeses, in particular, reaching a significantly different pH value of 6.69 at 6 months of storage ($p < 0.001$). Interestingly, non-treated and HPP treated paper wrapped cheeses, $Ch_C$+P and $Ch_P$+P, were the cheeses that revealed the highest increase in pH values over a 6 month storage period; in fact, both cheese types showed pH values higher than those normally reported in literature for this cheese, i.e. 4.82-5.66, which is aligned with the higher proteolytic indices discussed below – the release of free amino acids will increase the cheese pH value (Guiné *et al.*, 2016; Inácio *et al.*, 2014; Macedo *et al.*, 2004; Sousa and Malcata, 1997). Similarly to a previous study, $Ch_C$+V cheeses revealed the highest TA, probably due to higher indigenous microbial counts and consequently a higher metabolic activity (Macedo *et al.*, 1993). Non-treated and HPP treated paper wrapped cheeses maintained TA values relatively stable over 6 months of storage. We should highlight that the lack of reports in the literature concerning *Serra da Estrela* cheese storage aswell as that of other cheeses when wrapped in greaseproof paper do not enable further comparisons.

**Table 6.1: Moisture, protein content, pH values, and titratable acidity measured at 0, 3, 6 and 10 months of refrigerated storage of *Serra da Estrela* control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P).**

| | $Ch_C$+V | | $Ch_P$+V | | $Ch_C$+P | | $Ch_P$+P | |
|---|---|---|---|---|---|---|---|---|
| **Water Content** | **% (w/w) ± STD** | | **% (w/w) ± STD** | | **% (w/w) ± STD** | | **% (w/w) ± STD** | |
| 0 | 46.0 ± 2.43 | a,A | 46.1 ± 0.93 | a,A | 46.4 ± 0.39 | a,A | 43.3 ± 1.36 | b,A |
| 3 | 41.7 ± 0.65 | a,B | 41.8 ± 1.88 | a,B | 43.3 ± 0.39 | a,B | 42.9 ± 0.29 | a,A,B |
| 6 | 41.7 ± 0.73 | b,B | 40.4 ± 0.65 | c,B | 43.8 ± 0.77 | a,B | 41.8 ± 0.56 | b,B |
| 10 | 40.8 ± 1.47 | a,B | 40.9 ± 0.40 | a,B | | | | |
| **Protein Content** | **% (w/w) ± STD** | | **% (w/w) ± STD** | | **% (w/w) ± STD** | | **% (w/w) ± STD** | |
| 0 | 21.9 ± 1.83 | a,A | 22.2 ± 1.43 | a,A | 24.0 ± 0.73 | a,A | 22.1 ± 0.47 | a,A |
| 6 | 24.9 ± 1.20 | a,B | 24.7 ± 1.39 | a,A,B | 23.8 ± 1.29 | a,A | 24.6 ± 1.12 | a,B |
| 10 | 24.8 ± 0.48 | a,B | 25.3 ± 1.30 | a,B | | | | |
| **pH values** | **pH ± STD** | | **pH ± STD** | | **pH ± STD** | | **pH ± STD** | |
| 0 | 5.24 ± 0.01 | a,c,B | 5.19 ± 0.01 | b,A | 5.25 ± 0.02 | a,C | 5.19 ± 0.05 | b,B |
| 3 | 5.17 ± 0.05 | b,C | 5.18 ± 0.01 | b,A,B | 5.82 ± 0.05 | a,B | 5.19 ± 0.02 | b,B |
| 6 | 5.28 ± 0.05 | c,B | 5.13 ± 0.03 | b,B | 6.69 ± 0.09 | a,A | 5.71 ± 0.07 | b,A |
| 10 | 5.45 ± 0.03 | a,A | 5.27 ± 0.05 | b,A,B | | | | |
| **TA** | **$g_{lactic\ acid}$/100 g STD** | | **$g_{lactic\ acid}$/100 g STD** | | **$g_{lactic\ acid}$/100 g STD** | | **$g_{lactic\ acid}$/100 g STD** | |
| 0 | 1.43 ± 0.10 | a,b,B | 1.39 ± 0.04 | a,b,B | 1.30 ± 0.02 | b,A | 1.19 ± 0.04 | c,B |
| 3 | 1.69 ± 0.14 | a,A | 1.38 ± 0.11 | b,B | 1.20 ± 0.07 | c,B | 1.31 ± 0.08 | b,c,A |
| 6 | 1.58 ± 0.06 | a,A,B | 1.25 ± 0.11 | c,B | 1.39 ± 0.06 | b,A | 1.22 ± 0.10 | c,A,B |
| 10 | 1.72 ± 0.15 | a,A | 1.90 ± 0.05 | b,A | | | | |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 6.3.4. Changes in proteolytic indices

At the beginning of storage (0 months) the ripening extension and depth indices were not significantly affected neither by HPP treatment nor by packaging system type, being closer to 25–26 % and 6–7 %, respectively ($p > 0.05$) (Figure 6.2 A and B). These values are slightly lower than those reported in literature for *Serra da Estrela* cheese of about 29–37 % and 6–13 %, for WSN/TN and TCA/TN ratios, respectively (Sousa and Malcata, 1997; Tavaria *et al.*, 2003). However, during storage, a significant increase of the WSN/TN and TCA/TN ratios occurred, reaching 42.8 and 10.2 %, respectively in $Ch_C$+P cheeses at 6 months storage ($p < 0.001$). These higher proteolytic indices in non-vacuum paper wrapped cheeses were reflected in the higher pH values, as discussed above; higher pH values may favour proteolysis since the optimum pH for most proteinases and peptidases is close to 7 (Garde *et al.*, 2007a). These results seem to indicate a more intense post-ripening metabolic activity in $Ch_C$+P cheeses.

On the other hand, the HPP treated cheeses, independently of the packaging system, $Ch_P$+P and $Ch_P$+V, maintained the WSN/TN (27 and 26 %, respectively) and the TCA/TN (8 and 7 %, respectively) values relatively stable over 6 months of storage, values which were close to those obtained at the starting point (0 months) for the $Ch_C$+V cheeses, i.e. 25 and 6 %, respectively ($p > 0.05$). These results tend to indicate that the HPP treatment and the keeping of the cheeses under-vacuum led to a reduction in the production of medium- and small-size peptides included in the WSN and TCA fractions, respectively.

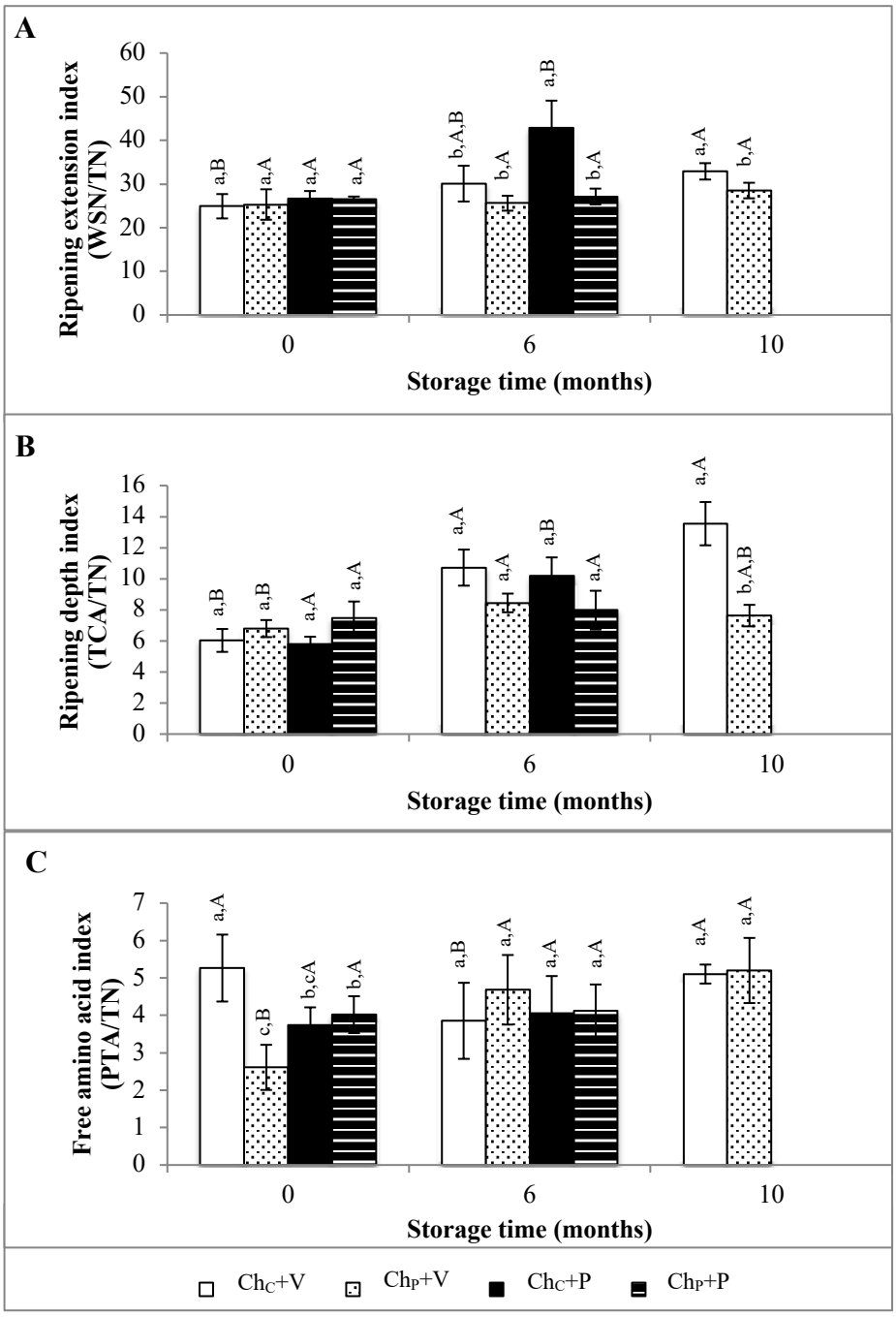



**Figure 6.2: Evolution of (A) Ripening extension index (WSN/TN), (B) ripening depth index (TCA/TN) and (C) free amino acid index (TCA/TN) of *Serra da Estrela* control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P) at 0, 6 and 10 months of storage. Different non-capital letters (a, b, c) indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) indicate statistically significant differences among the same condition ($p < 0.05$).**

Previous studies have shown that more intense HPP treatments at 600 MPa/6 min for *Serra da Estrela* cheeses (CHAPTER 4) and at 600 MPa/20 min for *Torta del Casar* cheeses (Delgado *et al.*, 2015) reduce proteolysis development during 500 and 240 days of storage under vacuum, confirming the possibility of HPP treatment to keep the ideal ripening characteristics during extended storage periods. The PTA/TN index was (unexpectedly) significantly affected by the interaction between HPP treatment and the vacuum packaging system used at 0 months ($p < 0.05$); HPP treatment lowered the PTA/TN ratio of $Ch_P$+V cheeses. Regardless of the effect of HPP on free amino acid index of *Serra da Estrela* cheeses, at 6 and 10 months of storage no significant changes in the PTA/TN ratios ($p > 0.05$) were verified for all 4 cheese types.

### 6.3.5. Colour

No significant effect of the HPP treatment and/or packaging system type on the cheese surface *L** and *a** colour parameters and cheese core *L** colour parameter (measured the lightness from black (0) to white (100)) ($p > 0.05$) was observed (Table 6.2). On the other hand, the *b** colour parameter of the cheese core, which measures the blue (−) to yellow (+) colour, was significantly higher for $Ch_P$+V, $Ch_C$+P and $Ch_P$+P than for $Ch_C$+V cheeses ($p < 0.001$), indicating these former cheeses as being yellower. Along the storage period, the *a** colour parameter of the cheese surface and core, which measures from green (−) to red (+) colour, was maintained constant in all 4 cheese types ($p > 0.05$). On the other hand, the surface of vacuum packaged in plastic film cheeses became lighter and the surface of non-vacuum paper wrapped cheeses became yellower (higher *L** and *b** parameters, respectively) ($p < 0.05$), having the latter gained a very heterogeneous and non-characteristic color at 6 months, as can be observed in (Figure 6.3), and so the color of these cheeses was not quantified. Delgado *et al.*, (2013) measured

also higher *L** and *b** colour parameter on *Ibores* cheese HPP (400 or 600 MPa/7 min) after 1 month under-vacuum. According to Voigt *et al.*, (2010), the colour changes induced by HPP can be related to: the effect of processing on hydrophobic bonds between casein molecules, which changes the light-scattering of the HPP treated cheese; or the HPP treatment might involve the release of pigments by the cheese moulds. It has been pointed out in literature that vacuum storage causes the rind to become whitish (Delgado *et al.*, 2013; Rodríguez-Pinilla *et al.*, 2015) . In order to try and overcome such limitation, the present work studied an alternative packaging system (non-vacuum and paper wrapping), which hypothetically could keep the achieved HPP advantages and avoid the changes in the colour of the rind. However, $Ch_C$+P and $Ch_P$+P cheeses rind revealed a high total colour variation of 8.26 and 10.46, respectively (data not shown) by 3 months of storage (relative to the beginning of storage), possibly due to higher oxygen availability in the case of $Ch_C$+P and $Ch_P$+P cheeses. For a short storage period (less than 3 months) the non-vacuum + greaseproof wrapping paper could be an interesting way to package cheese, but for longer storage periods the vacuum packaging in polyamide-polyethylene plastic film method is preferable. Curiously, when $Ch_C$+V and $Ch_P$+V cheeses were unpacked and kept some days at atmospheric pressure, they became yellower (data not shown).

**Table 6.2: Colour values of *Serra da Estrela* control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P) at 0, 3, 6 and 10 months of storage.**

| | | | $Ch_C$+V | | $Ch_P$+V | | $Ch_C$+P | | $Ch_P$+P | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Storage time (months) | STD | | STD | | STD | | STD | |
| Cheese Surface Colour | ***L**** | 0 | 71.9 ± 2.04 | a,C | 73.6 ± 2.02 | a,B | 70.6 ± 2.24 | a,B | 86.7 ± 0.92 | a,B |
| | | 3 | 77.3 ± 1.26 | a,B | 78.7 ± 2.04 | a,A | 78.7 ± 2.04 | a,A | 77.4 ± 1.93 | a,A |
| | | 6 | 79.8 ± 0.35 | a,A | 78.5 ± 2.05 | a,A | | | | |
| | ***a**** | 0 | -0.12 ± 1.20 | a,A | -0.43 ± 1.31 | a,A | 0.92 ± 0.93 | a,A | -2.93 ± 0.14 | a,B |
| | | 3 | -0.29 ± 1.08 | a,b.A | 0.57 ± 1.38 | b,A | 0.57 ± 1.38 | a,A | 0.04 ± 2.27 | a,b,A |
| | | 6 | -0.29 ± 0.58 | a,A | -0.88 ± 1.05 | a,A | | | | |
| | ***b**** | 0 | 24.4 ± 1.20 | c,A | 28.0 ± 1.40 | b,A | 25.7 ± 1.69 | c,A | 22.3 ± 0.74 | a,B |
| | | 3 | 22.1 ± 1.08 | b,B | 24.6 ± 1.78 | a,B | 24.6 ± 1.78 | a,A | 26.2 ± 1.02 | a,A |
| | | 6 | 20.9 ± 0.58 | b,B | 24.3 ± 2.05 | a,B | | | | |
| Cheese Core Colour | ***L**** | 0 | 85.1 ± 2.04 | a,b,A | 85.0 ± 1.37 | b,A | 86.3 ± 1.83 | a,b,A | 86.7 ± 0.92 | a,A |
| | | 3 | 83.7 ± 1.26 | b,A,B | 85.6 ± 1.14 | a,A | 85.6 ± 1.78 | a,b,A,B | 84.3 ± 1.55 | a,b,B |
| | | 6 | 82.3 ± 0.35 | b,B | 82.9 ± 1.14 | b,B | 84.4 ± 1.32 | a,B | 84.9 ± 0.82 | a,B |
| | ***a**** | 0 | -1.54 ± 1.20 | a,A | -1.88 ± 0.16 | b,A | -2.97 ± 0.22 | c,A | 0.12 ± 1.32 | c,A |
| | | 3 | -1.43 ± 1.08 | a,A | -1.34 ± 0.10 | a,A | -3.03 ± 0.20 | c,A | -2.81 ± 0.25 | b,A |
| | | 6 | -1.49 ± 0.58 | a,A | -1.30 ± 0.47 | a,A | -2.77 ± 0.26 | b,A | -2.47 ± 0.10 | b,A |
| | ***b**** | 0 | 18.8 ± 1.18 | b,B | 22.1 ± 1.08 | a,A | 22.6 ± 0.98 | a,A | 30.2 ± 0.94 | a,B |
| | | 3 | 19.7 ± 1.64 | b,A,B | 20.4 ± 1.75 | b,B | 23.5 ± 1.08 | a,A | 24.4 ± 0.69 | a,A |
| | | 6 | 21.0 ± 1.37 | b,A | 22.2 ± 1.44 | b,A | 23.9 ± 0.75 | a,A | 24.0 ± 0.94 | a,A |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

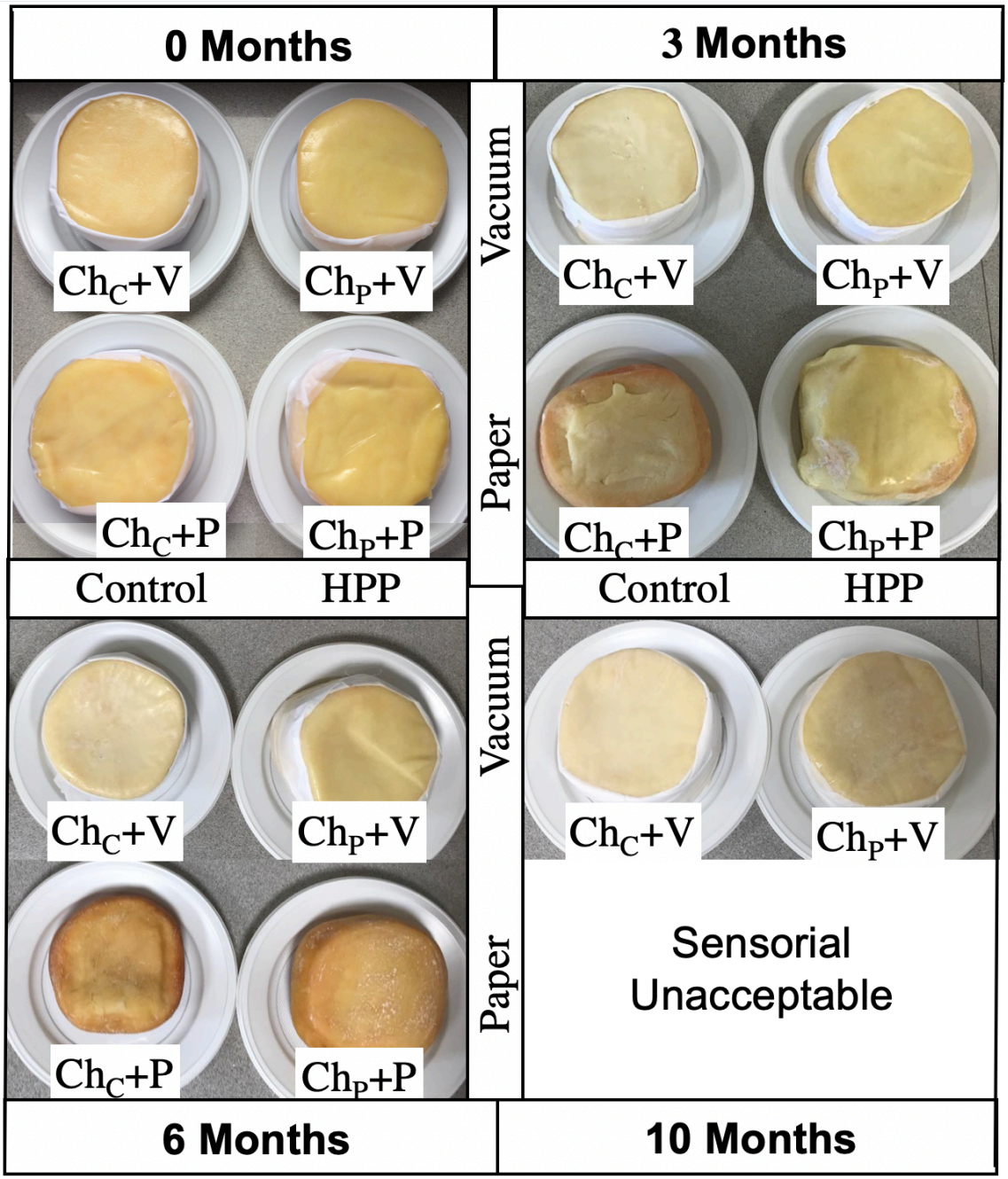



**Figure 6.3: Visual appearance of *Serra da Estrela* control cheeses stored under vacuum in plastic film (Ch$_C$+V) or wrapped in paper without vacuum (Ch$_C$+P) and HPP treated cheeses stored under vacuum in plastic film (Ch$_P$+V) or wrapped in paper without vacuum (Ch$_P$+P) at 0, 3, 6 and 10 months of storage.**

### 6.3.6. Changes in textural properties

HPP showed no significant effect on cheeses' textural properties at 0 months of storage (comparison of $Ch_C$+V with $Ch_P$+V and $Ch_C$+P with $Ch_P$+P cheeses, $p > 0.05$) (Table 6.3). On the other hand, cheeses vacuum packed in plastic film revealed significantly lower hardness (0.18-0.27 N vs 0.56-0.58 N) ($p < 0.001$), lower consistency (1.4-1.7 N/s vs 4.8-5.1 N/s) ($p < 0.01$), lower adhesiveness (0.3-0.6 N/s vs 1.3-1.4 N/s) ($p < 0.001$) and higher cohesiveness (5.5-6.6 vs 2.6-2.7) ($p < 0.01$) than non-vacuum paper wrapped cheeses at 0 months of storage. During the first 6 months of storage, both $Ch_C$+V and $Ch_P$+V cheeses revealed an increase in hardness and consistency features (to 0.49-0.59 N and 4.1-5.1 N/s, respectively) ($p < 0.01$) and a decrease in cohesiveness values (to 2.3-4.2) ($p < 0.05$); no significant differences in these parameters were reported among $Ch_C$+V and $Ch_P$+V ($p > 0.05$) in the first 3 months. The increase in these texture parameters could be related to the loss of moisture throughout refrigerated storage (Table 6.1). This behaviour was also verified in a previous study in HPP treated (at 600 MPa/6 min) and control cheeses stored under vacuum for 15 months (CHAPTER 4). A study performed on *Torta del Casar* cheeses revealed a similar behaviour, an increase in the consistency along 6 months under vacuum, without significant differences between vacuum-packaged control and HPP (600 MPa/5 min) treated cheeses, which could be related to the loss of moisture during storage (Delgado *et al.*, 2015). A study on *Ibores* raw goat milk cheese, showed a similar behavior for cheeses kept for 30 days under vacuum; HPP treated cheeses (600 MPa/7 min) revealed higher hardness and lower adhesiveness than control ones (Delgado *et al.*, 2013).

**Table 6.3: Textural properties of *Serra da Estrela* control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P) at 0, 3, 6 and 10 months of storage.**

| Property | Storage time (months) | $Ch_C$+V | | $Ch_P$+V | | $Ch_C$+P | | $Ch_P$+P | |
|---|---|---|---|---|---|---|---|---|---|
| **Hardness** | 0 | 0.27 ± 0.14 | b,B | 0.18 ± 0.04 | b,C | 0.58 ± 0.14 | a,A | 0.56 ± 0.11 | a,A |
| **(N)** | 3 | 0.47 ± 0.093 | a,A | 0.37 ± 0.041 | a,b,B | 0.28 ± 0.14 | b,B | 0.39 ± 0.046 | a,b,B |
| | 6 | 0.49 ± 0.085 | a,A | 0.59 ± 0.13 | a,A | 0.28 ± 0.079 | b,B | 0.59 ± 0.10 | a,A |
| | 10 | 0.30 ± 0.047 | a,B | 0.35 ± 0.084 | a,B | | | | |
| **Consistency** | 0 | 1.7 ± 0.41 | b,B | 1.4 ± 0.34 | b,C | 4.8 ± 1.36 | a,A | 5.1 ± 0.87 | a,A |
| **(N/s)** | 3 | 3.6 ± 0.67 | a,A | 2.9 ± 0.61 | a,B | 1.3 ± 0.47 | b,C | 3.1 ± 0.65 | a,B,B |
| | 6 | 4.1 ± 0.79 | a,A | 5.1 ± 0.67 | a,A | 2.8 ± 0.91 | b,B | 5.0 ± 0.92 | a,A |
| | 10 | 2.4 ± 0.33 | a,B | 2.7 ± 0.33 | a,B | | | | |
| **Adhesiveness** | 0 | 0.6 ± 0.19 | a,A | 0.3 ± 0.07 | a,A | 1.3 ± 0.61 | b,A,B | 1.4 ± 0.09 | b,A |
| **(N/s)** | 3 | 1.5 ± 0.44 | b,B | 1.1 ± 0.41 | a,b,B | 0.7 ± 0.12 | a,A | 1.4 ± 0.37 | b,A |
| | 6 | 2.4 ± 0.58 | a,b,C | 2.3 ± 0.47 | a,b,C | 1.6 ± 0.69 | a,B | 2.8 ± 0.61 | b,B |
| | 10 | 1.8 ± 0.39 | a,B,C | 1.8 ± 0.37 | a,C | | | | |
| **Cohesiveness** | 0 | 5.5 ± 2.2 | a,A | 6.6 ± 2.0 | a,A | 2.7 ± 0.56 | b,B | 2.6 ± 0.49 | b,B |
| **(dimensionless)** | 3 | 3.2 ± 0.65 | a,B | 4.2 ± 0.63 | a,C | 4.4 ± 1.8 | a,A,B | 3.9 ± 0.46 | a,A |
| | 6 | 4.2 ± 0.72 | a,A,B | 2.3 ± 0.68 | b,B | 5.6 ± 1.8 | a,A | 2.6 ± 0.32 | b,B |
| | 10 | 4.7 ± 1.7 | a,A,B | 4.2 ± 0.71 | a,B | | | | |
| **Gumminess** | 0 | 1.4 ± 0.33 | a,b,C | 1.1 ± 0.16 | b,B | 1.6 ± 0.16 | a,A | 1.4 ± 0.28 | a,b,A |
| **(N)** | 3 | 1.5 ± 0.052 | a,B,C | 1.5 ± 0.082 | a,A | 1.6 ± 0.13 | a,A | 1.5 ± 0.043 | a,A |
| | 6 | 2.0 ± 0.054 | a,A | 1.5 ± 0.085 | b,c,A | 1.3 ± 0.12 | c,B | 1.6 ± 0.16 | b,A |
| | 10 | 1.7 ± 0.042 | a,A,B | 1.6 ± 0.074 | b,A | | | | |

Values presented are means ± standard deviation of data from quintuplicate analysis on duplicate trials. Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 6.3.7. Changes in sensorial attributes

Paired comparison between $Ch_P$ and $Ch_C$ samples at 0 months storage revealed significant differences between non-vacuum paper wrapped control and HPP cheeses for rind defects and paste appearance (Table 6.4), with $Ch_P$+P having been judged to present less rind defects than $Ch_C$+P ($p < 0.05$), a paste with lighter colour tone and a less firm consistency ($p < 0.05$). These results were confirmed by results obtained in the rating test in which the four cheese samples were evaluated (Table 6.5). No significant differences in odour attributes were found at this storage time. As discussed in the previous section, TPA analysis did not reveal significant differences in hardness among $Ch_C$+V - $Ch_P$+V and $Ch_C$+P - $Ch_P$+P cheeses at 0 months storage (Table 6.3), which might be related to the particularities of sensorial and TPA analysis, like sensitivity. At 3 months storage, significant differences between the HPP treated and the control cheeses stored under vacuum in plastic film were found for most of the evaluated appearance, texture and flavor attributes; between the HPP treated cheeses and the control cheese stored non-vacuum paper wrapped differences were found for rind appearance and texture attributes. Relatively to appearance, $Ch_P$+V cheeses were judged to have a significantly darker rind and paste colour tone, with a less homogenous rind and a firmer paste consistency ($p < 0.05$) than $Ch_C$+V cheeses. The rind appearance of $Ch_P$+P cheeses revealed to have a darker colour tone and less defects ($p < 0.01$) (Table 6.4). The panel rated the $Ch_P$+P cheeses with a more intense lactic odour than $Ch_C$+P cheeses. The HPP treated cheeses were judged to have a firmer paste than controls, as verified in a previous study (CHAPTER 4). Nevertheless, instrumental measured texture revealed only significant lower consistency values for $Ch_C$+P cheeses ($p < 0.001$). Delgado *et al.*, (2013) verified that odour and flavour intensity were not affected by HPP (600 MPa/7 min), for *Ibores* raw goat milk cheeses evaluated 1 month after treatment.

**Table 6.4: Values of sensory attributes (scale from -10 to 10) of *Serra da Estrela* cheeses control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P) at 0, 3, 6 and 10 months of storage.**

| Storage time (months) | 0 | | 3 | | 6 | | 10 |
|---|---|---|---|---|---|---|---|
| | $Ch_P$+V Vs $Ch_C$+V | $Ch_P$+P Vs $Ch_C$+P | $Ch_P$+V Vs $Ch_C$+V | $Ch_P$+P Vs $Ch_C$+P | $Ch_P$+V Vs $Ch_C$+V | $Ch_P$+P Vs $Ch_C$+P | $Ch_P$+V Vs $Ch_C$+V |
| **Rind Appearance** | | | | | | | |
| Tonality | -0.59 | -0.86 | 2.41 * | 4.22 * | 1.86 * | -1.55 * | 2.37 * |
| Homogeneity | -1.38 | 1.61 | -4.58 * | -0.38 | 3.36 * | 0.62 | -0.86 |
| Defects | 0.38 | -2.59 * | 1.98 | -4.85 * | -2.67 * | 0.01 | -0.17 |
| **Paste Appearance** | | | | | | | |
| Colour | 0.23 | -1.48 * | 2.80 * | -0.71 | 1.63 * | -0.99 * | 2.38 * |
| Consistency | -1.24 | -2.35 * | 3.67 * | 2.32 * | 1.53 | 1.49 * | -0.12 |
| **Odour** | | | | | | | |
| Lactic | -0.53 | -0.92 | -1.60 | 3.52 * | -2.52 * | -2.04 * | 0.63 |
| Acid | -0.01 | 0.36 | -1.63 | 0.02 | -1.50 | -0.27 | 1.16 |
| Animal | -0.55 | 0.42 | -0.66 | -0.46 | -0.30 | -2.07 * | 0.88 |
| SCFA[#] | -0.26 | -0.24 | -1.11 | -0.42 | 0.69 * | -0.23 | 1.15 |
| **Texture** | | | | | | | |
| Consistency | | | 3.87 * | 2.60 * | 1.00 | 2.62 * | 2.52 * |
| Friability | | | 3.00 * | 0.27 | -0.49 | 0.56 | -0.02 |
| Unctuosity | | | 3.00 * | -2.66 * | -0.86 | -3.97 * | -0.03 |
| **Flavour** | | | | | | | |
| Salty | | | -2.47 * | -0.39 | -1.25 | -3.45 * | -0.17 |
| Acid | | | 3.72 * | 2.67 | 2.06 * | 3.74 * | 1.23 |
| Bitter | | | -1.90 | 1.31 | 0.13 | -0.85 | -0.77 |
| **After-taste** | | | 3.43 * | 0.06 | -0.61 | 0.01 | -0.88 |

Data expressed as mean (n =10); * significant difference (p-value < 0.05). [#] SCFA means short-chain fatty acids, vomit like odour.

**Table 6.5: Mean values of sensory attributes (scale from 0 to 10) by classification of *Serra da Estrela* control cheeses stored under vacuum in plastic film ($Ch_C$+V) or wrapped in paper without vacuum ($Ch_C$+P) and HPP treated cheeses stored under vacuum in plastic film ($Ch_P$+V) or wrapped in paper without vacuum ($Ch_P$+P) at 0, 3, 6 and 10 months of storage.**

| Storage time (months) | 0 | | | | 3 | | | | 6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $Ch_C$+V | $Ch_P$+V | $Ch_C$+P | $Ch_P$+P | $Ch_C$+V | $Ch_P$+V | $Ch_C$+P | $Ch_P$+P | $Ch_C$+V | $Ch_P$+V | $Ch_C$+P | $Ch_P$+P |
| **Rind Appearance** | | | | | | | | | | | | |
| Tonality | 2.81 $^{a,b}$ | 4.36 $^{a,b}$ | 4.36 $^{a}$ | 2.53 $^{b}$ | 1.13 c | 5.00 $^{b}$ | 7.96 $^{a}$ | 3.68 $^{b}$ | 0.91 $^{d}$ | 2.61 $^{c}$ | 9.1 $^{a}$ | 6.65 $^{b}$ |
| Homogeneity | 4.94 $^{a}$ | 2.26 $^{b}$ | 3.01 $^{a,b}$ | 4.31 $^{a,b}$ | 7.36 $^{a}$ | 7.66 $^{a}$ | 0.94 $^{b}$ | 6.66 $^{a}$ | 6.02 $^{a}$ | 6.11 $^{a}$ | 3.08 $^{b}$ | 3.33 $^{b}$ |
| Defects | 1.38 $^{b}$ | 4.20 $^{a}$ | 3.54 $^{a}$ | 1.20 $^{b}$ | 0.71 $^{b}$ | 0.69 $^{b}$ | 6.26 $^{a}$ | 1.04 $^{b}$ | 3.50 $^{c}$ | 1.72 $^{d}$ | 5.64 $^{b}$ | 8.15 $^{a}$ |
| **Paste Appearance** | | | | | | | | | | | | |
| Colour | 4.65 $^{a}$ | 2.44 $^{b}$ | 3.83 $^{a,b}$ | 2.24 $^{b}$ | 3.72 $^{b}$ | 4.78 $^{a,b}$ | 6.34 $^{a}$ | 5.10 $^{a,b}$ | 3.90 $^{a}$ | 4.39 $^{a}$ | 5.44 $^{a}$ | 5.91 $^{a}$ |
| Consistency | 5.09 $^{a}$ | 2.53 $^{b}$ | 4.95 $^{a}$ | 2.15 $^{b}$ | 4.42 $^{b}$ | 6.90 $^{b}$ | 3.56 $^{a}$ | 5.48 $^{a,b}$ | 4.38 $^{a}$ | 6.07 $^{a}$ | 5.30 $^{a}$ | 6.66 $^{a}$ |
| **Odour** | | | | | | | | | | | | |
| Lactic | 3.66 $^{a}$ | 2.69 $^{a}$ | 3.45 $^{a}$ | 2.28 $^{a}$ | 3.83 $^{a}$ | 4.08 $^{a}$ | 3.48 $^{a}$ | 2.99 $^{a}$ | 4.96 $^{a}$ | 3.63 $^{a}$ | 3.22 $^{a}$ | 2.60 $^{a}$ |
| Acid | 3.41 $^{a}$ | 2.19 $^{a}$ | 3.13 $^{a}$ | 2.32 $^{a}$ | 3.30 $^{a}$ | 3.59 $^{a}$ | 2.13 $^{a}$ | 2.74 $^{a}$ | 4.35 $^{a}$ | 3.24 $^{a,b}$ | 1.86 $^{b}$ | 1.90 $^{b}$ |
| Animal | 2.74 $^{a}$ | 2.56 $^{a}$ | 2.52 $^{a}$ | 2.73 $^{a}$ | 3.22 $^{a}$ | 2.22 $^{a}$ | 3.31 $^{a}$ | 2.97 $^{a}$ | 1.40 $^{a}$ | 0.94 $^{a}$ | 2.98 $^{a}$ | 2.33 $^{a}$ |
| SCFA | 2.59 $^{a}$ | 1.82 $^{a}$ | 2.16 $^{a}$ | 1.88 $^{a}$ | 2.61 $^{a}$ | 1.90 $^{a}$ | 1.59 $^{a}$ | 3.48 $^{a}$ | 1.06 $^{a}$ | 1.67 $^{a}$ | 1.43 $^{a}$ | 0.81 $^{a}$ |
| **Texture** | | | | | | | | | | | | |
| Consistency | | | | | 2.50 $^{b}$ | 5.64 $^{a}$ | 1.49 $^{b}$ | 4.61 $^{a}$ | 2.94 $^{b}$ | 3.96 $^{a,b}$ | 3.60 $^{a,b}$ | 5.19 $^{a}$ |
| Friability | | | | | 1.78 $^{a,b}$ | 3.60 $^{a}$ | 0.19 $^{b}$ | 2.04 $^{a,b}$ | 1.32 $^{a}$ | 1.32 $^{a}$ | 1.42 $^{a}$ | 1.28 $^{a}$ |
| Unctuosity | | | | | 6.66 $^{a}$ | 3.52 $^{b}$ | 6.31 $^{a}$ | 3.82 $^{b}$ | 5.77 $^{a}$ | 5.3 $^{a}$ | 5.31 $^{a}$ | 2.16 $^{b}$ |
| **Flavour** | | | | | | | | | | | | |
| Salty | | | | | 4.67 $^{a}$ | 4.49 $^{a}$ | 4.20 $^{a}$ | 3.59 $^{a}$ | 4.58 $^{a}$ | 3.60 $^{a}$ | 3.97 $^{a}$ | 2.26 $^{a}$ |
| Acid | | | | | 4.40 $^{a}$ | 2.84 $^{a}$ | 2.84 $^{a}$ | 2.64 $^{a}$ | 5.65 $^{a}$ | 3.75 $^{a,b}$ | 3.16 $^{a,b}$ | 1.40 $^{b}$ |
| Bitter | | | | | 3.93 $^{a}$ | 2.49 $^{a}$ | 3.59 $^{a}$ | 2.97 $^{a}$ | 3.00 $^{a}$ | 2.94 $^{a}$ | 3.62 $^{a}$ | 2.98 $^{a}$ |
| **After-taste** | | | | | 5.56 $^{a}$ | 3.70 $^{a}$ | 5.14 $^{a}$ | 3.33 $^{a}$ | 5.52 $^{a}$ | 4.31 $^{a}$ | 4.21 $^{a}$ | 2.57 $^{a}$ |

Different non-capital letters (a, b, c) in the same storage time indicate statistically significant differences (Tukey test $p < 0.05$).

At 6 months, the panel attributed lighter rind and paste colour tone for cheeses stored under vacuum packed in plastic film (Figure 6.3 and Table 6.5) particularly for $Ch_C$+V cheeses, being in agreement with instrumental colour evaluation. Similarly, a darker yellow colour appearance was attributed to *Ibores* raw goat milk cheeses 1 month after HPP treatment (600 MPa/7 min) than control ones (Delgado *et al.*, 2013). $Ch_C$+P and $Ch_P$+P cheeses at 6 months revealed significantly lower ($p < 0.05$) acid odour and flavour (Table 6.5). These results indicated that $Ch_C$+P and $Ch_P$+P cheeses were clearly outside of the expected characteristics for this type of cheese, and so the evaluation was ended at this point for these cheeses.

At 10 months, three attributes (rind tonality, colour paste and texture consistency) revealed that HPP had an effect on $Ch_P$+V cheeses, but with a positive effect, where the cheese rind and paste colour tone became darker (in accordance with lower $L^*$ colour parameter). Due to the absence of reports in the literature on sensorial analysis for longer cheese storage times, the comparison of results is not straightforward. Nevertheless, *Torta del Casar* raw ewe milk (Calzada *et al.*, 2014b, 2014a) and raw cows' milk cheeses (Calzada *et al.*, 2014d) were HPP treated (400 or 600 MPa/5 min) at 21 or 35 days of ripening, both were unpacked for ripening during 2 months, followed by storage at 4 ºC for 6 months. Along storage, raw ewe milk cheeses processed by HPP revealed significantly lower odour intensity, but significantly higher odour quality, as well as lower putrid and rancid odours than control ones (Calzada *et al.*, 2014b); the flavour intensity was slightly lower, but the flavour quality was significantly higher than that of the control cheeses (Calzada *et al.*, 2014a). In raw cow milk cheeses, HPP treated and control cheeses showed similar flavour intensity and quality, but the HPP cheeses revealed a more pronounced bitter flavour than controls (Calzada *et al.*, 2014d), in contrast to the results presented in this work. Overall, from the sensorial point of view, it can be concluded that

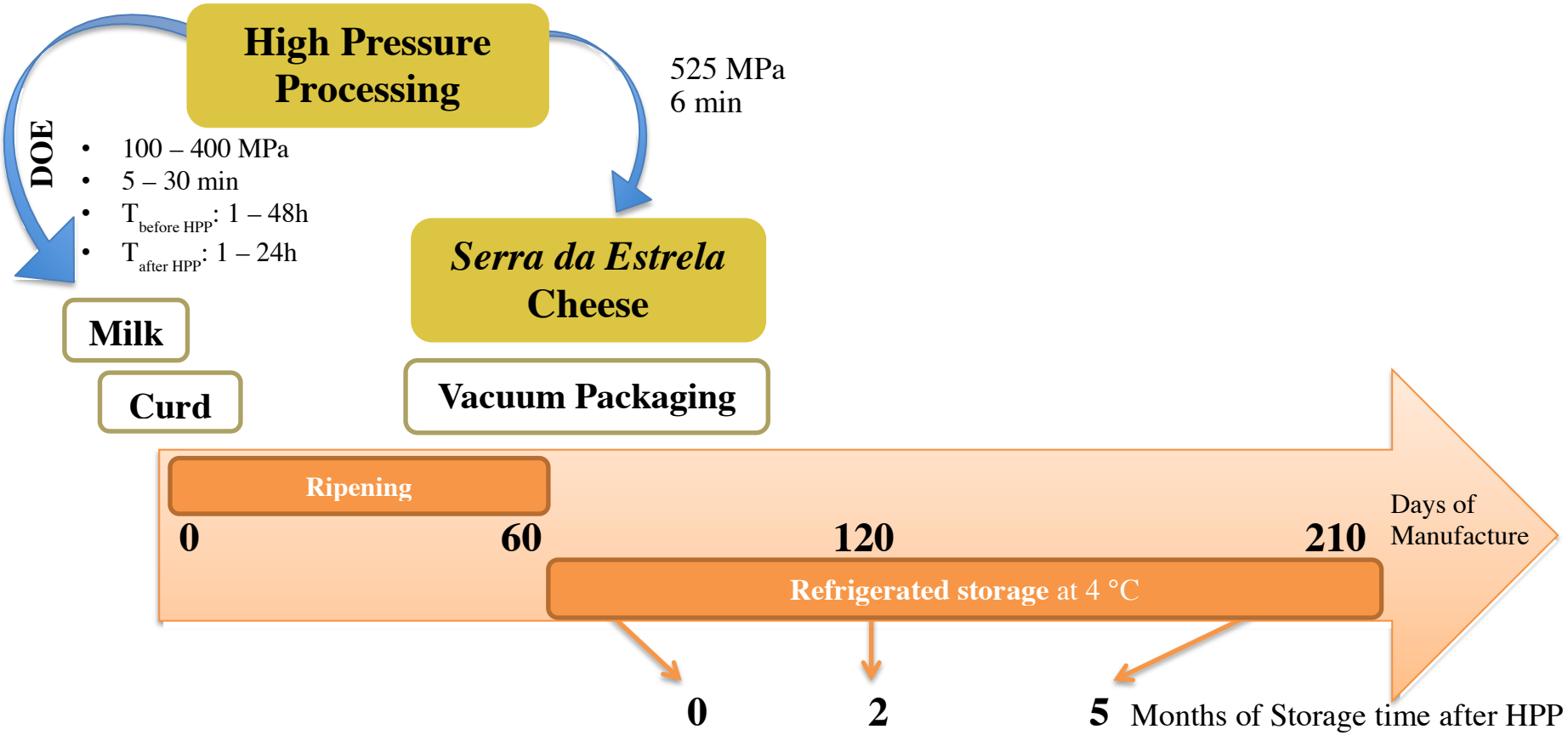
for longer storage times, cheese packaging in plastic film under vacuum is preferable, while for shorter periods, non-vacuum paper wrapping system is also a viable option.

## 6.4. Conclusion

Control cheeses revealed viable cell numbers of different microbial groups closer to values commonly reported for *Serra da Estrela* cheese. HPP treatment caused microbial reductions in the range of those reported in literature, being more pronounced for gram-negative bacteria such as *Enterobacteriaceae*, *Pseudomonas* spp. and *E. coli*, which were reduced to below the quantification limit, while for lactobacilli, lactococci, enterococci and total aerobic mesophilic microorganisms, a reduction of about 1 – 3 log cycle units was verified. In general, the packaging system did not have a significant impact on in viable cell numbers. However, yeasts and moulds grew more (> 5 log cfu/g) in non-vacuum paper wrapped cheeses in comparison to vacuum plastic film packed cheeses, whose presence was also perceived by the sensorial panel, while the rind of the latter cheeses became whitish. Ripening extension and depth indices were maintained relatively constant for long storage periods in HPP treated cheeses, independently of the packaging system type, leading to a harder texture. Sensorial analysis indicated that HPP cheeses stored under vacuum in plastic film kept the main attributes up to 10 months, while this was verified for only 3 months for non-vacuum paper wrapped cheeses.

Overall, these results allow to conclude that HPP has a positive effect on cheese safety and quality characteristics maintenance of raw ewe milk *Serra da Estrela* cheese, while storage under vacuum in plastic films is more adequate than non-vacuum paper wrapping for longer periods, avoiding the yeasts and moulds growth but leading to a more whitish rind.

# PART III – High pressure processing treatment from milk to cheese




$\mathbf{M_C+Ch_C}$ Milk Control and cheese control

$\mathbf{M_P+Ch_C}$ Milk HPP and cheese control

$\mathbf{M_C+Ch_P}$ Milk Control and cheese HPP

$\mathbf{M_P+Ch_P}$ Milk HPP and cheese HPP

**Study the effects on:**

- Microbial Composition
- Surrogate microbiota
- Physicochemical parameters
- Colour
- Textural parameters
- Proteolysis
- Visual appearance
- Sensorial atributtes

General schematic flow diagram of the work carried in Part III – CHAPTER 7, 8 and 9.

# CHAPTER 7 - Response surface methodology as a tool for optimisation of raw ewes' milk high pressure pre-treatment for improved production of raw milk cheese

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*This chapter has been submitted for publication.*

## Abstract

*Serra da Estrela* Protected Designation of Origin (PDO) cheese is manufactured with raw milk from Bordaleira and/or Churra Mondegueira da *Serra da Estrela s*heep breeds. Several socio-environmental shortcomings have reduced production capacity hence treatments that may contribute to its efficient transformation into cheese are welcome. High pressure processing (HPP) milk pre-treatment may contribute to cheese yield increment, yet processing conditions optimization is warranted.

An initial wide-scope screening experiment allowed pinpointing pressure intensity, holding time under pressure and time after HPP as the most important factors influencing curd yield. Based on this, a more targeted screening experiment allowed selecting the range of experimental conditions to be used for an experimental design study that revealed a HPP treatment at 121 MPa for 30 min, as the optimum for milk processing to improve curd yield (> 9 %) and effectively maintain the beneficial cheese microbiota; optimum was validated in a final experimental framework.

## 7.1. Introduction

In cheesemaking, the cheese yield (kg cheese/kg milk) is of particular economic interest since small differences in yield translate into big differences in both milk volume savings and final profits; the higher the solids percentage recovered, the greater the amount of cheese obtained thus reflecting economic gains. In the particular case of the Protected Designation of Origin (PDO) *Serra da Estrela* ewe cheese, the available milk is becoming scarcer due to limitations of various kinds – environmental and social cues. *Serra da Estrela* cheese is made solely with milk from Bordaleira *Serra da Estrela* and/or Churra Mondegueira ewe's breeds, and according to specifications the milk cannot undergo any thermal treatment (Freitas and Malcata, 2000; Macedo and Malcata, 1997a). High pressure processing (HPP) is a non-thermal food processing technology, wherein the food is subjected to a very high pressure range from 100-800 MPa during a holding period between 5–60 min. Different literature reports have indicated that HPP milk pre-treatment can increase cheese yield (Huppertz *et al.*, 2004c, 2005), as detailed in CHAPTER 2. Moreover, milk HPP processing has the potential to reduce viable cell numbers of undesirable contaminant microorganisms, without significant effects on flavour and nutritional components, contributing to safer high quality cheese products, however HPP processing may influence the physico-chemical and technological properties of milk (Chawla *et al.*, 2011; Dhineshkumar *et al.*, 2016; Trujillo *et al.*, 2002b). As reported in CHAPTER 2, the effect of HPP on cheese yield has been evaluated mainly in cows' milk, goats' milk, while only few studies have focused on ewes' milk (López-Fandiño and Olano, 1998a, 1998b). In general, milk HPP pre-treatments enabled an increase of the cheese yield in about 4–23 % in comparison to untreated milk. Huppertz *et al.*, (2005) studied cow milk HPP pre-treatment between 100 and 600 MPa, having verified higher yield values (13-18%) at 100 and 250 MPa. On the other hand, one year

before, the same group had verified lower values for HPP treated milk at 250 MPa (exception for treatment for 60 min with 4 % yield increasing) and higher values for HPP treatment at 400 and 600 MPa (4-23%) (Huppertz *et al.*, 2004c). Furthermore, higher cow cheese yield values were verified after a holding time at 20 °C for 24 h post HPP treatment (Huppertz *et al.*, 2004c). The same study revealed that a longer holding time under pressure (from 5 to 30 minutes) also increased the curd yield. In ewes' milk, HPP treatments at 100 MPa for 30 min revealed a similar yield compared to untreated milk and an increase of about 5, 5 and 16 % for 200, 300 and 400 MPa, respectively (López-Fandiño and Olano, 1998b). Ewes' milk HPP treatment at 300 MPa with a holding time of 10, 20 and 30 min showed similar yield values, but lower values when processing was for 5 min (López-Fandiño and Olano, 1998b). Similar results were verified in a further study by the same research group; López-Fandiño and Olano (1998b) observed a higher yield after HPP at 40 °C than at 25 °C (about 23% vs 9%), but reported that such treatment caused deleterious effects on gel firmness.

Several questions remain unanswered, hence the main objectives of the current research were to use design of experiments (DoE) and response surface methodology (RSM) to determine the optimum HPP milk pre-treatment conditions that maximize cheese yield, while maintaining the beneficial microbiota of the cheese at most desirable levels for cheese biochemical properties development.

## 7.2. Materials and methods

### 7.2.1. Screening experimental design and rationale for choice of conditions

Firstly, an initial wide screening study - two level full factorial design in triplicate for four factors ($2^4$ = 16*3 = 48 runs – see Table 7.1) was performed, in a randomized

order, to identify the variables/factors with main effect and the interaction of factors on *Serra da Estrela* cheese yield and time of coagulation. Based on previous studies in the literature, the four variables selected were: pressure intensity (range 200–400 MPa), holding time under pressure (range 5–60 min), time before HPP (1–48 h) and time after HPP (1–24 h), as shown in Figure 7.1. The holding time before HPP and after HPP allowed understanding if the storage time of milk prior to HPP and after HPP treatment influenced cheese yield. The outcome parameters measured were yield and coagulation time. At the same time, untreated milk was studied as a control in order to compare with HPP treated milk.

Considering that the results of this initial wide screening study revealed more interesting results for lower pressures, 100 MPa was also studied and the processing time fixed on 5 min, because the processing time was found to have minor or no effects; further analyses were also carried out, namely, pH, titratable acidity, and microbiological enumeration.

**Table 7.1: Factors and levels for the initial wide screening and focused screening studies and optimization design of experiment.**

| Factors | Level (-1) | Level (0) | Level (+1) |
|---|---|---|---|
| **Wide screening study: two level full factorial design** | | | |
| Pressure intensity (MPa) | 200 | | 400 |
| Holding time (min) | 5 | | 30 |
| Before HPP time (h) | 1 | | 48 |
| After HPP time (h) | 1 | | 24 |
| **Focused screening study** | | | |
| Pressure intensity (MPa) | 100 | | 400 |
| Holding time (min) | 5 | | |
| Before HPP time (h) | | | 48 |
| After HPP time (h) | | | 24 |
| **Optimization design of Experiment: central composite design** | | | |
| Pressure intensity (MPa) | 100 | 200 | 300 |
| Holding time (min) | 5 | 17.5 | 30 |
| Before HPP time (h) | | 24 | |
| After HPP time (h) | | | 24 |

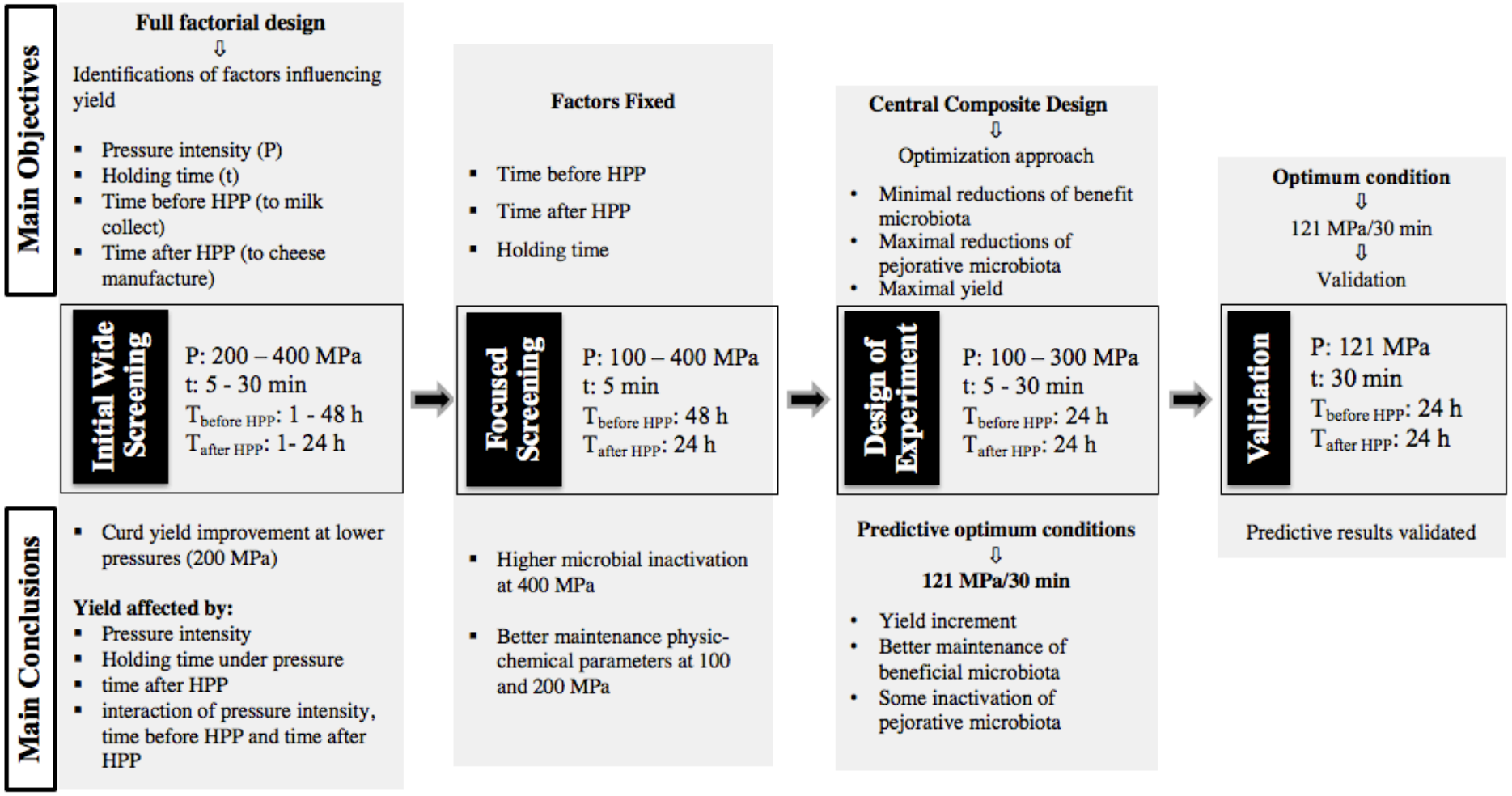


**Figure 7.1: Schematic representation of initial wide screening, focused screening, design of experiment and validation**.

### 7.2.2. Surface model - optimization experiment design - central composite design

Upon selection of the most important factors in the initial wide and the focused studies, an optimization design for cheese yield improvement was established using a central composite design (Figure 7.1). This design consisted in a factorial design with two factors at two levels: pressure intensity ranged between 100–300 MPa and holding time under pressure between 5– 30 min, additional axial and 5 central points were considered, as shown in Table 7.1. The dependent variables were technological cheese parameters (curd yield and coagulation time) and milk microbiota viable cell numbers (lactococci, lactobacilli, enterococci, *Enterobacteriaceae*, total coliforms, *E. coli,* staphylococci, yeasts and moulds counts).

### 7.2.3. Validation experiment design

The theoretical optimum conditions 121 MPa/30 min (obtained for the optimization experiment design) were applied to raw ewes' milk samples, in a validation experiment in quintuplicate for greater validation robustness (untreated milk was also studied for data normalization).

### 7.2.4. Milk supply

Raw ewes' milk (from three farms in *Serra da Estrela* cheese PDO region, Portugal) was kept in a refrigerated tank until use and prior to sampling milk was well mixed to ensure a homogeneous sample. Five litres of milk were used for the initial wide screening and another 5 L for the focused screening experiments which were performed

in December 2017 and January 2018, respectively. For the response surface design 8 L of milk were used in February and another 8 L in March 2018 for the model validation.

### 7.2.5. Sample packaging

In the dairy, milk aliquots (≈75 ml) were placed into polyamide-polyethylene (PA-PE) bags (Plásticos Macar – Indústria de Plásticos Lda, Santo Tirso, Portugal) and heat sealed. The milk bags were stored under refrigeration (4 °C) before and after HPP treatment until analysis.

### 7.2.6. High pressure processing

HPP was performed in a 55-liter capacity industrial scale high pressure equipment (model 55, Hyperbaric, Burgos, Spain). For all experiments, the initial temperature of the water used as transmitting fluid was 8 °C. For the initial wide screening study: HPP was performed on the day of milk collection and after 48 h, as shown in Table 7.1, and Figure 7.1, having the milk been treated at 200 and 400 MPa for 5 and 30 min. For the focused screening study: the milk was treated after 48 h of collection and the curd transformation occurred after 24 h of HPP treatment, and milk samples were treated at 100, 200, 300 and 400 MPa for 5 min. For design of experiment, the milk was treated after 24 h of collection and the curd transformation occurred after 24 h of HPP treatment according to Table 7.1. The validation step occurred using the optimum HPP processing conditions obtained in the design of experiment study, i.e. 121 MPa for 30 min.

### 7.2.7. Yield and coagulation time

Yield was estimated by centrifugation. Prewarmed milk (30 mL) to 32 ºC was treated with 50 µL of standard vegetable rennet (*Cyanara cardunculus*, strength 1:15000, Enzilab, Maia, Portugal). After 1 h at 32 º C, the curd was cut and 10 min later centrifuged at 1 500x*g* for 15 min at 5 ºC. The curd and whey were then separated and weighed. Coagulation time was evaluated by placing a spatula in the tubes every 10 minutes to see when the spatula came out of the curd free of any curd granules.

### 7.2.8. Microbiological analyses

Milk samples were added to and decimally diluted in 13.5 mL of sterile 0.1% (w/v) aqueous peptone and then plated, in triplicate, on several culture media The microbial groups were enumerated according to 6.2.4 Microbiological analyses. Petri dishes containing 10 – 100 colony forming units (cfu) were selected for counting. The results were converted into logarithmic decimals of the number of cfu per mL of milk.

### 7.2.9. Physicochemical analyses

The pH values of the milk and cheese were measured, at room temperature, in random points using a properly calibrated pH/temperature penetration pH meter (Testo 205, Testo, Inc., New Jersey, USA). The titratable acidity was determined according to AOAC 947.05 (2002) procedure for milk, using an automatic titrator with pH meter (Crison – Titromatic 1S with pH electrode 50 14, Barcelona, Spain), by titration to a pH value of 8.9. Physicochemical analyses were performed in triplicate per milk and cheese samples.

### 7.2.10. Colour

Colour parameters were measured as previously described in section 3.2.6 Colour.

### 7.2.11. Statistical analyses

For experimental design Minitab version 17 and JMP version 9 software's were used. SPSS software version 24.0 was used to evaluate the effect of factors and interactions in the initial wide screening study. For the focused screening, one-way analysis variance (ANOVA) was performed to establish the effect of different conditions (four HPP and untreated milk). The significant difference Tukey's test was applied to compare the mean values of parameters, with the significance assigned at $p < 0.05$.

## 7.3. Results and discussion

### 7.3.1. Initial wide screening study

In order to identify which factors may influence cheese yield, a full factorial design was chosen where all possible combinations of all the input variables and their levels were included (Table 7.1 and Figure 7.1). Immediately after HPP treatment, the milk processed at 200 MPa was still liquid, however the milk treated at 400 MPa revealed a more viscous texture, and after 24 h under refrigeration these samples revealed curd and whey separation. In literature a linear increase in skim milk viscosity was verified after HPP between 100 and 400 MPa for 30 min by Huppertz *et al*., (2003).

In general, firmer curds were obtained from HPP processed milk during 5 min, while the milk treated for 30 min resulted in a curd/paste similar to a granular whey cheese (Figure A. 7.1). López-Fandiño *et al*., (1997) also verified higher curd firmness for cow

HPP treated milk for 10 than for 30 min at 400 MPa. Results in the literature indicate that for ewes' milk, curd firmness was not affected by the HPP conditions (100–400 MPa for 30 min), while for goats' milk firmness increased at 300 and 400 MPa (López-Fandiño and Olano, 1998b). In the present study, control samples revealed intermediate firmness, compared to the HPP treated samples. Faster coagulation occurred in HPP treated milk at 400 MPa, particularly for 30 min holding time (Figure 7.2 A). The effect of HPP milk pre-treatment on coagulation time has been reported in the literature as being mainly dependent of pressure intensity and holding time. In this regard, an research group in this dominion treated bovine milk by HPP using different binomials pressure intensity/holding time (López-Fandiño *et al.*, (1996); López-Fandiño *et al.*, (1997); López-Fandiño and Olano (1998b)). These researchers were able to verify a reduction in coagulation time for HPP up to 200 MPa with treatment times within the range 10–60 min, while HPP treatment at 400 MPa only registered lower coagulation time when applied for 10 min; longer HPP holding times under such pressure increased coagulation time to values close to those of unprocessed milk. In a study performed with ovine milk, authors achieved results that support those presented in the current study; they were able to show that coagulation time decreased slightly after HPP 100 MPa for 30 min and increased significantly after HPP at 200–300 MPa to values 14-28 % higher than untreated milk samples, albeit a new decrease for HPP at 400 MPa although to values that remained slightly higher than those for untreated milk - notably, gel firmness was not affected in the whole range of pressure studied (100–400 MPa) (López-Fandiño and Olano, 1998b).

The timespan between HPP treatment and milk transformation into curd could also be a factor influencing coagulation time. In the present study, a lower coagulation time was verified when milk was transformed immediately into curd compared to curd

production after 24 h storage of the HPP treated milk under refrigeration (Figure 7.2) Zobrist, Huppertz *et al*., (2005) also reported a lower coagulation time for cows' milk stored for short (after 0 and 4 h) than for long periods (after 24 and 48 h). Since HPP leads to an increase in size and number of casein micelles, due to weakening of hydrophobic and electrostatic interactions between sub micelle and further aggregation of sub micelle to bigger clusters, with changes to form chains or clusters of sub-micelles ((Dhineshkumar *et al.*, 2016) cited Huppertz, Kelly, and Fox, (2006)).

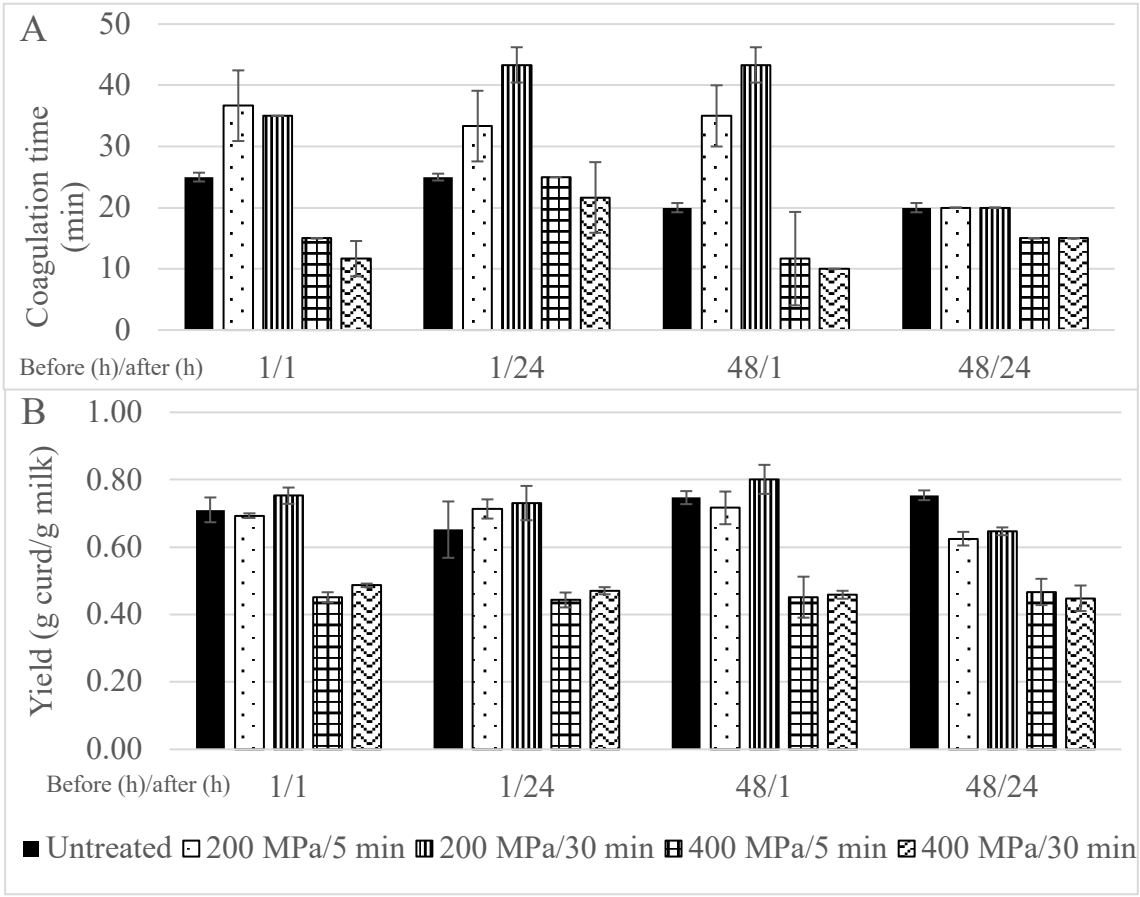


**Figure 7.2: Initial wide screening results for: (A) coagulation time (in min) and (B) yield expressed in g of curd per g of milk; 1/1 - milk HPP treated and transformed within the day of collection; 1/24 - milk HPP treated on day of collection and transformed the next day; 48/1 - milk HPP treated after 48 h of collection and subsequently transformed on the same day; 48/24 - milk HPP treated after 48 h of collection and transformed after 24 h; control samples are untreated milk.**

Syneresis occurred only in those cheeses manufactured from HPP milk treated at the lower pressure under study (200 MPa) and also in control cheeses (see Figure A. 7.1). Low syneresis was reported in the literature for milk HPP treated at higher intensity, e.g. treatments at 676 MPa/5 min at 10 ºC for bovine milk (San Martín-González *et al.*, 2007), 600/15 min for skim milk (Needs *et al.*, 2000), while treatments at 200 and 400, did not show significant differences (Needs *et al.*, 2000).

Yield was improved by milk HPP pre-treatment at 200 MPa, as shown in Figure 7.2 B, in particular, when the milk was treated for 30 min, after 48 h of refrigeration upon collection and transformed 1 h after HPP, a 12% increase in yield was achieved. In contrast to what has been reported in the literature (Huppertz *et al.*, 2004; López-Fandiño and Olano, 1998a), in this study the milk HPP treatment at higher pressure intensity, i.e. 400 MPa led to lower cheese yields. Huppertz *et al*., (2004a) reported a higher yield for cheeses made from HPP treated cows' milk (100-400 MPa) upon storage for 24 h at 20 ºC than those produced immediately after HPP milk treatment. Furthermore, a higher curd formation yield was obtained with ewes' milk HPP treated at 200 MPa/30 min than with control milk; nevertheless, a considerable increase in curd yield was achieved after milk was HPP treated at 400 MPa/30 min (15.6 %) (López-Fandiño and Olano, 1998b). A similar HPP treatment (400 MPa/30 min) on cows' milk led to a curd yield increase of 20 % (López-Fandiño *et al.*, 1996). The increase in curd/cheese yield may be due to greater moisture retention, but also to the incorporation of some denatured ß-lactoglobulin (López-Fandiño *et al.*, 1996).

Statistical analysis of data showed that the curd yield was affected by the pressure intensity ($p < 0.001$), holding time under pressure ($p < 0.05$), time after HPP ($p < 0.05$) as single factors and by the interaction of pressure intensity, time before HPP and time after HPP ($p < 0.001$). This first step, i.e. the initial wide screening design, was crucial to

determine that the pressure intensity, holding time under pressure and time after HPP were the most important factors when cheese yield increment was desired. To the best of our knowledge, this is the first research work where all four factors and their interactions were studied for milk; furthermore, only Zobrist *et al.*, (2005) studied the effect of cows' milk storage after HPP and prior to rennet addition on coagulation time, having also verified different coagulation times after different milk HPP storage time 4, 24 and 48 h at 4 and 20 ºC.

Based on the results obtained in the wide screening study and discussed above, milk holding time before and after HPP treatment was fixed at 48 and 24 h, respectively, since the time before HPP showed no individual effects on curd yield.

### 7.3.2. Focused screening design

As mentioned above, the initial wide screening design revealed more interesting results in milk HPP pre-treated at low pressure intensity (200 MPa) than at high pressure intensity (400 MPa). Therefore, in order to rule out any possibility of lower pressures bringing on more favourable results, in the focused screening design, the range of pressure intensity was widened to include also 100 MPa, and additional analyses were also carried out, namely, pH, titratable acidity, and microbiological data.

As in the previous screening, HPP treated milk at lower pressures, i.e. 100 and 200 MPa, remained in its liquid form and the milk treated at 300 and 400 MPa became viscous and yellower, and presented phase separation with time (Figure A. 7.2). This visual analysis is in agreement with the obtained curds (Figure A. 7.3) and milk pH values measured, as shown in Figure 7.3 B. HPP treated milk at 300 and 400 MPa revealed significantly higher pH values (6.38 and 6.42, respectively) than control milk (5.74) ($p < 0.001$). Closer to the pH values of control milk were those of milk HPP treated at 100 and

200 MPa (5.81 and 5.89), although statistically different ($p < 0.001$). In the literature, HPP goat milk treatment (500 MPa/15 min) led to significantly higher milk pH values in comparison to thermally pasteurized milk (6.66 *vs* 6.54, respectively) (Trujillo *et al.*, 1999b). Raw whole bovine milk revealed a similar effect, with HPP treatments (100, 250 and 400 MPa/15 min) inducing increments in milk pH values (to 6.73-6.75 vs 6.66 of control milk), but without significant differences among HPP treatments (Zobrist *et al.*, 2005). These pH changes brought about by HPP can be due to dissolution of colloidal calcium phosphate (CCP), due to its dissociation from the casein micelle (Schrader *et al.*, 1997), possibly due to weakening of hydrophobic and electrostatic interactions between submicelles.

Titratable acidity was in agreement with the changes in pH values (Figure 7.3 B. Relatively to curd yield, similar values were obtained for milk HPP treated at 100 MPa and untreated milk ($p > 0.05$) (0.55 *vs* 0.53 g milk/g curd), as shown in Figure 7.3 B.

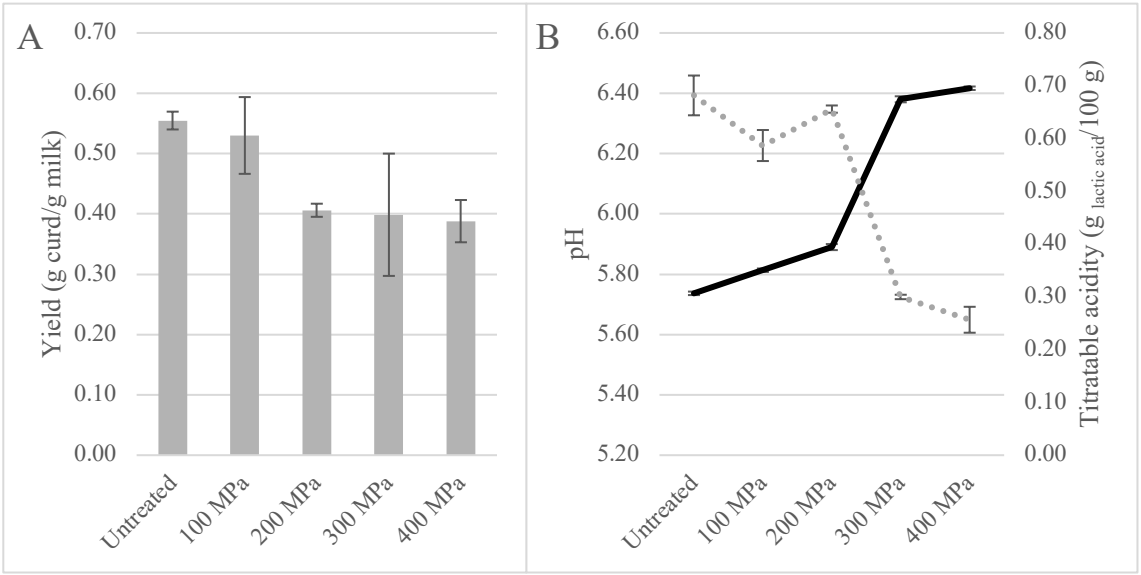


**Figure 7.3: Focused screening study results: (A) yield expressed in g of curd per g of HPP milk at 100, 200, 300 and 400 MPa for 5 min after 48 h of collection and transformed after 24 h storage; (B) milk pH values (——) and titratable acidity (• • • •).**

As expected, HPP treatment at 400 MPa strongly affected microbial cell viability, in particular the beneficial microbiota that contribute positively to the cheese ripening process. On the other hand, many of the microbial groups tested, namely, lactobacilli, enterococci, total mesophilic microorganisms, staphylococci, coliforms and *Enterobacteriaceae* counts were only slightly affected when milk was treated at 100 MPa (data not shown). Thus, a lower pressure intensity kept the beneficial microbiota and could improve the yield, but the minimization of spoilage bacteria such as staphylococci, coliforms and *Enterobacteriaceae* was only slightly affected.

### 7.3.3. Optimization design of experiment by central composite design

Based on the results obtained in the two screening studies, an optimization approach followed, where factors to be studied included pressure intensity between 100 and 300 MPa, time of HPP treatment between 5 to 30 min (Table 7.1 and Figure 7.1) after 24 h of milk collection (note that this time period was reduced due to high viable cell numbers quantified in the focused screening design) and curd transformed after 24 h of HPP treatment.

Visual analysis of the milk bags upon treatment revealed that samples treated at 300 MPa for 5, 30 and 17.5 minutes (samples 3, 4 and 6, respectively, in Figure A. 7.4) were yellower. Instrumental colour analysis confirmed these colour variations, since these HPP treated milks revealed higher *b**-values (Figure 7.4 C); Gervilla *et al.,* (2001) reported similar results for HPP treated (100 – 300 MPa/15 min) raw ewes' milk where an increase of *b** values were observed. Overall, HPP treatments at higher pressure intensity (200 and 300 MPa) led to a yellower milk colour (higher *b**-values), particularly for longer holding times under pressure (17.5 and 30 min). Higher *L**-values were measured for HPP treated milk at 100 MPa for 5 and 17.5 min, while the other treatments led to similar or slightly

lower *L**-values than control milk. Literature reports that HPP induces changes in *L**-values mainly due to casein micelles disintegration into small fragments that increase the milk translucidity (Gaucheron *et al.*, 1997; Gervilla *et al.*, 2001; Huppertz *et al.*, 2004a).

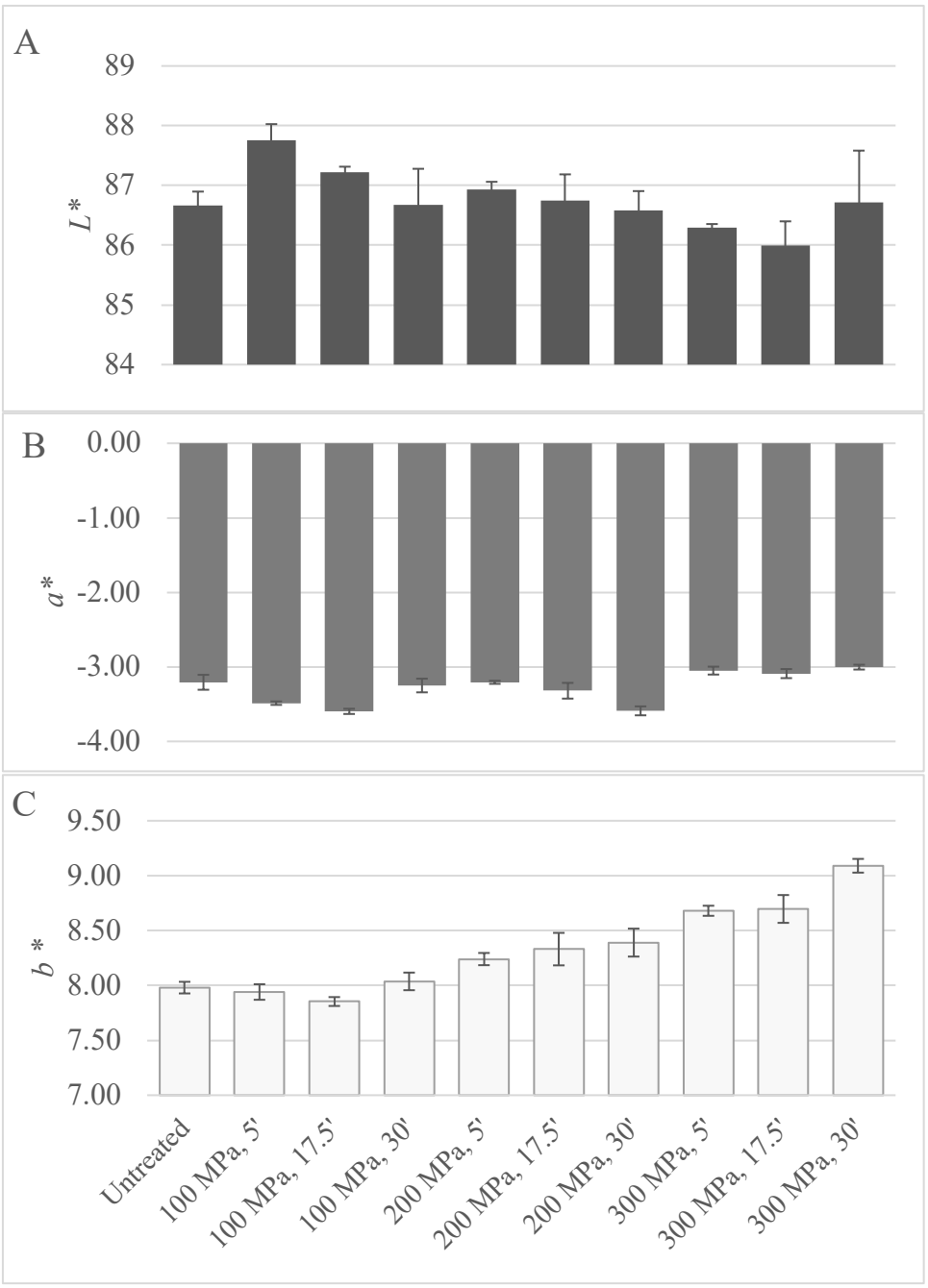


**Figure 7.4: Design of experiment results: colour CIE (A) *L**, (B) *a** and (C) *b** parameters measured in milk HPP treated according to central composite design.**

HPP treated milk resulted in curds (Figure A. 7.5) with increased yield between 5 and 24% in comparison to control milk (Figure 7.5 A), being the highest values achieved with milk treated at 300 MPa/17.5 min. To the best of our knowledge there is only one work that studied HPP application on ewes' milk, revealing a similar behaviour, but reporting lower curd yields of about 5% for HPP treated ewes' milk at 200 and 300 MPa for 30 min, while at 100 MPa a yield similar to untreated milk was verified (10, 20 and 30 min of treatment time at 300 MPa showed no effect on yield) (López-Fandiño and Olano, 1998b). In the present study, the model analysis of the results revealed that the effect of the studied variables on yield could be described by a linear model, where pressure has the greatest contribution ($p < 0.03$ with lack of fit $p=0.067$).

Since during the two previous screening studies it was visually observed that syneresis showed a clearly different behaviour among samples, syneresis was also studied (Figure 7.5 B). Initially after centrifugation, minor whey release was quantified for curds obtained from HPP milk pre-treatment, particularly for treatments at 100 and 300 MPa for 17.5 min (about 34 and 29 %, respectively against 45 % for untreated milk), but syneresis after 24 h revealed lower, yet statistically insignificant, values for the control milk curds ($p > 0.05$). As previously mentioned, HPP may induce water retention in curd (Molina *et al.*, 2000; Saldo *et al.*, 2002, 2000; Trujillo *et al.*, 2000), a situation that appears to be related to a change in the structure of the *para*-caseinate network (Saldo *et al.*, 2002), an observation that may help explain the different syneresis behaviours observed for the HPP treated samples. Relatively to coagulation time, pre-treated HPP milk revealed at least 12% faster coagulation than untreated milk as reported in the literature (López-Fandiño *et al.*, 1997; López-Fandiño and Olano, 1998b, 1998a; Zobrist *et al.*, 2005).

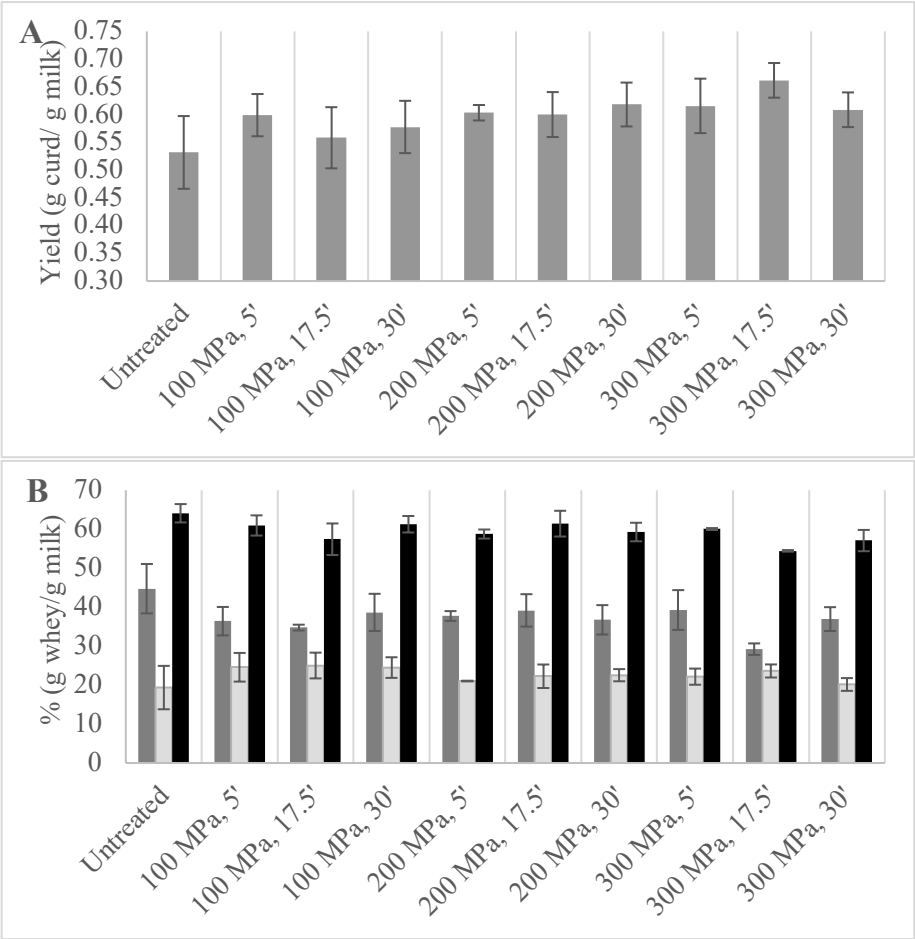


**Figure 7.5: Design of experiment results: (A) yield expressed in g of curd per g of milk; (B) whey release immediately after centrifugation: first whey (■); syneresis (24 h) (■); whey+syneresis (■); from milk HPP treated according to central composite design. Control samples are untreated milk.**

The pH values were also analysed in untreated and HPP pre-treated milk and in the curds obtained therefrom, as shown in Figure 7.6. HPP treated milk revealed higher pH values (6.4-6.5) than the untreated milk (6.29), a trend even more noticeable in milk pressurized at the highest pressure intensity (300 MPa), corroborating the results reported above which can be justified by colloidal calcium phosphate solubilization (Schrader *et al.*, 1997). Higher pH values were registered in curds resulting from HPP treated milk (5.18-6.42) than untreated milk (5.13), being significantly higher in curd resulting from milk treated at 300 MPa ($p < 0.001$) (Figure 7.6). Similarly, about 0.6 pH units above that

of the control cheese were reported for curd from HPP goat milk (400 MPa/5 min) (Saldo *et al.*, 2002). However, higher intensity HPP treatments (586 MPa/1 min and 400-600 MPa/10 min) in bovine milk revealed no effect on curd pH (Drake *et al.*, 1997) or led to a decrease (Voigt *et al.*, 2010b).

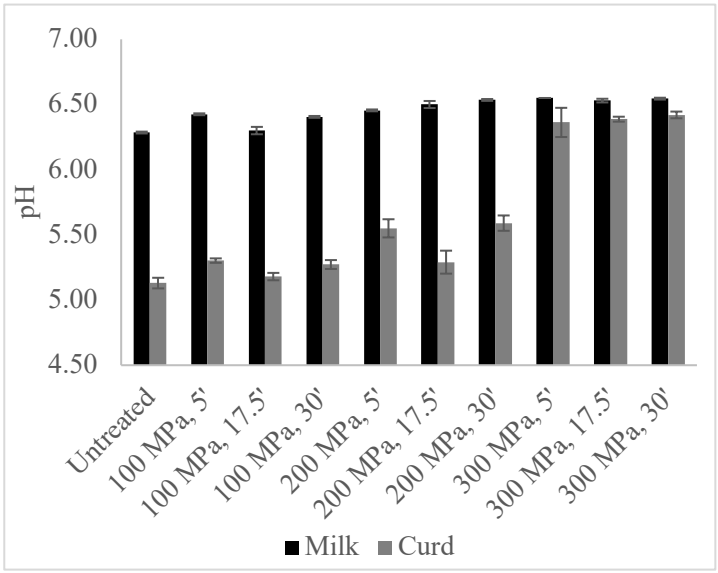


**Figure 7.6: Design of experiment results: milk (■) and curd (■) pH values from milk HPP treated according to central composite design.**

Milk microbiota viable cell numbers are shown in Figure 7.7. In untreated milk samples, lactobacilli, lactococci and enterococci were found at 7.25, 4.28 and 5.35 log cfu/mL, respectively (Figure 7.7 A). *Enterobacteriaceae* and total coliforms viable cell numbers were found to be at a similar level, 6.53 and 6.69 log cfu/mL, respectively. *Escherichia coli* and *Staphylococc*us spp. were detected at 4.34 and 4.48 log cfu/mL, respectively. Yeasts and moulds were detected at 5.63 log cfu/mL (Figure 7.7 B). As expected, a higher pressure intensity led to a higher microbial inactivation, particularly for longer holding times under pressure, as shown in Figure 7.7. Pressure lethal effect on microorganisms was also reported to be higher as pressure increased from 100 to 300

MPa for 30 min in bovine milk (López-Fandiño *et al.*, 1996), having the total aerobic counts achieved an approximately 0.9 log reduction.

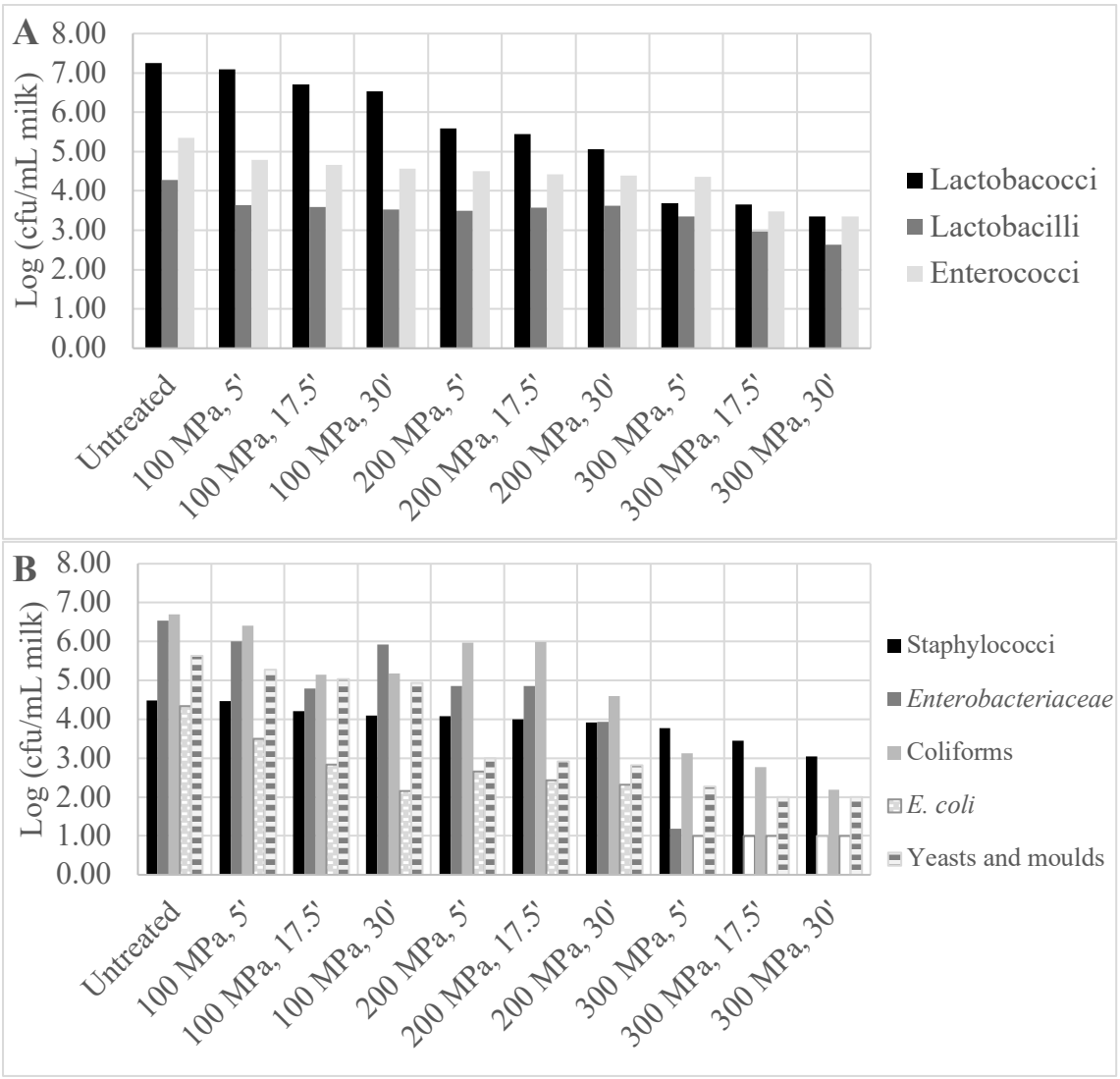


**Figure 7.7: Design of experiment microbiota results: (A) lactobacilli, lactococci, enterococci, and (B) staphylococci, *Enterobacteriaceae*, total coliforms, *Escherichia coli* and yeasts and moulds viable cell numbers in ewe milk samples HPP treated according to central composite design. Empty bars represent microbial loads below the quantification limit (1.0 log cfu/mL).**

HPP has been reported as an alternative to traditional thermal pasteurisation in order to increase the microbial milk quality, but then lactic starter cultures needed to be added. This is an approach not possible for PDO cheeses, like *Serra da Estrela* cheese, since the

use of starter cultures is not allowed, and so a balance between spoilage microbiota inactivation, while keeping as much as possible the beneficial microbiota was necessary, and became one of the objectives of the present work. Drake *et al*., (1997), Buffa *et al*., (2001) and Trujillo *et al*., (1999) treated bovine and goat milks at 586 MPa/1 min and 500 MPa/15 min and the total viable cells numbers were reduced in 0.87-2.2 log cycles, coliforms in > 1.3 log cycle and *Enterobacteriaceae* > 1.9-3.8 log cycles relativity to control milk. This same HPP treatment in goat milk led to lactobacilli reduction to below the quantification limit (> 2.36 log cycles) (Buffa *et al.*, 2001b).

The design of experiment results analysis allowed to optimize the desirability answers (Table 7.2). In what concerns the microbiota, viable cell numbers reduction data were normalized by dividing by the mean of the microbial load of the untreated samples, expressing microbial inactivation percentage. The model was then optimized in order to have: (1) minimum values of normalized logarithmic reductions of lactobacilli, lactococci and enterococci (this family was added as a group to benefit, since its relevance in the development of cheese flavour is well known); (2) maximum values of normalized logarithmic reductions of *Enterobacteriaceae*, total coliforms, *E. coli*, staphylococci and yeasts and moulds (known to be spoilage microorganisms), and (3) highest yield possible.

In this analysis it was taken into account that not all the microbial groups have equal relevance to cheese maturation. Different importance levels were considered in the optimization design of experiment analysis to determine the optimal conditions, as shown in Table 7.2. An equal relevance attribution revealed as optimal HPP conditions 288.38 MPa for 5 min. Considering the lactobacilli and lactococci values 5-fold more important, enterococci 3-fold more important and 1-fold for the other microbial groups under study, it was achieved as optimum conditions the HPP treatment at 121.5 MPa for 30 min (the predicted results using these conditions are shown in in Figure 7.8).

**Table 7.2: Parameters considered in model optimization of design of experiment (microbiota and yield) with response goals, with the respective different importance attributed and values expected to achieve by the modulation design at predicted optimized conditions.**

| | Response goal | Importance | Expected value | Importance | Expected value | Importance | Expected value | STD adjusted |
|---|---|---|---|---|---|---|---|---|
| **Lactococci** | *Low* | 1 | 45.4 | 20 | 16.9 | 25 | 14.2 | 1.38 |
| **Lactobacilli** | *Low* | 1 | 22.2 | 20 | 33.7 | 25 | 15.2 | 2.25 |
| **Enterococci** | *Low* | 1 | 20.1 | 15 | 14.9 | 15 | 13.4 | 2.13 |
| ***Enterobacteriaceae*** | *High* | 1 | 71.3 | 5 | 43.8 | 5 | 16.1 | 5.42 |
| **Coliforms** | *High* | 1 | 45.4 | 5 | 22.2 | 5 | 23.2 | 4.05 |
| ***Escherichia coli*** | *High* | 1 | 71.9 | 5 | 52.2 | 5 | 46.8 | 2.43 |
| **Staphylococci** | *High* | 1 | 15.1 | 10 | 9.9 | 5 | 8.5 | 1.70 |
| **Yeasts and moulds** | *High* | 1 | 61.1 | 5 | 53.9 | 5 | 20.9 | 1.98 |
| **Yield** | *High* | 1 | 0.62 | 5 | 0.62 | | 0.58 | |
| **Optimized conditions:** | | **P (MPa)** | **Tempo (min)** | **P (MPa)** | **Tempo (min)** | **P (MPa)** | **Tempo (min)** | |
| | | 288.4 | 5 | 245.1 | 5 | 121.5 | 30 | |
| **Desirability** | | 0.59 | | 0.56 | | 0.61 | | |

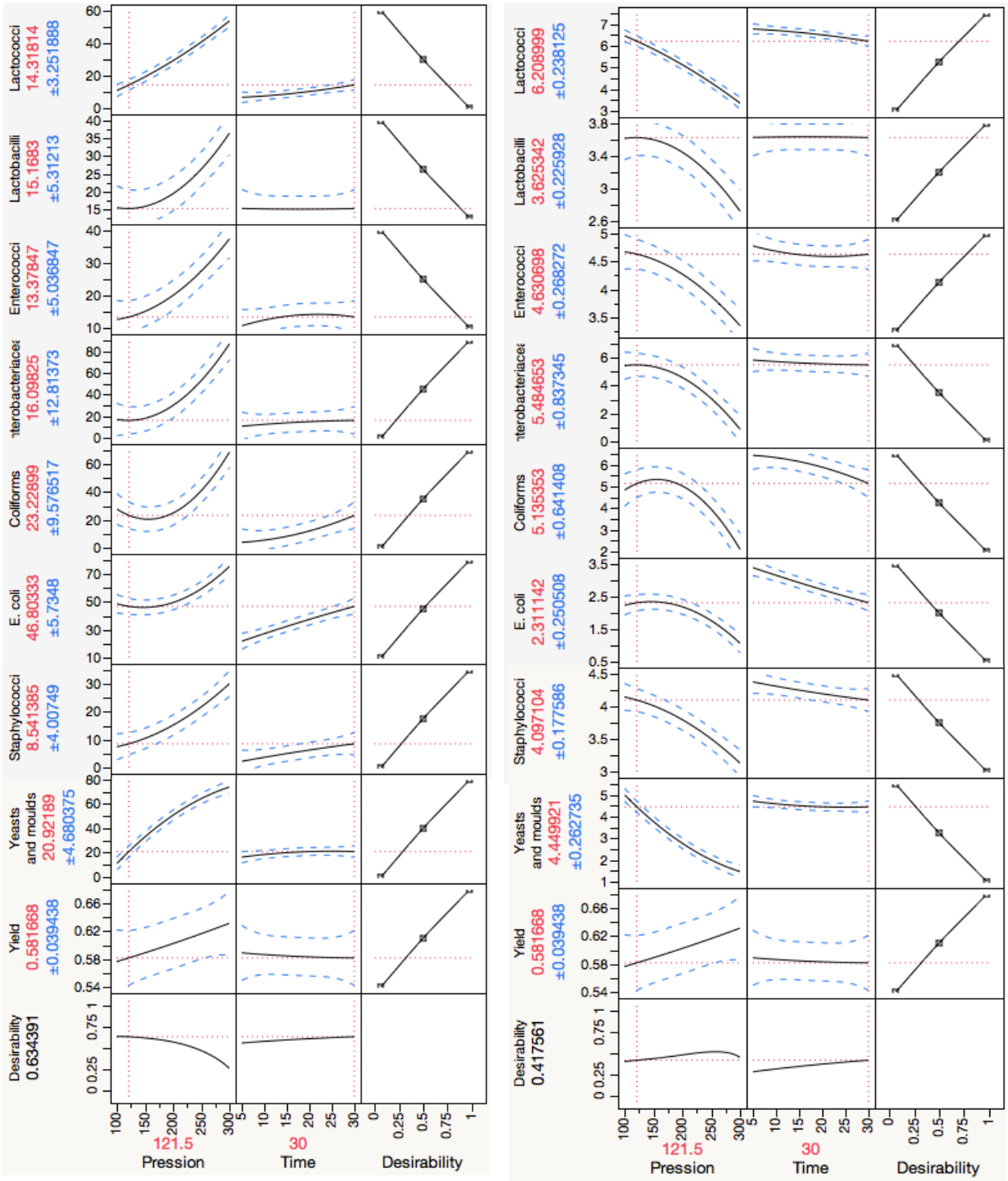


**Figure 7.8: Design of experiment results: prediction profile of results optimization (by JMP9 software) of the surface model assuming greater importance for lactobacilli and lactococci (quintuple of importance), and to enterococci (triple of importance). Equal importance was attributed to the remaining microbial groups/species. (A) standard results are expressed in decimal reductions relative to the control load; (B) microbial viable cells numbers.**

### 7.3.4. Model validation

The optimum condition obtained in the optimization study considering different importance levels – 121 MPa/30 min was subsequently applied to new batch of raw ewes' milk, in quintuplicate, for a greater robustness to validate the predicted results. Untreated milk was also studied to allow data normalization and Table 7.3 and Figure A. 7.6 present all obtained results: curd yield, microbiota viable cell numbers, whey quantification, and pH values. Statistical analysis of all data obtained during model validation revealed that lactococci, lactobacilli, enterococci, *Enterobacteriaceae*, total coliforms and yeasts and moulds inactivation percentages ($p > 0.05$) were according to the predicted statistic prevision, thus validating these parameters (Table 7.3). However, *E. coli* and staphylococci inactivation was not validated ($p < 0.05$). Curd yield and released whey were also validated by the model ($p > 0.05$).

Thus the HPP conditions 121 MPa for 30 min, when applied to ewes' raw milk after 24 h collection and transformed within the next 24 h to curd, were validated as optimal conditions to combine the best possible inactivation of spoilage microbial viable cells, with a very low reduction of viable cell numbers of beneficial microbiota and simultaneously achieve a better cheese yield.

**Table 7.3: Results for model validation: microbiota viable cell numbers were normalized and expressed in inactivation percentage (log reductions/log counts untreated milk samples). Prevision values and adjusted standard deviation determined by Minitab Software.**

| | Lactococci | Lactobacilli | Enterococci | *Enterobacteriaceae* | Coliforms | *Escherichia coli* | Staphylococci | Yeasts and moulds |
|---|---|---|---|---|---|---|---|---|
| **121 MPa, 30' (A)** | 11.37 | 15.06 | 13.11 | 25.57 | 22.27 | 43.14 | 27.32 | 19.34 |
| **121 MPa, 30' (B)** | 16.61 | 12.03 | 15.10 | 20.65 | 23.12 | 37.30 | 15.24 | 22.55 |
| **121 MPa, 30' (C)** | 12.55 | 15.95 | 12.01 | 23.02 | 22.82 | 44.64 | 22.68 | 23.27 |
| **121 MPa, 30' (D)** | 15.17 | 15.06 | 14.88 | 18.75 | 17.34 | 40.53 | 25.15 | 23.10 |
| **121 MPa, 30' (E)** | 12.55 | 15.42 | 15.56 | 12.68 | 21.59 | 39.41 | 23.23 | 24.00 |
| **Prevision** | 14.23 | 15.17 | 13.38 | 16.10 | 23.22 | 46.80 | 8.54 | 20.92 |
| **STD (adjusted)** | 1.38 | 2.25 | 2.13 | 5.42 | 4.05 | 2.43 | 1.70 | 1.98 |
| **IC 95%** | 11.07 | 9.86 | 8.34 | 3.28 | 13.65 | 41.07 | 4.53 | 16.24 |
| | 17.57 | 20.48 | 18.42 | 28.91 | 32.80 | 52.54 | 12.55 | 25.60 |
| | Validated | Validated | Validated | Validated | Validated | Not validated | Not validated | Validated |
| **p-value** | 0.278 | 0.645 | 0.432 | 0.096 | 0.325 | 0 | 0 | 0.083 |

## 7.4. Conclusion

When screening the factors that affect cheese yield, it is very important to test as many factors as possible in order to identify the significance of each of them. The experimental design allowed to determine that the most influential factors on *Serra da Estrela* cheese production, from high-pressure treated milk, were pressure intensity, holding time under pressure and time after HPP. A focused screening design was able to pinpoint that the viable cell numbers in milk HPP treated at 400 MPa were considerably affected, while the lower pressure intensity kept the beneficial microbiota and improved the curd yield. For identification of optima, a response surface design was performed and higher pressure intensity led to a higher microbial inactivation, which was more pronounced at longer holding times under pressure. Nevertheless, placing as main targets, an equilibrium between the best inactivation level for spoilage bacteria without hindering (lowest reduction possible) beneficial microbiota viable cell numbers, coupled to an increased yield led to determine HPP milk pre-treatment at 121 MPa for 30 min as the optimum condition and model validation confirmed the predicted results.

In conclusion, HPP treatment of ewes' raw milk prior to cheese manufacture can enable *Serra da Estrela* cheese yield increment and improve the microbial profile important from both a safety and quality points of view.

# CHAPTER 8 - Effect of HPP pre-treatment of raw ewes' milk and on subsequently produced cheese throughout ripening

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*This chapter has been submitted for publication.*

## Abstract

Raw ewes milk, used to manufacture *Serra da Estrela* Protected Designation of Origin (PDO) cheese, was pre-treated by high pressure processing (HPP), using previously predicted optimum conditions (121 MPa/30 min), to study its effect on milk technological properties for subsequent cheese production: impact on resulting curd, whey, and cheese throughout ripening was assessed. The cheese yield was found to increase by 10.4% with HPP milk pre-treatment. Although HPP pre-treated milk cheese revealed some inactivation of beneficial microbial groups, at 60 days of ripening treated and control cheeses showed no significant differences ($p > 0.05$) in quantified microbial load nor in basic physicochemical quality parameters. Hence, HPP can be seen as a promising non-thermal treatment for ewes milk to inactivate contaminant bacteria yet with no negative effect on lactic acid bacteria, which are very important for the unique characteristics of *Serra da Estrela* cheese.

## 8.1. Introduction

*Serra da Estrela* cheese, holding a Protected Designation of Origin (PDO) status, is made only with milk from Bordaleira *Serra da Estrela* and/or Churra Mondegueira ewe's breeds, salt and cardoon flower (*Cynara cardunculus* L.) extract (Macedo *et al.*, 1993). The milk from these ewe breeds is known to give a good yield, about 1 kg of cheese from 5.0 to 6.0 L of milk (Macedo *et al.*, 1993). Different literature reports have indicated that cheese yield can be increased in about 4 – 23 % through a non-thermal high pressure processing (HPP) pre-treatment of bovine milk (Huppertz *et al.*, 2005, 2004c). Moreover, HPP can substantially reduce the microbial pathogenic/spoilage microorganisms present in the raw milk used to produce *Serra da Estrela* cheese, thus possibly improving the safety of this traditional dairy product.

In the CHAPTER 7, a design of experiments (DoE) approach was used to construct experimentally efficient factor screening and optimization studies, in order to identify the best HPP conditions to be applied to raw ewes milk for subsequent *Serra da Estrela* cheese production, envisaging cheese yield improvement and at least maintenance of the principal quality characteristics of the cheese. The use of DoE allowed concluding that 121 MPa for 30 min was the treatment that enabled the most efficient maintenance of the beneficial microbiota responsible for biochemical and sensory attributes of the cheese (lactococci, lactobacilli and enterococci), while causing inactivation of the spoilage microorganisms *Enterobacteriaceae*, coliforms, *Escherichia coli*, staphylococci, yeasts and moulds. Several studies have used the application of more intense HPP conditions for milk pasteurisation (>345 MPa/15 min) in order to cause higher microbial inactivation, but, in such cases, starter cultures needed to be added to manufacture cheese (Buffa *et al.*, 2001b; Drake *et al.*, 1997; Trujillo *et al.*, 1999b). In this present study, focus is on defining a HPP pre-treatment of the cheese-making milk, at HPP conditions that

will allow cheese manufacture therewith without the need of starter cultures addition, and to the best of our knowledge, there is no study available in literature related with this aspect. Hence, the main goal of the present research was to understand the effects of a HPP pre-treatment (121 MPa for 30 min) on raw ewe milk used to produce *Serra da Estrela* cheese and on subsequently produced curd, whey, and ripened cheese.

## 8.2. Materials and methods

### 8.2.1. Milk supply, cheese manufacture and yield determination

One hundred and sixty litres of raw ewes milk were collected, in the morning, from different dairy farms located in *Serra da Estrela* PDO region, Portugal, pooled and transported to an artisanal dairy facility that produces commercial *Serra da Estrela* cheese, according to the PDO procedure. The bulk milk was kept in a refrigerated reservoir until use and prior to sampling, milk was well mixed to ensure sample homogeneity. The bulk milk was then divided into two batches: 82 L were used, in the same morning, to manufacture 35 cheeses according to the PDO procedure (Macedo *et al.*, 1993) considered as milk control cheeses ($M_c$). After coagulation, cutting and pressing of the curd, aliquots of curd control and whey control were collected (1.5 h after milk coagulation initiated). The remaining milk was packaged in portions of 8 L into polyamide-polyethylene (PA-PE, Plásticos Macar, Indústria de Plásticos Lda, Santo Tirso, Portugal) bags that were heat sealed and stored under refrigeration (4 °C) before HPP pre-treatment (121 MPa for 30 min) ($M_P$), which occurred in the afternoon of the same day. The next day, in the morning, 77 L of the pre-treated milk were used to produce 34 cheeses cheeses, according to the PDO procedure. Aliquots of curd and whey were collected from the cheese manufacture process with $M_P$ milk similarly to that of control cheeses. All 69 cheeses manufactured from $M_C$ and $M_P$ milks, of about 500 g each, were

ripened at 7 ± 2 ºC and 95% relative humidity (RH) for 15 days and then at 10 ± 2º C and 85% RH, for 60 days at the artisanal dairy. During the ripening period, the cheeses were washed and weighed periodically/weekly (according to the procedures implied by the PDO status). Cheese yield and percentage weight loss was determined weekly taking into account the litres used in the manufacture of each batch and the cheese weight upon surface cleaning.

### 8.2.2. High pressure processing

HPP treatments were performed in a 55-liter capacity industrial scale high pressure equipment (model 55, Hiperbaric, Burgos, Spain) at 121 MPa for 30 min (as already explained above, this condition was selected based on a previous experimental design study, performed to find the optimum HPP pre-treatment to be applied to ewes milk for cheese production), with the initial temperature of the water used as transmitting fluid being 8 °C.

### 8.2.3. Microbiological analyses

As previously described in section 7.2.8 Microbiological analyses.

### 8.2.4. Physicochemical analyses

As previously described in section 3.2.5 Physicochemical analyses. For calcium content, cheese samples were dried at 105 ºC for 24 h and subsequently subjected to mineralization in a microwave system. About 0.5 g of dry, ground sample was introduced into the digestion vessel and added with 5 mL of concentrated nitric acid. The vessels were capped and placed in a microwave pressure digestor Speedwave MWS-3+ (Berghof) and subjected to microwave radiation at 20 bar according to the following program: room temperature was raised first to 130 ºC at 22 ºC/min and 30% of irradiation power, then to

160 ºC at 6 ºC/min and 40% of irradiation power, remaining 5 min at this temperature, and to 170 ºC at 5 ºC/min and 50% of irradiation power, remaining 5 min at this temperature. The cooling process consisted in decreasing temperature first to 100 ºC for 4 min and then to room temperature. After cooling, acid digests were made up to 20 mL with Milli-Q water. Three replicates were performed for each cheese sample as well as blanks. The content of calcium is expressed as the mean plus standard deviation. The calcium composition was determined using an inductively coupled plasma (ICP) optical emission spectrometer model Optima™ 7000 DV ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with radial plasma configuration. Standard plasma conditions were used namely 1300 W for radio-frequency power, 1.5 mL/min pump rate, and 15.0, 0.2 and 0.8 L/min for plasma, auxiliary and nebulizer gas flow, respectively. Detection wavelength was 317.933 nm. A standard containing up to 3000 mg/L of Ca was used and prepared in 2% HNO3. Successive dilutions of the standard solution were prepared and used for calibration and the concentration of calcium was determined by direct interpolation in the standard curve within its linear dynamic range.

### 8.2.5. Statistical analyses

All analytical results are presented as average values with the standard deviation. The *t*-student test using SPSS software, version 24.0 (SPSS Inc., Chicago, IL, USA) was used to determine the significant differences, at significance level of $p < 0.05$, between control milk ($M_C$) and HPP pre-treated milk ($M_P$) and resulting curd and cheese samples.

## 8.3. Results and discussion

### 8.3.1. Milk, curd and whey composition

Raw ewes milk ($M_C$) and HPP pre-treated milk ($M_P$) showed similar ($p > 0.05$) moisture and protein contents, about 81% and 5.8 %, respectively (Table 8.1). Trujillo *et al.*, (1999) found similar protein and fat contents in goat cheese produced from thermal pasteurized and HPP pre-treated milk (500 MPa/15 min). Although statistically different ($p < 0.05$), the moisture, fat and protein contents of $M_P$ milk curd differed only around 1 % from those of $M_C$ milk curd. Literature reports 5 % lower moisture content for the curd from bovine milk treated at 100 or 250 MPa compared to that from untreated milk (Huppertz *et al.*, 2004c). Nevertheless, in another study, raw whole bovine milk curd and HPP pre-treated milk curd (400 or 600 MPa/10 min), revealed no significant differences in moisture, protein, fat, and salt contents (Voigt *et al.*, 2010b).

The protein content of the whey obtained from the $M_P$ milk cheese manufacture procedure was significantly ($p < 0.05$) higher (+19.7 %) than that of the Mc milk cheese counterpart (Table 8.1). An opposite behaviour has been reported in the literature for HPP pre-treated bovine milk (250-600 MPa/0-60 min), having the protein content of whey from HPP pre-treated bovine milk cheese decreased progressively with increasing treatment pressure (Huppertz *et al.*, 2004c); on the other hand, no changes in whey obtained from cheeses production with HPP pre-treated bovine milk at 400 MPa were reported by Voigt *et al*., (2010). Furthermore, $M_P$ milk had a higher calcium content than $M_C$ milk ($p < 0.05$), which can be the result of the effect of HPP on the weakening of hydrophobic and electrostatic interactions between sub-micelles leading to the dissolution of colloidal calcium phosphate (Schrader *et al.*, 1997).

**Table 8.1: Average values for moisture, fat, protein and calcium contents, pH and titratable acidity of control and HPP pre-treated milk, and resulting curd, whey, and cheese with 60 days of ripening (cheese production took place in an artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).**

| | | Control | HPP pre-treated milk |
|---|---|---|---|
| **Moisture Content % (w/w)** | Milk | 80.8 ± 0.04 [a] | 80.9 ± 0.04 [a] |
| | Curd | 66.6 ± 0.04 [b] | 65.4 ± 0.13 [a] |
| | Whey | 90.4 ± 0.02 [b] | 89.8 ± 0.02 [a] |
| | Cheese | 41.2 ± 0.86 [a] | 40.3 ± 0.99 [a] |
| **Fat content % (w/w)** | Milk | 7.93 ± 0.02 [b] | 7.53 ± 0.00 [a] |
| | Curd | 13.8 ± 0.02 [a] | 14.9 ± 0.10 [b] |
| | Whey | 0.85 ± 0.03 [a] | 0.91 ± 0.01 [a] |
| **Protein content % (w/w)** | Milk | 5.83 ± 0.05 [a] | 5.84 ± 0.04 [a] |
| | Curd | 11.4 ± 0.05 [a] | 11.9 ± 0.08 [b] |
| | Whey | 1.32 ± 0.04 [a] | 1.58 ± 0.03 [b] |
| | Cheese | 23.9 ± 0.36 [a] | 22.2 ± 0.47 [a] |
| **Calcium content % (w/w)** | Milk | 0.115 ± 0.007 [a] | 0.128 ± 0.003 [b] |
| **pH values** | Milk | 6.46 ± 0.01 [a] | 6.58 ± 0.02 [b] |
| | Curd | 6.37 ± 0.01 [a] | 6.41 ± 0.01 [b] |
| | Whey | 6.33 ± 0.01 [a] | 6.37 ± 0.03 [b] |
| | Cheese | 5.16 ± 0.01 [b] | 5.11 ± 0.03 [a] |
| **Titratable acidity ($g_{lactic\ acid}$/100 g)** | Milk | 0.33 ± 0.013 [a] | 0.32 ± 0.010 [a] |
| | Curd | 0.46 ± 0.002 [a] | 0.47 ± 0.064 [a] |
| | Whey | 0.26 ± 0.020 [a] | 0.25 ± 0.003 [a] |
| | Cheese | 1.38 ± 0.044 [a] | 1.31 ± 0.045 [a] |

Different letters for the same analysis and product (milk, curd, whey, and cheese) indicate statistically significant differences (*t*-Student test $p < 0.05$).

### 8.3.2. Curd and cheese yield and weight loss along ripening

The curd yield obtained from HPP treated milk ($M_P$) of 0.302 $kg_{curd}/L_{milk}$, increased 10.4 % in comparison to that obtained with untreated milk ($M_C$), 0.274 $kg_{curd}/L_{milk}$ (Figure 8.1). This difference amplitude was basically maintained throughout ripening and by 60 days ripening, the $M_P$ milk cheeses reported 8.0 % more cheese yield. All cheeses revealed a similar weight loss trend throughout ripening as illustrated in Figure 8.1**.** According to these results, a yield improvement is achieved by HPP milk pre-treatment. In contrast, a study in literature refers that bovine milk cheese yield is not influenced by treatment at HPP < 250 MPa (Huppertz *et al.*, 2004c), and to the best of

our knowledge there are no publications with application of HPP pre-treatment at the range applied in this work (121 MPa for 30 min) on ewes milk. Higher cheese yields from HPP pre-treated cow milk were reported for more intensive HPP treatments (> 250 MPa), being the effect attributed to a higher moisture content (Drake *et al.*, 1997; López-Fandiño *et al.*, 1996), possibly due to the formation of a finer structural network and due to the water-binding properties of denatured ß-lactoglobulin, which was incorporated into the protein matrix (López-Fandiño *et al.*, 1996). However, in the present study, cheese moisture content was not affected by the milk pre-treatment (Table 8.1), but still, cheese yield increased about 10 %; other factors are possibly affecting the cheese yield.

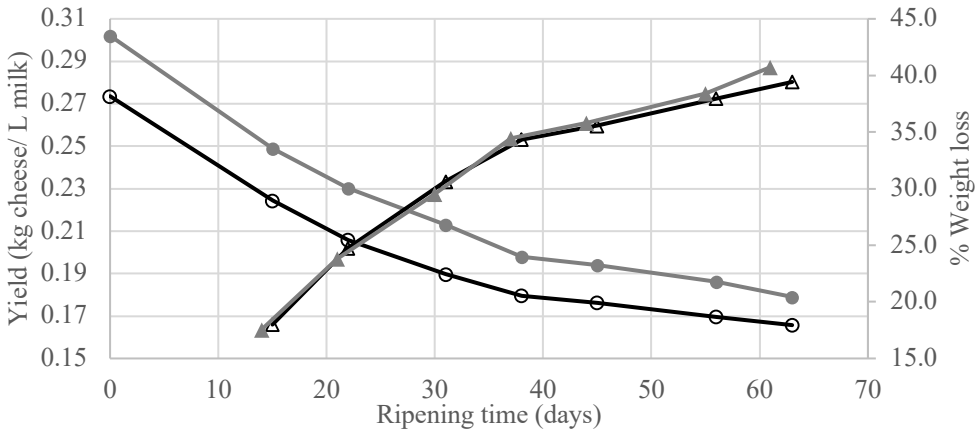


**Figure 8.1: Chesses' yield (kg of cheese L/of milk) from control milk (○) and HPP pre-treated milk (●), and weight loss percentage from control milk (△) and made from HPP pre-treated milk (▲) (cheese production took place in a real artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).**

### 8.3.3. Microbial composition of milk, curd and cheese

In $M_C$ ewes milk samples, lactococci and lactobacilli were found at 7.02 and 2.38 log cfu/mL, total mesophiles at 6.06 log cfu/mL, enterococci at 4.43 log cfu/mL and *Enterobacteriaceae,* total coliforms and staphylococci were all found at similar levels of 5.51, 5.57 and 5.06 log cfu/mL, respectively (Table 8.2). *Escherichia coli* and yeasts and

moulds were detected at 4.34 and 4.10 log cfu/mL, respectively. The application of the previously determined HPP ewes milk pre-treatment led to a reduction of microbiota viable cell numbers although of less order of magnitude than that reported in literature for many of the microbial groups. Nonetheless, it must be recalled that the main aim in this study is to apply such HPP conditions that may maximize the inactivation of pejorative microorganisms while minimizing the reduction of beneficial microbiota viable cell numbers in order to produce *Serra da Estrela* Cheese with a higher yield, maintaining as much as possible the characteristics of this cheese (CHAPTER 7). In $M_P$ ewes milk a total mesophiles suffered a reduction of 0.66 log cycles, similar to what was reported for bovine milk treated at 100 and 200 MPa/30 min (about 0.2-0.5 log cycles reduction) (López-Fandiño *et al.*, 1996). Higher reductions were observed for more intensive HPP milk treatments, *e.g.* HPP treatments at 586 MPa/1 min and 500 MPa/15 min in bovine and caprine milks revealed viable cell numbers' reduction between 0.87-2.22 log cycles (Buffa *et al.*, 2001b; Drake *et al.*, 1997; Trujillo *et al.*, 1999b). Minor numerical reductions in viable cell numbers (< 1 log), statistically not significant ($p > 0.05$), were observed for yeasts and moulds, total coliforms, and *Enterobacteriaceae* in $M_P$ milk. Higher reductions in viable cell numbers of coliforms and *Enterobacteriaceae* (> 1.32 log units) have been reported in caprine and bovine milks that underwent far more intensive HPP treatments (586 MPa/1 min and 500 MPa/15 min) (Buffa *et al.*, 2001b; Drake *et al.*, 1997; Trujillo *et al.*, 1999b). Gram-positive bacteria lactococci, lactobacilli, enterococci and staphylococci were less affected in $M_P$ milk, having been observed < 0.8 log cycle reductions in viable cell numbers in comparison to $M_C$ control values. More intense HPP treatments (500 MPa/15 min and 600 MPa/10 min) applied to caprine and bovine milks, led to more than 2.36 log cycles reductions of lactobacilli viable cell numbers (Buffa *et al.*, 2001b; Voigt *et al.*, 2012).

**Table 8.2: Microbiota quantification in control and HPP pre-treated milk, and resulting curd, whey, and cheese with 60 days of ripening (cheese production took place in an artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).**

| | | Control | | HPP | |
|---|---|---|---|---|---|
| | | Log cfu/mL or g | | Log cfu/mL or g | |
| **Total mesophilic** | Milk | 6.06 ± 0.13 | b | 5.34 ± 0.07 | a |
| | Curd | 6.22 ± 0.08 | a | 6.66 ± 0.31 | a |
| | Cheese | 8.33 ± 0.16 | a | 8.48 ± 0.41 | a |
| **Lactococci** | Milk | 7.02 ± 0.45 | b | 6.26 ± 0.08 | a |
| | Curd | 7.09 ± 0.41 | a | 6.42 ± 0.34 | a |
| | Cheese | 9.10 ± 0.16 | a | 9.11 ± 0.12 | a |
| **Lactobacilli** | Milk | 2.38 ± 0.03 | | < 2.00 | |
| | Curd | < 3.00 | | 3.84 ± 0.07 | |
| | Cheese | 8.62 ± 0.18 | a | 9.03 ± 0.17 | a |
| **Enterococci** | Milk | 4.33 ± 0.09 | a | 4.22 ± 0.06 | a |
| | Curd | 5.46 ± 0.05 | b | 4.79 ± 0.07 | a |
| | Cheese | 8.31 ± 0.08 | a | 8.32 ± 0.13 | a |
| **Staphylococci** | Milk | 5.06 ± 0.13 | b | 4.43 ± 0.19 | a |
| | Curd | 5.65 ± 0.16 | a | 6.24 ± 0.09 | b |
| | Cheese | 7.68 ± 0.06 | b | 7.51 ± 0.08 | a |
| ***Enterobacteriaceae*** | Milk | 5.51 ± 0.14 | b | 4.85 ± 0.13 | a |
| | Curd | 5.75 ± 0.11 | a | 5.77 ± 0.07 | a |
| | Cheese | 5.78 ± 0.08 | a | 6.06 ± 0.22 | b |
| **Coliforms** | Milk | 5.57 ± 0.12 | b | 4.86 ± 0.07 | a |
| | Curd | 6.06 ± 0.09 | a | 6.58 ± 0.38 | b |
| | Cheese | 7.32 ± 0.00 | b | 7.15 ± 0.00 | a |
| ***Escherichia coli*** | Milk | 4.34 ± 0.11 | a | 4.10 ± 0.11 | a |
| | Curd | 4.20 ± 0.51 | a | 4.79 ± 0.45 | a |
| | Cheese | 5.59 ± 0.10 | a | 5.76 ± 0.28 | a |
| **Yeasts and moulds** | Milk | 4.10 ± 0.19 | a | 3.58 ± 0.20 | a |
| | Curd | 4.06 ± 0.13 | b | 3.84 ± 0.13 | a |
| | Cheese | 4.24 ± 0.18 | a | 4.62 ± 0.38 | b |

Different letters for the same microorganism indicate statistically significant differences between the milk, curd and cheese from control and HPP treated milk (*t*-student test $p < 0.05$).

In general, the curd samples obtained from $M_P$ milk differed statistically in enterococci, staphylococci, coliforms and yeasts and moulds viable cell numbers relatively to curds obtained from $M_C$ milk ($p < 0.05$), however the differences were, once again very low, less than 0.6 log units. Different studies have demonstrated that the decrease in microbial groups' viable cell numbers brought about by the HPP milk pre-

treatment is subsequently reflected in the curd microbiota, but for more intensive treatments. For example, curds from HPP pre-treated goat milk (500 MPa/5 or 30 min; 500 MPa/15 min) showed about 1.8-2.0 log cycle reductions of total aerobic bacteria (Trujillo *et al.*, 2000), 2.46 log cycle reductions of *Enterobacteriaceae* and approximately 3 log cycle reductions of lactobacilli viable cell numbers (Buffa *et al.*, 2004), while curd from HPP pre-treated bovine milk (400 MPa/15 min) revealed 1.4 log cycle reductions of total microbiota viable cell numbers (Molina *et al.*, 2000).

At 60 days of ripening, cheeses manufactured from $M_C$ and $M_P$ milks showed no significant differences in lactococci, lactobacilli and enterococci viable cell numbers, with values around 9 log cfu/g (Table 8.2), these values being close to those previously reported for *Serra da Estrela* cheese (Macedo *et al.*, 2004; Tavaria and Malcata, 2000). Likewise, at 60 days of ripening, Buffa *et al.*, (2001) observed similar total bacteria, lactococci, lactobacilli and enterococci viable cell numbers for cheeses manufactured from HPP (500 MPa/15 min) treated and non-treated goat milk, exception for *Enterobacteriaceae* viable cell numbers that showed about 2 log cycle reductions in cheeses made from HPP pre-treated milk. For more intense HPP treatments, goat cheeses at 60 days of ripening, revealed reductions in viable cell numbers of total aerobic bacteria of about 3 and 6 log cycles for 500 MPa/5 and 30 min (Trujillo *et al.*, 2000), while for lactobacilli approximately 2 log cycle reductions were observed for 500 MPa/15 min (Buffa *et al.*, 2004). On the other hand, other authors observed less than 1 log cycle reduction in lactobacilli viable cell numbers after 60 days of ripening for bovine cheese produced from HPP milk pre-treated at 400 and 600 MPa/10 min (Voigt *et al.*, 2012).

According to the results of the present study, one of the first to be done on ewe's milk, to the best of our knowledge, and the first to be done on Bordaleira ewes' milk to manufacture *Serra da Estrela* cheese, a milder milk pre-treatment by HPP at 121 MPa for

30 min was able to cause ca. 1 log cycle reductions in viable cell numbers of pejorative microbiota in milk, while basically maintaining the beneficial microbiota load; yet by 60 days of ripening similar values were found for cheeses produced form control and HPP pre-treated milk, independently of the microbial group.

### 8.3.4. pH variation in milk, curd and cheese

The pH variations in cheese are mainly due to the formation and consumption of lactic acid, due to the metabolism of LAB and other microorganisms. In this work, a statistically significant increase of pH was verified in $M_P$ milk relatively to $M_C$ milk ($p < 0.05$), although the difference was only about 0.12 units. Even lower variations ($\leq 0.05$ pH units) were verified for curd, whey and cheese produced from $M_C$ and $M_P$ milks. The cheese pH values observed in the present study were within the range of those reported in the literature (4.82-5.66) (Macedo *et al.*, 2004; Sousa and Malcata, 1997). Similar low variations in pH were observed in a study with bovine milk treated by HPP (100, 250 and 400 MPa/15 min) (Zobrist *et al.*, 2005). These changes in pH caused by the HPP treatment were associated by the authors to dissolution of colloidal calcium phosphate (CCP), possibly due to the weakening of hydrophobic and electrostatic interactions between sub micelles. For curd, a small pH increment of 0.04 units was found when obtained with HPP (500 MPa/5 min) treated goat milk (Trujillo *et al.*, 2002a). Higher differences (0.16-0.36 pH units decrease) were reported for curd from HPP treated bovine milk (400 and 600 MPa/10 min) (Voigt *et al.*, 2010b). Concerning cheese, a small increment of 0.13 pH units was verified by Buffa *et al.*, (2004) for goat chesses manufactured from HPP treated milk (500 MPa/15 min).

As expected, HPP milk treatment showed no effect on the titratable acidity, being determined similar values for milk, whey, curd and cheese ($p > 0.05$) (Table 8.1).

## 8.4. Conclusion

*Serra da Estrela* cheese production, in a real artisanal dairy facility, from HPP (121 MPa for 30 min) pre-treated milk resulted in an increased cheese yield of 10.4 %. Furthermore, HPP milk pre-treatment led to a mild reduction of microbial load in milk, a small effect on curd and without significant differences in ripened cheese microbiota load. HPP pre-treated milk showed higher pH values, while cheese manufactured from HPP pre-treated milk had lower pH values; nevertheless being of small order of magnitude these differences were not reflected on titratable acidity values and no significant differences in moisture and protein content were verified. This study has been able to demonstrate, for the first time, that HPP treatment of raw *Bordaleira* ewes' milk prior to cheese manufacture can be used to increase *Serra da Estrela* cheese yield and to enable an improved microbial profile which is important from a safety and quality point of view, thus contributing positively to the production of such an important cheese.

# CHAPTER 9 - Effect of HPP on ewe cheese quality produced from previously HPP treated milk

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*This chapter has been submitted for publication.*

## Abstract

A high pressure treatment (121 MPa/30 min), selected as optimum in a previous optimization study) was applied as a pre-treatment to ewe raw milk and the subsequently produced cheese was processed by high pressure at 525 MPa/6 min, by the 60$^{th}$ day of ripening and then stored for 5 months at 4 ºC. Milk pre-treatment had little effect on cheese microbiota, while the application of pressure in the ripened cheese led to a decrease of the microbial loads, including to below the quantification limit for surrogate inoculated microorganisms (*Staphylococcus aureus, Listeria innocua*, and *Salmonella enterica*). Milk pre-treatment increased the cheese proteolytic index, while HPP applied to cheese allowed maintaining the ripening extension index along storage. A higher amount of volatile free fatty acids was observed for HPP-treated cheeses and the cheeses made from pre-treated milk were perceived to be softer and more unctuous by panellists.

## 9.1. Introduction

Cheeses made from raw milk are known to have strong flavours, due to the presence of diverse native microbiota that together play an important role during cheese ripening. One such case is *Serra da Estrela* Cheese with Protected Designation of Origin (PDO) certification that is manufactured only with raw ewes milk, salt, and a crude extract of vegetable rennet from the dried thistle flowers of *Cynara cardunculus* L. (Macedo *et al.*, 1993).

High pressure processing (HPP) is known as a cold pasteurization technology at 400 – 600 MPa, being capable of producing microbiologically safe products, with minimal changes on food characteristics. On the other hand, the application of lower pressure treatments (< 400 MPa) can induce other effects on foods, such as modification of biochemical features that may be of interest, as is the case of pressure treatment of milk to subsequently produce cheese. Regarding this possibility, a previous work based on an experimental design study was performed to find the optimum HPP pre-treatment to be applied to raw ewes milk for *Serra da Estrela* cheese production maximising cheese yield while minimising negative impact on beneficial lactococci, lactobacilli and enterococci; the predicted optimum milk HPP pre-treatment at 121 MPa/30 min resulted in a cheese yield increase of 8.0 % (CHAPTER 8).

There is only one study in the literature, as far as the authors are aware, that actually covers the HPP pre-treatment of milk, raw bovine milk in this case, at 300 - 600 MPa/10-30 min/40-45 ºC and subsequently uses the milk to produce cheese at large scale, having considered the processing conditions of 300 - 330 MPa/30 min/40–45 ºC as the most adequate to preserve the biochemical properties of milk. This study also reported that HPP led to a higher vitamin preservation and a positive influence in the organoleptic properties of the cheese (Sukmanov and Kiiko, 2016).

Envisaging cheese pasteurization, more intense HPP treatments (> 450 MPa/6 or 9 min and 600 MPa/6 min) were applied to ripened *Serra da Estrela* cheese produced from raw ewes' milk. The treatment at 450 MPa/6 min caused a minimal impact on microbial population with important metabolic activity to cheese (lactobacilli, lactococci, enterococci) (CHAPTER 3), while simultaneously inactivating considerably pathogenic microorganisms. On the other hand, the HPP treatment at 600 MPa/6 min kept the ripening extension index along the 500 days of refrigerated storage closer to that of control cheese at 45 days of ripening (CHAPTER 4).

Taking into account the above considerations, the combination of HPP on raw ewes' milk prior to large scale cheese manufacture in a certified dairy, followed by HPP of ripened cheese was studied. To the best of our knowledge this is the first study using such novel approach which may allow us to glean new insights into the impact of HPP on milk and cheese and simultaneously gain important features for *Serra da Estrela* cheese: a higher cheese yield, which is important from an economical point of view, alongside an improved microbial profile of the cheese, important from a safety and a quality points of view. Hence, in the present study, *Serra da Estrela* cheese was evaluated over a five month refrigerated storage period (equal novelty) in order to determine the effects of HPP milk pre-treatment and of HPP on the subsequently produced cheeses in terms of microbiota profiles, proteolysis, volatiles profile, texture and sensorial attributes.

## 9.2. Materials and methods

### 9.2.1. Milk supply and cheese manufacture

In March 2018, 165 litres of raw ewes' milk (from two farms in *Serra da Estrela* cheese PDO region) were collected, in the morning, pooled and transported to a certified artisanal dairy facility that produces commercial *Serra da Estrela* cheese, according to

the conditions set forth by the PDO legal procedure (Planning and Political Office, 2011). The bulk milk was kept in a refrigerated reservoir until use and prior to sampling, milk was well mixed to ensure sample homogeneity. The bulk milk was then divided into two batches: 82 L were used, in the same morning, to manufacture 35 cheeses (of about 500 g each) according to the PDO legal procedure (Macedo *et al.*, 1993). The remaining milk was packaged and processed by HPP in the afternoon of the same day. In the next morning, 77 litres of HPP pre-treated milk were used to produce the second batch of 34 cheeses following the conditions set forth in the PDO legal procedure. All 69 cheeses were ripened in the dairy during 60 days according to the PDO practices (Macedo *et al.*, 1993), to reach the optimum organoleptic level. The ripened cheeses were placed into low permeability polyamide-polyethylene (PA/PE) bags (Plásticos Macar – Indústria de Plásticos Lda, Santo Tirso, Portugal) and vacuum sealed (vacuum packaging machine HenkoVac E-193, Albipack, Águeda, Portugal) in order to be subsequently HPP treated. Transportation of the milk and cheeses between artisanal dairy.

### 9.2.2. High pressure processing

HPP treatments were performed in a 55-liter capacity industrial scale high pressure equipment (model 55, Hiperbaric, Burgos, Spain) at 121 MPa for 30 min for milk, while cheeses were subject to 525 MPa for 6 min (this condition was selected based on previous results obtained for *Serra da Estrela* cheese pasteurization, where minimal impact on microbial population with important metabolic activity to cheese, while simultaneously inactivating pathogenic microorganisms was envisaged (CHAPTER 3-6).The initial temperature of tap water (used as pressurization fluid) was 8 °C.

### 9.2.3. Samples identification and sampling

For the sake of easiness of samples identification by the reader, samples' abbreviations used in this work for the four types of cheeses produced are (Figure 9.1) shows the sample pipeline representation of milk HPP pre-treatment, cheese production and cheese HPP):

Untreated **M**ilk or milk **C**ontrol for the effect of HPP on milk: $\mathbf{M_C}$

**M**ilk pre-treated by H**P**P: $\mathbf{M_P}$

**Ch**eese **C**ontrol for the effect of H**P**P on cheese produced from untreated milk ($\mathbf{M_C}$): $\mathbf{M_C}$+$\mathbf{Ch_C}$

**Ch**eese **C**ontrol for the effect of H**P**P on cheese produced from milk pre-treated by HPP ($\mathbf{M_P}$): $\mathbf{M_P}$+$\mathbf{Ch_C}$

**Ch**eese pasteurized by H**P**P and produced from untreated milk ($\mathbf{M_C}$): $\mathbf{M_C}$+$\mathbf{Ch_P}$

**Ch**eese pasteurized by H**P**P and produced from milk pre-treated by HPP ($\mathbf{M_P}$): $\mathbf{M_P}$+$\mathbf{Ch_P}$

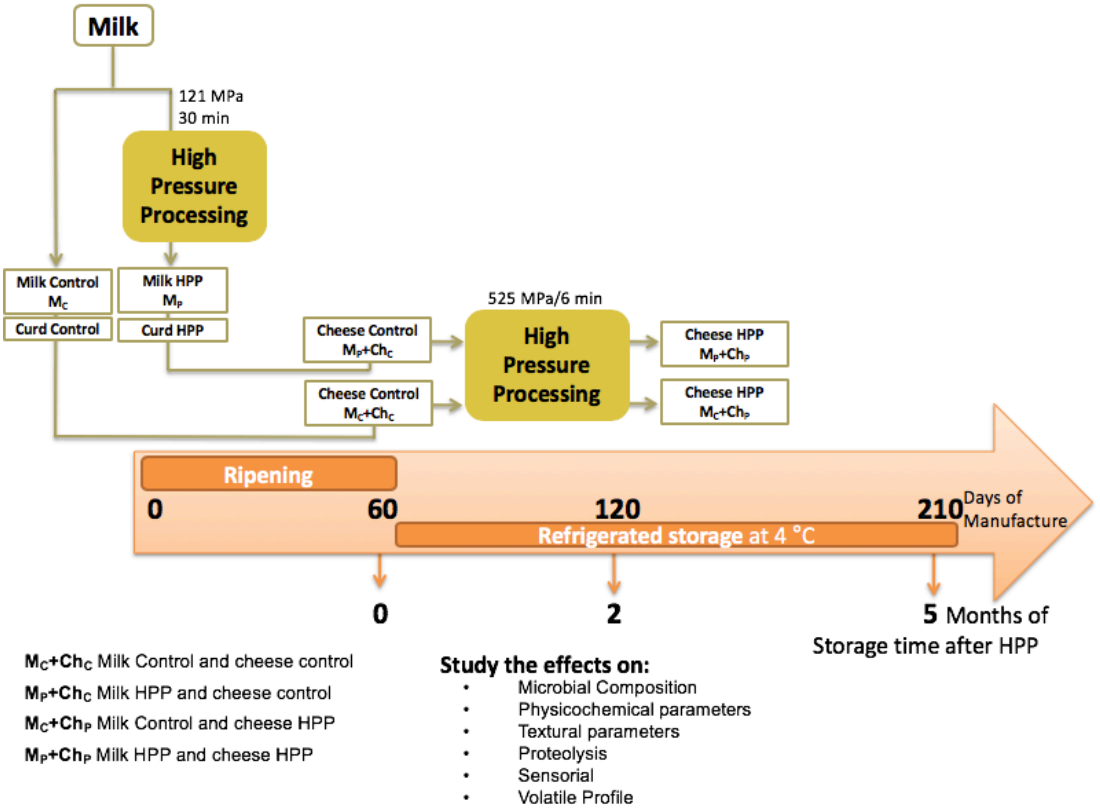


**Figure 9.1: Schematic representation of samples processing, cheese production, and samples identification using the abbreviations explained in 9.2.3.**

All chesses were kept under refrigeration conditions for a storage period of up to 5 months. Aliquots of each homogenised cheese (≈35 g per sample) were taken at 0, 2 and 5 months of storage for physicochemical characterization and were stored at −80 °C until analyses were performed.

### 9.2.4. Microbiological analyses

As previously described in section 6.2.4 Microbiological analyses.

Cheese samples with inoculated microorganisms were submitted to pre-enrichment in peptone water for *Salmonella enterica* and enrichment in Listeria fraser broth for *Listeria innocua*. *Salmonella* were monitored via plating on xylose lysine deoxycholate agar (XLD agar, Himedia, India) and *Listeria* on PALCAM agar with selective supplement for PALCAM (Liofilchem, Italy) and on CHROMagar Listeria with CHROMagar Listeria supplement (CHROMagar, France), both using the spread plate technique. Petri dishes containing 30-300 and 10–100 colony forming units (cfu) were selected for enumeration according to pour plate and Miles and Misra methods, respectively. The results were converted into logarithmic decimals of the number of cfu *per* g of cheese sample, and the values were considered below the limit of quantification of 2.0 log cfu/g for pour plate technique and 3.0 log cfu/g for Miles and Misra technique.

Cheese samples with inoculated microorganisms were submitted to pre-enrichment in peptone water for *S. enterica* and enrichment in Listeria fraser broth for *L. innocua*. Spread plate was used on xylose lysine deoxycholate agar (XLD agar, Himedia, India) for *Salmonella* and on PLACAM with selective supplement for PALCAM (Liofilchem, Italy) and CHROMagar Listeria with CHROMagar Listeria supplement (CHROMagar, France) for *Listeria*. The results were expressed in all cases as log of colony forming unit per cheese gram (cfu/g).

### 9.2.5. Microbial inoculation

*Staphylococcus aureus* ATCC 29213, *Listeria innocua* 2030c and *Salmonella enterica* serovar *enteritidis* ATCC 13076 from *Escola Superior de Biotecnologia da Universidade Católica Portuguesa* (Porto) were in early stationary growth phase when inoculated, according to Inácio *et al.,* (2014). An amount of about 2200 g of homogenised cheese paste was prepared from 4 $M_C$+$Ch_C$ cheeses (without rind) that was then divided into 2 aliquots. One aliquot was used as control samples (untreated and uninoculated) and the second was inoculated at 8.13, 6.48 and 5.17 log cfu/g cheese of *St. aureus*, *L. innocua* and *S. enterica*, respectively. About half the amount of the second aliquot was treated by HPP 525 MPa/6 min to see the effect of HPP and the other half used as control (untreated and inoculated).

### 9.2.6. Physicochemical analyses

As previously described in section 3.2.5 Physicochemical analyses.

### 9.2.7. Colour

As previously described in section 3.2.6 Colour.

### 9.2.8. Proteolysis

As previously described in section 4.2.2 Proteolytic indices.

### 9.2.9. Volatile compounds

Volatile compounds were assessed using solid-phase micro-extraction (SPME) of the headspace atmosphere. At each sampling point, 1 g of cheese was weighed in a headspace screw vial with Magnetic Precision-Thread Screw Caps and volume of 10 µL of an internal standard, octan-3-ol in methanol solution at 50.80 mg/L was added. After equilibration at 60 ºC, the SPME fibre containing a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre coating (Supelco, Bellefonte, PA) was introduced, and left for 1h to trap the volatiles. After this period, the fibre was introduced in the injector port and left to desorb the trapped volatiles for 15 min. The volatile components adsorbed from the SPME fibre were analysed using a Perkin-Elmer gas chromatograph (AutoSystem XL, Norwalk CT, USA) with a CP-Wax 58 FFRAP 50 m column (0.25 mm × 0.39 mm × 0.20 µm) from J & W Scientific (Folsom CA, USA). Data acquisition and analysis was accomplished using the TurbochromePC software from Perkin-Elmer. Identification was achieved via comparison of the mass spectra obtained for a sample containing a mixture of pure standards injected under the same conditions to the mass spectra of the NIST 98 MS library database. A semi quantification was performed for identified compounds based on pure standards data; other compounds detected were calculated from internal standard data.

### 9.2.10. Instrumental texture profile analysis (TPA)

As previously described in section 4.2.4 Instrumental texture profile analysis (TPA).

### 9.2.11. Sensory evaluation

Sensory sessions were carried out in the conditions previously described in section 4.2.5 Sensory evaluation. Panellists were asked to rate de intensity of the following attributes on the four cheeses, using a continuous anchored scale (0=non detectable, 10=strong): rind appearance (tonality, homogeneity and defects), paste appearance (colour and consistency), odour intensity (lactic, acid, animal/stable and short-chain fatty acids (SCFA)/vomit), texture (consistency, unctuosity and friability), taste intensity (salty, acid and bitter) and after-taste intensity. Description of the defects encountered were also recorded.

### 9.2.12. Statistical analyses

Analysis of variance (ANOVA) was performed to establish the effect of different processing conditions, the effect of storage and interactions on cheese outputs (microbiological, physicochemical and volatiles features). The significant difference Bonferroni's test was applied to compare the mean values of parameters, with the significance assigned at $p < 0.05$. Sensory data was analysed by one-way ANOVA and a Tukey's post-hoc test was applied to compare the mean values of attributes for each storage time. SPSS software version 24.0 was used for the statistical analysis.

## 9.3. Results and discussion

### 9.3.1. Changes in microbial composition induced by HPP milk pre-treatment and HPP of ripened cheeses

It has been well established that lactic acid bacteria (LAB) and *Enterobacteriaceae* are the predominant microbial groups in *Serra da Estrela* cheese; in general, viable numbers of the latter decrease, whereas those of lactobacilli, lactococci and enterococci increase

throughout ripening (Inácio *et al.*, 2014; Macedo *et al.*, 1993; Tavaria *et al.*, 2006; Tavaria and Malcata, 2000). The evolution with storage time of lactic acid bacteria, enterococci and total aerobic microorganisms in the four types of cheeses is depicted in Figure 9.2.

Indeed, lactobacilli, lactococci and enterococci were the predominant groups at 60 days of ripening in $M_C$+$Ch_C$ cheeses (control cheese) reaching viable cell numbers of 8.62, 9.10 and 8.31 log cfu/g, respectively (Figure 9.2 A-C), which are similar to those obtained in previous studies (Inácio *et al.*, 2014; Macedo *et al.*, 1993; Tavaria *et al.*, 2006; Tavaria and Malcata, 2000). No significant differences ($p > 0.05$) in viable cell numbers of these microbial groups were found between $M_C$+$Ch_C$ cheeses and those made with HPP pre-treated milk ($M_P$+$Ch_C$ cheeses). This is an important observation given the fact that these microbial groups are partly responsible for the biochemical changes that occur in cheese during ripening and need to be safeguarded against microbial loss. Similar results pertaining to maintenance of viable cell numbers of lactic acid bacteria independently of HPP pre-treatment of milk were observed by Voigt *et al.,* (2012) for 60-day old ripened Cheddar cheese, where both control and cheese made from HPP pre-treated milk (400 or 600 MPa/10 min) showed similar lactobacilli viable cell numbers since HPP treatment took place before starter inoculation. In their study on goat cheese, Buffa *et al.*, (2001b) also showed maintenance of enterococci viable numbers in both control and cheeses made from milk HPP pre-treated at 500 MPa/15 min, both manufactured with addition of starters, but lactobacilli viable cell numbers were 3 log cycles lower in the HPP pre-treated milk cheeses, which can reflect a higher number of lactobacilli in control milk.

On the other hand, HPP of ripened cheeses was determinant upon reduction of the viable cell numbers of these microbial groups, immediately after application ($p < 0.05$). $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses registered significant reductions of 1.39 and 1.95 log cycles

in lactococci viable cell numbers, of 2.19 and 3.03 log cycles in lactobacilli viable cell numbers ($p < 0.001$) and of 1.14 and 1.47 log cycles in enterococci viable cell numbers ($p < 0.05$) in comparison to $M_C$+$Ch_C$ and $M_P$+$Ch_C$, respectively. Similarly, 1.21-1.24 log cycle reductions in enterococci viable cell numbers were obtained in a previous study where *Serra da Estrela* cheeses were treated at 450 MPa/6 and 9 min, yet in this case higher reductions in lactococci and lactobacilli viable cell numbers (> 2.71 and > 3.20 log units, respectively) were observed (CHAPTER 3). On the other hand, a lower reduction in lactobacilli viable cell numbers (1.64 log cycles) was verified in *Torta del Casar* cheeses (also produced from raw ewes' milk) after HPP at 400 MPa/5 min at 35 days of ripening.

Over the 5 months of refrigerated storage, lactococci and lactobacilli viable cell numbers decreased steadily ($p < 0.05$) in all cheeses, exception for lactococci viable cell numbers ($p > 0.05$) in $M_C$+$Ch_C$ and $M_P$+$Ch_C$ cheeses, whereas enterococci maintained their viable cell numbers constant throughout storage ($p > 0.05$) in all four types of cheese. As expected, the trends observed for total aerobic microorganisms reflect those observed for lactococci, lactobacilli and enterococci (Figure 9.2 D). While no significant differences were observed between $M_C$+$Ch_C$ and $M_P$+$Ch_C$ cheeses (8.48 and 8.61 log cfu/g) ($p > 0.05$), similarly to that observed by Buffa *et al.*, (2001b) and to Molina *et al.*, (2000) (both added starts cultures), in the case of $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses the use of HPP on cheeses caused significant 1.11 and 1.51 log cycle reductions in viable cell numbers ($p < 0.01$). Rodríguez-Pinilla *et al.,* (2015) also reported small microbial load reductions (1.29 - 1.44) in total aerobic bacteria viable cell numbers in *Torta del Casar* cheese. However, a higher total aerobic bacteria decrease (2.4 log cycles) was achieved in a previous study on *Serra da Estrela* cheese at 45 days of ripening with HPP at 450 MPa for 6 and 9 min (CHAPTER 3). Along storage the total aerobic bacteria remained

stable ($p > 0.05$) in each cheese, except for a significant decrease of 0.68 log cycles ($p < 0.05$) observed in $M_P$+$Ch_P$ cheeses at 0 - 2 months of storage.

In what concerns yeasts and moulds, similar viable cell numbers were found in both $M_C$+$Ch_C$ and $M_P$+$Ch_C$ throughout the 5 months of storage, being close to 4 log cfu/g ($p > 0.05$) (data not shown). Yeasts and moulds, are reported to present lower resistance towards high pressure (Georget *et al.*, 2015), showing a reduction to below the quantification limit in $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses throughout the whole storage period, similarly to that reported by Rodríguez-Pinilla *et al.,* (2015).

The gram-negative bacteria *Enterobacteriaceae*, *Pseudomonas* spp. and *E. coli* also revealed low resistance to HPP, and corresponding viable cell numbers were reduced to below the quantification limit (data not shown) in $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses, whereas, these microorganisms were quantified in $M_C$+$Ch_C$ and $M_P$+$Ch_C$ cheeses (6.2, 5.2 and 5.6 log cfu/g, respectively); indeed, *Enterobacteriaceae* accounted for one of the major components of the microbiota in 60-d old ripened cheeses. Nonetheless, coliforms were quantified in $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses (7.3 and 7.1 log cfu/g, respectively) but without significant differences ($p > 0.05$) between the two types of cheeses, and viable cell numbers were 0.98 and 0.64 log cycles lower ($p < 0.05$) compared to the control and HPP pre-treated milk cheese ($M_C$+$Ch_C$ and $M_P$+$Ch_C$), respectively, similarly to previous observations (CHAPTER 3) for HPP at 450 MPa/6 min, but differently from the higher reductions reported by Arqués *et al.,* (2006) and Calzada *et al.,* (2013).

Viable cell numbers of *Staphylococcus* spp. were not affected by HPP milk pre-treatment ($p > 0.05$), but were reduced by about 1.5 log cycles to viable cell numbers below 5 log cfu/g when the cheeses were processed by HPP ($p < 0.05$). According to the European Commission (2005) the established limit for staphylococci in cheeses made from raw milk is of $10^5$ cfu/g.

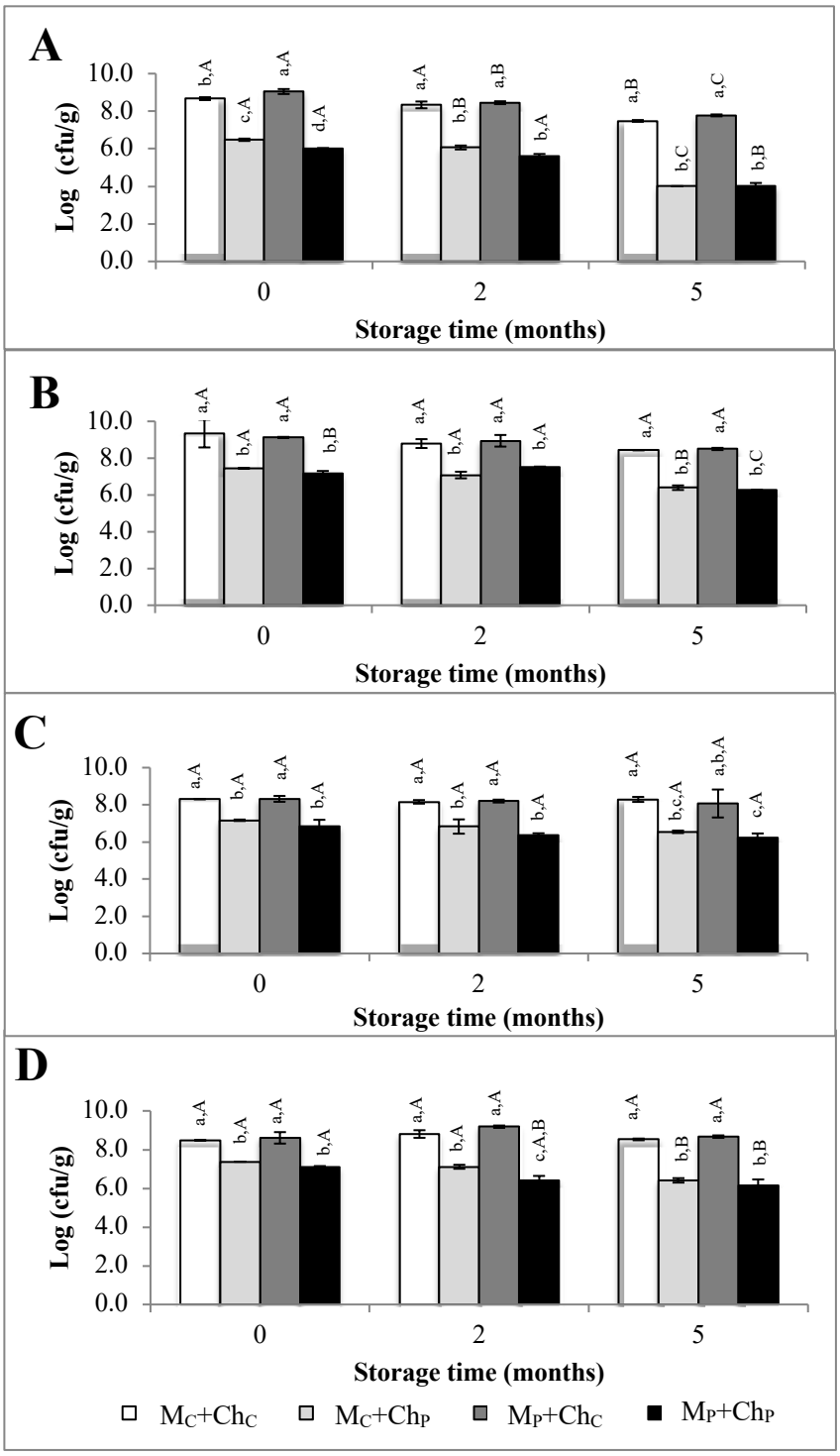


**Figure 9.2: (A) Evolution of (A) lactobacilli, (B) lactococci, (C) enterococci, (D) total aerobic microorganisms in *Serra da Estrela* cheese at 0, 2 and 5 months of refrigerated storage, produced from control raw ewes' milk ($M_C$) and HPP pre-treated milk ($M_P$), combined without ($Ch_C$) and with HPP in cheeses ($Ch_P$). Error bars represent standard deviations of the corresponding means. Different non-capital letters (a, b, c) indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) indicate statistically significant differences among the same experimental condition over storage ($p < 0.05$).**

### 9.3.2. Changes in inoculated microbiota

In order to better understand the impact of HPP on pathogenic organisms that may be found in raw milk cheeses a combined surrogate cocktail of *Staphylococcus aureus* ATCC 29213, *Listeria innocua* 2030c and *Salmonella enterica* serovar *enteritidis* ATCC 13076, more representative of the eventual competition between strains in a cheese environment, was inoculated in *Serra da Estrela* cheese paste and HPP was applied. As a gram-positive bacterium, *St. aureus* showed the highest resistance to HPP, and viable cell numbers were reduced by 2.93 log cycles (inoculated at the initial level of 8.13 log cfu/g). However, as storage progressed, viable cell numbers decreased, and by the 2$^{nd}$ and 5$^{th}$ months of storage, viable cell numbers of *Staphylococcus* in HPP cheese were found to be below the quantification limit (< 3 log cfu/g).

In the case of *L. innocua* and *S. enterica*, HPP led to a significant reduction in corresponding viable cell numbers to below the detection limit, with the enrichment procedure confirming the absence up to 5 months, while inoculated samples revealed a decrease in the viable cells numbers from 6.48 and 5.17 log cfu/g to < 3.00 log cfu/g, respectively. A similar effect was reported for *Listeria monocytogenes* strains NCTC 11994 and Scott A, inoculated in cheese and treated at 500 MPa/5 min (López-Pedemonte *et al.*, 2007). *Salmonella enteritidis* and *S. typhimurium* were also reduced in a model cheese samples (1.98 and 2.66 log) treated at 400 MPa/10 min, nevertheless they were capable of total recovery along refrigerated storage (15 days) (De Lamo-Castellví *et al.*, 2007).

### 9.3.3. Changes in physicochemical characteristics

Evolution of moisture, total protein content, pH and titratable acidity throughout storage of cheeses is shown in Table 9.1.

**Table 9.1: Moisture, protein content, pH values, titratable acidity measured in *Serra da Estrela* cheese at 0, 2 and 5 months of refrigerated produced from control milk ($M_C$) and HPP milk pre- treatment ($M_P$), combined without ($Ch_C$) and with the HPP in cheeses ($Ch_P$).**

| | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ |
|---|---|---|---|---|
| **Water Content** | **% (w/w) ± STD** | **% (w/w) ± STD** | **% (w/w) ± STD** | **% (w/w) ± STD** |
| 0 | 41.2 ± 0.86 $^{a,A}$ | 40.9 ± 0.25 $^{a,A}$ | 40.3 ± 0.99 $^{a,C}$ | 40.5 ± 1.41 $^{a,B}$ |
| 2 | 37.7 ± 1.71 $^{a,B}$ | 38.4 ± 1.02 $^{a,B}$ | 38.7 ± 0.42 $^{a,B}$ | 39.7 ± 1.47 $^{a,A}$ |
| 5 | 37.7 ± 1.60 $^{a,b,B}$ | 39.2 ± 1.76 $^{a,A,B}$ | 36.1 ± 0.95 $^{b,A}$ | 36.2 ± 2.01 $^{b,A}$ |
| **Protein Content** | **% (w/w) ± STD** | **% (w/w) ± STD** | **% (w/w) ± STD** | **% (w/w) ± STD** |
| 0 | 23.9 ± 0.36 $^{a,B}$ | 23.2 ± 1.29 $^{a,B}$ | 22.2 ± 0.47 $^{a,b,B}$ | 20.7 ± 0.10 $^{b,B}$ |
| 2 | 26.1 ± 0.52 $^{a,A}$ | 25.3 ± 0.66 $^{a,A}$ | 24.1 ± 0.07 $^{b,A}$ | 24.2 ± 0.52 $^{b,A}$ |
| 5 | 25.9 ± 0.21 $^{a,A}$ | 24.2 ± 0.64 $^{b,A,B}$ | 24.6 ± 0.17 $^{b,A}$ | 24.7 ± 0.96 $^{a,b,A}$ |
| **pH values** | **pH ± STD** | **pH ± STD** | **pH ± STD** | **pH ± STD** |
| 0 | 5.16 ± 0.01 $^{a,C}$ | 5.11 ± 0.01 $^{b,C}$ | 5.11 ± 0.03 $^{b,C}$ | 5.13 ± 0.03 $^{a,b,B}$ |
| 2 | 5.28 ± 0.01 $^{a,b,B}$ | 5.26 ± 0.02 $^{b,B}$ | 5.29 ± 0.01 $^{a,B}$ | 5.30 ± 0.02 $^{a,A}$ |
| 5 | 5.40 ± 0.03 $^{a,A}$ | 5.36 ± 0.02 $^{b,A}$ | 5.39 ± 0.02 $^{a,b,A}$ | 5.31 ± 0.01 $^{c,A}$ |
| **Titatrable acidity** | **$g_{lactic\ acid}$/100 g STD** | **$g_{lactic\ acid}$/100 g STD** | **$g_{lactic\ acid}$/100 g STD** | **$g_{lactic\ acid}$/100 g STD** |
| 0 | 1.38 ± 0.04 $^{a,b,C}$ | 1.41 ± 0.03 $^{a,B}$ | 1.31 ± 0.04 $^{b,C}$ | 1.45 ± 0.06 $^{a,B,B}$ |
| 2 | 1.49 ± 0.04 $^{b,B}$ | 1.47 ± 0.05 $^{b,B}$ | 1.71 ± 0.06 $^{a,B}$ | 1.63 ± 0.10 $^{a,B,B}$ |
| 5 | 1.72 ± 0.08 $^{c,A}$ | 1.75 ± 0.16 $^{c,A}$ | 2.38 ± 0.06 $^{a,A}$ | 2.10 ± 0.19 $^{b,A}$ |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

Recall that the four cheese types were manufactured from untreated or HPP pre-treated raw ewe's milk which is characterized by high protein content. The gross composition of $M_C$+$Ch_C$ cheeses at 0 months storage (60 day old ripened cheeses) presented in Table 9.1 for moisture (41.2 % (w/w)) and protein content (23.9 % (w/w)) is, in general, in agreement with the range of values reported in literature for both moisture (40.2-48.4 %) and protein (14.5-19.9 %) contents (Correia *et al.*, 2016). No significant differences ($p > 0.05$) in moisture content were found between $M_C$+$Ch_C$ cheeses and those made with HPP pre-treated milk ($M_P$+$Ch_C$ cheeses) or HPP treated upon ripening ($M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses) at the beginning and throughout 5 months of storage; note that the moisture content decreased slightly ($p < 0.05$) over storage. Similarly, protein content did not differ among the different cheeses, exception for a significantly lower protein content registered for $M_P$+$Ch_P$ cheeses at the beginning of storage ($p < 0.05$); nevertheless, such difference was dissipated by 5 months storage ($p > 0.05$) in relation to $M_C$+$Ch_C$ cheeses (Table 9.1). Several studies on goat (Buffa *et al.*, 2001b; Saldo *et al.*, 2000), cow (Molina *et al.*, 2000) and Cheddar cheese (San Martín-González *et al.*, 2007) reported similar trends for moisture and protein contents in control and cheeses made with HPP pre-treated milk (at 50 MPa/72 h, 500 MPa/15 min, 400 MPa/15 min and 483 MPa/5 min, respectively).

The four cheese types were characterized by different pH patterns (Table 9.1), in which HPP pre-treatment of milk or HPP application to ripened cheeses resulted in lower pH values, unlike the control; indeed, $M_C$+$Ch_C$ cheeses revealed higher pH values throughout 5 months storage (from 5.16 to 5.40), being only different from pH values of $M_P$+$Ch_C$ cheeses at the beginning of storage ($p < 0.05$), of $M_P$+$Ch_P$ at 5$^{th}$ month of storage ($p < 0.001$) and along storage for $M_C$+$Ch_P$ cheeses ($p < 0.01$). A significant increase in pH values ($p < 0.05$) was verified over the 5 months of storage, with exception of $M_P$+$Ch_P$

cheeses where pH values stabilized during the last 3 months of storage (Table 9.1). Similarly, higher pH values were observed for control Cheddar cheeses compared to cheeses made from pre-treated milk (400 MPa/10 min) (Voigt *et al.*, 2012), while goat and bovine cheeses revealed no significant differences between control and cheese made with milk pre-treated at 50 MPa/2 h (Saldo *et al.*, 2002) and 586 MPa/1 min (Drake *et al.*, 1997), respectively. An opposite behaviour was observed for goat milk cheese (Buffa *et al*., 2001b; Saldo *et al*., 2000; Trujillo *et al*., 2002a). In agreement with pH values, $M_C$+$Ch_C$ cheeses revealed, in general, lower titratable acidity (TA) values, which were comparable to those of $M_C$+$Ch_P$ cheeses ($p > 0.05$) (Table 9.1). On the other hand, HPP milk pre-treatment affected cheese titratable acidity, not at month 0 ($p > 0.05$), but during storage leading to a significant increase in TA ($p < 0.001$).

### 9.3.4. Changes in proteolytic indices

Evolution of proteolysis in terms of ripening extension, ripening depth and free amino acid indices, throughout storage time, for all four cheese types is presented in Figure 9.3. The proteolytic indices reported for $M_C$+$Ch_C$ cheeses at 60 days of ripening (0 months storage) were consistent with previous studies pertaining to *Serra da Estrela*, exception for the ripening extension index (23 %, Figure 9.3 A) which was slightly lower (about 27 to 40 % reported in previous studies) (Macedo and Malcata, 1997a, 1997c; Tavaria *et al*., 2003), possibly due to seasonal effects. HPP milk pre-treatment led to a significant increase of the ripening extension index to 27 % ($p < 0.01$) in $M_P$+$Ch_C$ and $M_P$+$Ch_P$ cheeses relatively to $M_C$+$Ch_C$ cheeses, reflecting an increase of the WSN fraction. This fraction has a heterogeneous composition that includes whey proteins, and high-, medium- and low-molecular weight peptides, as well as free amino acids (FAA) (Tavaria *et al.*, 2003). Studies performed on Cheddar cheese (Voigt *et al.*, 2012); and on

goat cheese (Trujillo *et al*., 2002a); made from pre-treated milk at 400 MPa/10 min and 500 MPa/15 min, respectively also reported a higher WSN/TN ratio; on the other hand, no differences in the ripening extension index were observed between raw goats' milk cheese and the same cheese made from pre-treated milk at 50 MPa/72 h (Saldo *et al.*, 2000) and at 500 MPa/15 min (Buffa *et al.*, 2005). Interestingly, HPP use on untreated milk cheeses ($M_C$+$Ch_P$) had no impact on the ripening extension index ($p > 0.05$) registering values similar to those of $M_C$+$Ch_C$ cheeses at 0 months and maintaining these during 2 months of storage ($p > 0.05$) (Figure 9.3 A); differences in WSN/TN ratios between cheeses were dissipated by 5 months of storage ($p > 0.05$).

HPP caused different impacts on the ripening depth index dependent on application condition; a single application, either in milk ($M_P$+$Ch_C$ cheeses) or cheese ($M_C$+$Ch_P$ cheeses), did not significantly affect the TCA/TN ratio in comparison to that of $M_C$+$Ch_C$ cheeses registering values between 4-6% ($p > 0.05$), while a coupled application in both milk and ripened cheese ($M_P$+$Ch_P$ cheeses) provoked a significant increase of this proteolytic index to 7-9 % *(*$p < 0.001$) (Figure 9.3 B). The TCA fraction expresses the small peptides (with a chain length among 2 and 20 amino acids residues) and FAA and was found naturally in higher amounts in $M_P$+$Ch_P$ cheeses. *Ibores* cheese manufactured from raw goats' milk and HPP treated at 50 days ripening (400 and 600 MPa/7 min) revealed a higher TCA/TN ratio than control cheeses, possibly due to intracellular release of proteinases/peptidases (Delgado *et al*., 2012).

A similar free amino acid index (~ 2.6%) was determined for all cheeses at 0 month ($p > 0.05$) (Figure 9.3 C); this trend was maintained throughout storage except for $M_C$+$Ch_C$ cheeses which registered higher values (~4.3 %) by 5 months of storage.

Overall, the HPP at 525 MPa/6 min of *Serra da Estrela* cheeses can keep the ripening extension, depth and free amino acid indices closer to those of control cheeses,

while the HPP milk pre-treatment at 121 MPa/30 min leads to an increase of the ripening extension index.

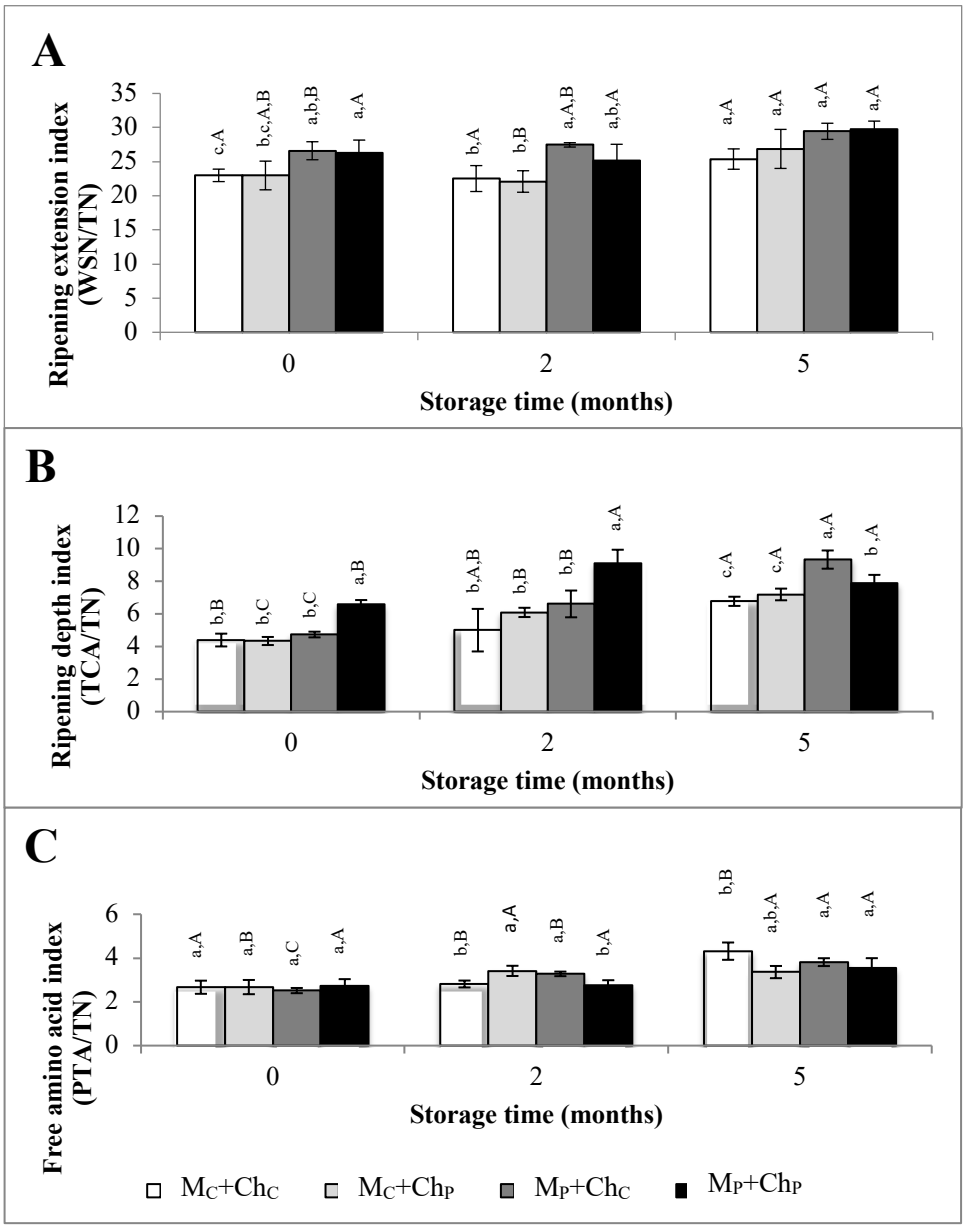


**Figure 9.3: Evolution of (A) ripening extension index (WSN/TN), (B) TCA/TN as ripening depth index and (C) PTA/TN as free amino acid index of *Serra da Estrela* cheese produced from control raw ewes' milk ($M_C$) and HPP pre-treated milk ($M_P$), combined without ($Ch_C$) and with HPP in cheeses ($Ch_P$) at 0, 2 and 5 months of storage. Error bars represent standard deviations of the corresponding means. Different non-capital letters (a, b, c) indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) indicate statistically significant differences among the same experimental condition over storage ($p < 0.05$).**

### 9.3.5. Changes in colour

HPP milk pre-treatment did not significantly affect the *L** and *b** colour parameters in cheese surface and core when compared with $M_C$+$Ch_C$ cheeses along the 5 months of storage ($p > 0.05$) (Table A. 10.2). Similarly, Voigt *et al.,* (2012) did not find differences in *L**, *b** and *a** parameters between control Cheddar and that made from milk pre-treated at 400 MPa/10 min. Furthermore, HPP application on ripened cheeses ($M_C$+$Ch_P$) did not affect the *L** of cheese surface and core comparatively to $M_C$+$Ch_C$ cheeses ($p > 0.05$); but led to a significant decrease ($p < 0.01$) in yellowness of cheese surface at month 0, but without differences at month 5 ($p > 0.05$) (Table A. 10.2). Along storage the cheese core of $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses were revealed to be yellower (*b** 19 - 22) than those of $M_C$+$Ch_C$ and $M_P$+$Ch_C$ cheeses (*b** 15 - 18). In general, $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses revealed higher chroma (*C**), an indicator of higher intensity in cheese core and surface colour than $M_C$+$Ch_C$ cheeses.

### 9.3.6. Changes in textural properties

Changes in the textural properties as obtained via texture profile analysis of the different cheeses throughout storage, are depicted in Table 9.2. In general, the textural parameters (hardness, consistency, adhesiveness, cohesiveness and gumminess) of $M_C$+$Ch_P$, $M_P$+$Ch_C$ and $M_P$+$Ch_P$ cheeses showed no significant differences compared to those of $M_C$+$Ch_C$ cheeses at 0 months and after 5 months of refrigerated storage ($p > 0.05$) (Table 9.2). In the case of raw bovine cheeses, results showed no significant differences in hardness and gumminess in comparison to cheeses made from milk pre-treated at 400 or 600 MPa/10 min and 400 MPa/15 min at 60 days of ripening (Molina *et al.*, 2000; Voigt *et al.*, 2012).

**Table 9.2: Evolution of textural properties of *Serra da Estrela* cheese produced from control milk ($M_C$) and HPP pre-treated milk ($M_P$), combined without ($M_C$+$Ch_C$ and $M_P$+$Ch_C$) and with the HPP of 60-day old ripened cheeses ($M_C$+$Ch_P$ and $M_P$+$Ch_P$) at 0, 2 and 5 months of storage.**

| Property | Storage time (months) | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ |
|---|---|---|---|---|---|
| **Hardness (N)** | 0 | 0.41 ± 0.11 $^{a,b,B}$ | 0.50 ± 0.08 $^{a,A}$ | 0.40 ± 0.03 $^{a,b,B}$ | 0.33 ± 0.05 $^{b,B}$ |
| | 2 | 0.67 ± 0.09 $^{a,A}$ | 0.49 ± 0.08 $^{b,A}$ | 0.32 ± 0.02 $^{c,B}$ | 0.35 ± 0.04 $^{c,B}$ |
| | 5 | 0.67 ± 0.15 $^{a,A}$ | 0.60 ± 0.11 $^{a,A}$ | 0.54 ± 0.10 $^{a,A}$ | 0.58 ± 0.07 $^{a,A}$ |
| **Consistency (N/s)** | 0 | 2.8 ± 1.0 $^{a,b,B}$ | 3.9 ± 0.74 $^{a,A}$ | 2.4 ± 1.0 $^{b,B}$ | 2.1 ± 0.35 $^{b,B}$ |
| | 2 | 6.0 ± 1.5 $^{a,A}$ | 3.5 ± 1.3 $^{b,A}$ | 1.0 ± 0.4 $^{c,C}$ | 2.5 ± 0.54 $^{b,c,B}$ |
| | 5 | 5.2 ± 1.2 $^{a,A}$ | 4.7 ± 1.1 $^{a,A}$ | 4.5 ± 1.1 $^{a,A}$ | 4.4 ± 0.64 $^{a,A}$ |
| **Adhesiveness (N/s)** | 0 | 0.52 ± 0.35 $^{a,A}$ | 1.2 ± 0.11 $^{a,A}$ | 0.82 ± 0.59 $^{a,A}$ | 0.50 ± 0.35 $^{a,A}$ |
| | 2 | 0.38 ± 0.23 $^{a,A}$ | 0.70 ± 0.48 $^{a,A}$ | 0.28 ± 0.27 $^{a,A}$ | 0.84 ± 0.20 $^{b,B}$ |
| | 5 | 2.5 ± 0.76 $^{a,B}$ | 2.1 ± 0.53 $^{a,B}$ | 2.5 ± 0.65 $^{a,B}$ | 2.6 ± 0.44 $^{a,B}$ |
| **Cohesiveness (dimensionless)** | 0 | 0.88 ± 0.21 $^{a,A}$ | 0.70 ± 0.03 $^{a,A}$ | 0.76 ± 0.16 $^{a,A}$ | 0.82 ± 0.12 $^{a,A}$ |
| | 2 | 0.49 ± 0.13 $^{c,B}$ | 0.78 ± 0.08 $^{a,b,A}$ | 0.91 ± 0.20 $^{a,A}$ | 0.71 ± 0.07 $^{b,A}$ |
| | 5 | 0.76 ± 0.09 $^{a,A}$ | 0.73 ± 0.07 $^{a,A}$ | 0.78 ± 0.06 $^{a,A}$ | 0.78 ± 0.06 $^{a,A}$ |
| **Gumminess (N)** | 0 | 0.37 ± 0.10 $^{a,B}$ | 0.36 ± 0.06 $^{a,A}$ | 0.32 ± 0.03 $^{a,B}$ | 0.27 ± 0.06 $^{a,B}$ |
| | 2 | 0.32 ± 0.06 $^{a,b,B}$ | 0.38 ± 0.06 $^{a,A}$ | 0.30 ± 0.06 $^{b,B}$ | 0.25 ± 0.02 $^{b,B}$ |
| | 5 | 0.50 ± 0.10 $^{a,A}$ | 0.44 ± 0.08 $^{a,A}$ | 0.42 ± 0.08 $^{a,A}$ | 0.45 ± 0.04 $^{a,A}$ |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

### 9.3.7. Changes in volatiles profile

The mean composition of the various compounds in the volatile fraction throughout the 5 month storage period, grouped by chemical families and pertaining to the four cheese types are listed in Table 9.3. Overall, the volatile profile of the different *Serra da Estrela* cheeses is in agreement with the results already reported by Tavaria, Silva Ferreira, and Malcata, (2004).

**Table 9.3: Semi quantitative volatiles analysis of *Serra da Estrela* cheese produced from control raw ewes' milk ($M_C$) and HPP pre-treated milk ($M_P$), combined without ($M_C$+$Ch_C$ and $M_P$+$Ch_C$) and with the HPP in 60-day old ripened cheeses ($M_C$+$Ch_P$ and $M_P$+$Ch_P$) at 0, 2 and 5 months of storage.**

| Storage time (months) | | | 0 | | | | 2 | | | | 5 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RT# (min) | Ion | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ |
| **Ethyl esters** | | | | | | | | | | | | | | |
| Ethyl acetate | 5.398 | 88 | 0.57±0.27 | 0.93±0.1 | 0.62±0.23 | 0.59±0.1 | 1.5±0.63 | 1.01±0.18 | 0.66±0.46 | 0.70±0.13 | 1.65±0.69 | 1.11±0.48 | 1.54±0.64 | 1.78±0.92 |
| Ethyl butanoate | 8.352 | 88 | 7.14±3.65 | 11.89±2.23 | 7.06±1.55 | 8.77±1.95 | 29.02±9.07 | 22.81±1.52 | 13.27±1.79 | 13.31±2.4 | 45.87±12.95 | 38.61±12.58 | 37.08±23.76 | 39.53±16.75 |
| Ethyl isovalerate | 9.243 | 88 | 0.52±0.17 | 0.72±0.13 | 0.86±0.21 | 0.75±0.15 | 1.38±0.14 | 1.22±0.11 | 1.5±0.22 | 1.18±0.18 | 1.39±0.2 | 1.17±0.18 | 1.79±0.40 | 1.71±0.24 |
| Ethyl valerate/Ethyl pentanoate | 11.257 | 88 | 1.22±0.58 | 1.95±0.19 | 1.63±0.31 | 0.83±0.18 | 3.54±0.8 | 3.44±0.53 | 2.18±0.13 | 2.34±0.23 | 6.06±0.96 | 5.89±2.04 | 6.11±1.84 | 5.26±1.61 |
| Ethyl hexanoate | 14.444 | 88 | 0.40±0.19 | 0.82±0.16 | 0.74±0.15 | 0.53±0.07 | 1.76±0.54 | 1.76±0.19 | 0.99±0.13 | 1.03±0.24 | 4.15±1.01 | 3.52±1.45 | 4.53±1.52 | 3.75±1.13 |
| Ethyl octanoate | 21.169 | 88 | 0.02±0.01 | 0.04±0.01 | 0.05±0.02 | 0.03±0.01 | 0.07±0.02 | 0.08±0.01 | 0.06±0.01 | 0.05±0.01 | 0.2±0.02 | 0.16±0.08 | 0.23±0.06 | 0.18±0.06 |
| Ethyl decanoate | 27.309 | 88 | 0.01±0.00 | 0.01±0.00 | 0.02±0.01 | 0.01±0.00 | 0.02±0.00 | 0.02±0.00 | 0.02±0.00 | 0.02±0.01 | 0.03±0.01 | 0.03±0.01 | 0.03±0.02 | 0.05±0.02 |
| Ethyl laurate/dodecanoate | 33.03 | 88 | 0.01±0.00 | 0.01±0.01 | 0.01±0.01 | 0.01±0.00 | 0.02±0.00 | 0.02±0.01 | 0.01±0.01 | 0.04±0.01 | 0.06±0.01 | 0.07±0.02 | 0.06±0.02 | 0.06±0.01 |
| Total | | | 9.88 | 16.38 | 10.98 | 11.52 | 37.31 | 30.36 | 18.68 | 18.66 | 59.41 | 50.55 | 51.37 | 52.31 |
| **Ketones** | | | | | | | | | | | | | | |
| 2-Heptanone | 12.777 | 58 | 1.35±0.65 | 3.31±2.34 | 5.01±0.77 | 3.05±0.71 | 2.44±1.11 | 9.51±2.78 | 1.64±0.28 | 4.22±4.33 | 1.51±0.58 | 11.16±2.3 | 2.32±1.02 | 11.37±1.39 |
| 2-Octanone | 16.264 | 58 | 0.02±0.01 | 0.09±0.08 | 0.10±0.02 | 0.07±0.02 | 0.07±0.03 | 0.36±0.14 | 0.06±0.02 | 0.44±0.39 | 0.08±0.04 | 0.58±0.14 | 0.16±0.07 | 0.43±0.07 |
| 2-Nonanone | 19.715 | 58 | 0.06±0.03 | 0.29±0.25 | 0.41±0.09 | 0.24±0.06 | 0.30±0.08 | 1.29±0.57 | 0.28±0.13 | 0.19±0.29 | 0.69±0.21 | 2.73±1.00 | 1.17±0.46 | 2.02±0.33 |
| 2-Undecanone | 26.223 | 58 | 0.02±0.02 | 0.40±0.29 | 0.53±0.19 | 0.29±0.11 | 0.28±0.07 | 0.68±0.24 | 0.37±0.19 | 0.21±0.05 | 1.11±0.32 | 1.97±1.19 | 3.23±0.87 | 2.83±0.35 |
| Total | | | 1.45 | 4.09 | 6.05 | 3.66 | 3.09 | 11.84 | 2.34 | 5.06 | 3.38 | 16.45 | 6.87 | 16.65 |
| **Phenolic Compounds** | | | | | | | | | | | | | | |
| Benzoic acid | 47.078 | 105 | 0.01±0.01 | 0.03±0 | 0.04±0 | 0.04±0.01 | 0.02±0.01 | 0.03±0 | 0.03±0.01 | 0.04±0.01 | 0.03±0 | 0.04±0.01 | 0.04±0.02 | 0.05±0.01 |
| **Alcohols** | | | | | | | | | | | | | | |
| Ethanol | 7.98 | 45 | 60.65±16.42 | 94.28±48.91 | 51.32±11.01 | 51.65±9.08 | 65.21±58.36 | 88.68±19.98 | 63.97±10.26 | 53.4±13.87 | 92.94±37.0 | 71.41±14.67 | 98.48±30.15 | 97.74±41.13 |
| 1-Butanol, 3-methyl-, acetate | 10.877 | 70 | 0.38±0.17 | 0.61±0.09 | 0.48±0.11 | 0.44±0.08 | 0.82±0.13 | 0.79±0.04 | 0.56±0.08 | 0.53±0.09 | 0.70±0.16 | 0.77±0.19 | 0.68±0.16 | 0.82±0.24 |
| 1-Butanol, 3-methyl- | 17.152 | 70 | 4.96±1.77 | 5.73±1.38 | 7.57±1.73 | 6.64±1.32 | 6.57±1.18 | 6.8±0.51 | 6.85±1.08 | 5.97±0.83 | 4.77±1.02 | 5.18±0.70 | 5.19±1.26 | 6.32±0.77 |
| 3-Heptanol, 3-methyl- | 20.075 | 73 | 0.16±0.12 | 0.24±0.11 | 0.17±0.05 | 0.12±0.04 | 0.24±0.06 | 0.19±0.05 | 0.14±0.02 | 0.19±0.05 | 0.21±0.04 | 0.19±0.05 | 0.13±0.05 | 0.19±0.02 |
| Phenol | 37.397 | 94 | 0.01±0.00 | 0.01±0.00 | 0.01±0.00 | 0.01±0.00 | 0.02±0.00 | 0.02±0.00 | 0.01±0.00 | 0.01±0.00 | 0.02±0.01 | 0.02±0.00 | 0.02±0.01 | 0.02±0 |
| Phenylethyl Alcohol | 39.284 | 91 | 0.16±0.05 | 0.51±0.08 | 0.76±0.05 | 0.63±0.23 | 0.38±0.05 | 0.68±0.11 | 0.69±0.17 | 0.76±0.10 | 0.48±0.03 | 0.52±0.23 | 0.75±0.21 | 0.7±0.12 |
| Total | | | 66.41 | 101.49 | 60.41 | 59.58 | 73.34 | 97.28 | 72.31 | 60.97 | 99.19 | 78.16 | 105.34 | 105.89 |
| **Aldehydes** | | | | | | | | | | | | | | |
| Acetaldehyde | 3.926 | 44 | 72.02±34.97 | 4.36±0.89 | 275.55±43.99 | 33.00±10.14 | 131.25±30.62 | 4.62±0.55 | 232.41±33.36 | 44.96±9.93 | 77.23±39.83 | 26.03±13.53 | 121.46±52.1 | 34.88±5.15 |
| **Sulfur derivates** | | | | | | | | | | | | | | |
| Dimethylsulfone | 34.875 | 79 | 2.07±0.28 | 3.89±0.9 | 2.2±1.53 | 2.27±0.47 | 1.93±1.03 | 4.87±1.17 | 2.07±1.43 | 4.62±2.41 | 2.99±0.74 | 3.42±1.38 | 2.81±1.55 | 3.30±0.54 |
| **Free Fatty Acids** | | | | | | | | | | | | | | |
| Acetic acid (C2) | 21.791 | 60 | 2.79±1.31 | 4.44±0.49 | 3.36±1.39 | 1.49±0.52 | 3.74±0.91 | 4.06±0.42 | 2.57±0.63 | 2.93±0.63 | 2.83±0.87 | 3.19±1.21 | 2.99±1.37 | 3.74±1.64 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isobutyric acid (iC4) | 25.259 | 60 | 0.22±0.16 | 0.36±0.07 | 0.35±0.15 | 0.36±0.16 | 0.27±0.03 | 0.44±0.03 | 0.35±0.07 | 0.48±0.11 | 0.31±0.09 | 0.35±0.03 | 0.29±0.15 | 0.44±0.09 |
| Butyric acid (C4) | 27.076 | 60 | 88.66±55.91 | 166.39±48.35 | 114.95±45.89 | 121.11±51.59 | 153.12±33.09 | 332.48±70.43 | 147.58±46.98 | 332.69±62.02 | 348.69±68.78 | 450.11±124.26 | 346.43±101.74 | 444.25±88.66 |
| Isovaleric acid (iC5) | 28.215 | 60 | 90.18±29.95 | 175.98±13.21 | 183.49±116.54 | 212.96±97.09 | 116.71±23.51 | 229.54±29.38 | 203.73±49.44 | 316.73±61.25 | 137.3±19.45 | 157.61±17.2 | 155.79±46.28 | 226±61.83 |
| Valeric acid (C5) | 30.17 | 60 | 0.92±0.41 | 1.85±0.35 | 1.45±0.40 | 1.36±0.58 | 1.23±0.55 | 2.46±0.69 | 1.39±0.41 | 3.07±0.68 | 3.91±0.68 | 4.46±1.07 | 3.57±0.94 | 3.77±0.6 |
| Caproic acid (C6) | 33.038 | 60 | 4.48±2.18 | 11.37±2.53 | 10.31±4.20 | 7.22±2.73 | 11.53±1.13 | 25.94±7.41 | 10.59±4.67 | 30.12±5.78 | 44.13±8.09 | 56.2±14.16 | 43.48±11.61 | 49.9±7.51 |
| Heptanoic acid (C7) | 35.77 | 60 | 0.32±0.14 | 0.58±0.13 | 0.62±0.32 | 0.51±0.18 | 0.44±0.06 | 0.83±0.28 | 0.40±0.16 | 0.87±0.17 | 1.63±0.30 | 1.91±0.62 | 1.44±0.32 | 1.36±0.24 |
| Undecanoic acid (C11) | 35.886 | 60 | 25.7±12.3 | 48.97±11.88 | * | * | 39.48±3.38 | * | * | * | * | * | * | * |
| Caprylic acid (C8) | 38.392 | 60 | 2.03±0.87 | 5.4±1.39 | 8.59±4.96 | 5.37±2.26 | 4.00±0.59 | 9.94±3.63 | 4.84±1.94 | 9.34±1.68 | 14.66±2.07 | 21.09±7.63 | 13.68±7.59 | 14.89±3.67 |
| Nonanoic acid (C9) | 40.898 | 60 | 0.06±0.03 | 0.07±0.02 | 0.06±0.02 | 0.04±0.02 | 0.03±0.01 | 0.06±0.02 | 0.04±0.01 | 0.05±0.01 | 0.09±0.02 | 0.09±0.04 | 0.07±0.02 | 0.06±0.01 |
| Capric acid (C10) | 43.276 | 60 | 28.47±9.86 | 122.56±35.3 | 237.29±118.3 | 175.53±77.73 | 55.93±16.88 | 184.31±62.52 | 180.22±95.9 | 280.11±33.34 | 225.91±41.51 | 364.4±172.85 | 401.9±78.83 | 402.85±68.75 |
| Lauric acid (C12) | 47.72 | 60 | 0.31±0.15 | 0.42±0.25 | 0.49±0.24 | 0.51±0.33 | 0.31±0.11 | 0.45±0.18 | 0.51±0.18 | 0.60±0.15 | 0.58±0.13 | 0.68±0.29 | 1.10±0.27 | 0.97±0.30 |
| Total | | | 244.13 | 538.41 | 560.95 | 526.46 | 386.79 | 790.5 | 552.22 | 976.99 | 780.05 | 1060.09 | 970.74 | 1148.24 |
| **Other** | | | | | | | | | | | | | | |
| Caprolactam[§] | 37.567 | 113 | 0.001±0.000 | 0.002±0.000 | 0.002±0.000 | 0.003±0.001 | 0.002±0.000 | 0.003±0.000 | 0.002±0.000 | 0.003±0.001 | 0.002±0.001 | 0.002±0.001 | 0.003±0.000 | 0.003±0.001 |
| Butyrolactone | 27.472 | 86 | 0.004±0.001 | 0.001±0000 | 0.017±0.006 | * | 0.012±0.002 | 0.003±0.001 | * | 0.024±0.005 | 0.001±0.000 | 0.003±0.002 | * | 0.006±0.004 |
| Dehydromevalonic lactone | 37.975 | 82 | 0.008±0.002 | 0.019±0.008 | 0.01±0.002 | 0.005±0.001 | 0.010±0.003 | 0.025±0.006 | 0.015±0.003 | 0.012±0.005 | 0.031±0.004 | 0.028±0.011 | 0.024±0.008 | 0.032±0.009 |
| δ-Nonalactone | 41.727 | 99 | 0.003±0.002 | 0.009±0.002 | 0.013±0.002 | 0.012±0.003 | 0.008±0.002 | 0.014±0.001 | 0.014±0.002 | 0.017±0.003 | 0.009±0.001 | 0.015±0.003 | 0.018±0.003 | 0.019±0.003 |
| δ-lactone | 36.423 | 99 | 0.002±0.001 | 0.004±0.001 | 0.006±0.001 | 0.005±0.001 | 0.003±0.001 | 0.004±0.000 | 0.005±0.001 | 0.005±0.001 | 0.002±0.001 | 0.003±0.000 | 0.005±0.001 | 0.006±0.001 |
| Total | | | 0.04 | 0.07 | 0.27 | 0.07 | 0.19 | 0.13 | 0.31 | 0.20 | 0.14 | 0.19 | 0.30 | 0.23 |

# means retention time; * means below the limit of quantification; Levels are expressed in picograms relative to the standard per cheese gram, as mean±STD of triplicate determinations from two different cheeses; [§] indicated in the literature as arising from migration from the packaging material (AFSSA, 2009).

All identified ethyl ethers were affected by the storage time ($p < 0.001$), but only ethyl isovalerate and ethyl laurate were significantly affected by HPP ($p < 0.05$). In general, esters contribute to a pleasant cheese flavour, leading to sweet, fruity and floral notes and mask the sharpness and bitterness of other compounds (Calzada *et al*., 2014b). Volatile ketones increased significantly in $M_C$+$Ch_P$ cheeses ($p < 0.001$) along storage, while in $M_C$+$Ch_C$ cheeses they were essentially maintained ($p > 0.05$).

The dominant volatile fraction in the experimental cheeses were the volatile free fatty acids (FFA) both quantitatively and qualitatively; similar results were obtained by Tavaria *et al* (2004) and FFA up to 12 carbon atoms have been often detected in ewe's cheeses (Pinho *et al.*, 2003). Several volatile free fatty acids (FFA) are produced from lipolysis, be biosynthesized from acetyl CoA, appear as a result of microbial metabolism of amino acids (e.g. branched chain isovaleric acid) or lactose and lactic acid (e.g. acetic and butyric acids), with some FFA arising from hydrolysis of milk triglycerides during cheese ripening (Ganesan and Weimer, 2017). The total concentration of all FFAs increased between 0 and 5 months storage ($p < 0.05$) for all cheese types, reaching higher values at 5 months storage in $M_P$+$Ch_P$ and $M_C$+$Ch_P$ cheeses than in $M_C$+$Ch_C$ cheeses (Table 9.3); individually, the most abundant FFA were isovaleric, butyric, capric and caproic acids. Butyric acid may be generated by lactococci via lactose fermentation and by lipolysis, being associated to rancid and acid flavours (Ganesan and Weimer, 2017). A higher content of butyric acid was verified in $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses (332 μg/g) at 2 months compared to $M_C$+$Ch_C$ cheeses (153 μg/g) ($p < 0.001$), which revealed lower lactococci viable cell numbers. $M_P$+$Ch_C$ and $M_P$+$Ch_P$ cheeses showed significantly higher capric acid amounts, ranging from 175 – 403 μg/g, compared to $M_C$+$Ch_C$ cheeses that varied from 28-226 μg/g along storage ($p < 0.05$).

In terms of less predominant volatile free fatty acids, acetic acid which can also serve as precursor of aroma compounds (Partidário *et al.*, 1998) was not influenced by HPP; no significant differences in acetic acid values were observed at 0 months storage, between $M_C$+$Ch_C$ and the other experimental cheeses ($p > 0.05$). A similar observation was registered for 35 day old ripened *Torta del Casar* cheeses which were HPP treated at 400 or 600 MPa/5 min where acetic acid concentrations did not differ between processed and non-processed cheeses (Calzada *et al*., 2014b). Valeric acid, which is not present in milk, but is present in cheese due to microbial metabolism in cheese, is associated with a nutty flavour. Although present at relatively low concentrations, flavour threshold is important; HPP influenced valeric acid concentrations upon application as reflected in the fact that $M_C$+$Ch_P$ cheeses revealed significantly higher valeric acid values at month 0 (1.85 μg/g) in comparison to $M_C$+$Ch_C$ cheeses (0.92 μg/g) ($p < 0.05$), with the difference decreasing along storage to similar values (~4 μg/g) at 5 months ($p > 0.05$).

### 9.3.8. Changes in sensory attributes

Table 9.4 shows the sensory analysis results of the control and experimental *Serra da Estrela* cheeses. Significant differences ($p < 0.05$) across samples at each storage time were observed only for rind tonality and homogeneity, paste consistency, texture consistency and unctuosity.

$M_P$+$Ch_C$ cheeses showed a darker rind colour ($p < 0.05$) than $M_C$+$Ch_P$ cheeses at 0 months of storage, $M_P$+$Ch_C$ and $M_C$+$Ch_P$ cheeses showed a darker tonality than $M_C$+$Ch_C$ cheeses at 2 months storage and, $M_P$+$Ch_C$ and $M_P$+$Ch_P$ cheeses a darker rind tonality than $M_C$+$Ch_C$ cheeses at 5 months of storage. Differences in rind appearance homogeneity were also found for $M_P$+$Ch_C$ cheeses, being $M_P$+$Ch_C$ cheeses more homogeneous than $M_C$+$Ch_C$ counterparts at 0 months storage time and $M_P$+$Ch_P$ cheeses were rated more

homogeneous than $M_C$+$Ch_C$ counterparts at 2 months storage. These results are correlated with the instrumental lower $L^*$ colour parameter (less luminous) measured in $M_P$+$Ch_C$ cheeses in comparison to $M_C$+$Ch_C$ cheeses. Although all cheeses revealed similar paste appearance colour, the instrumental colour evaluation indicated the $M_C$+$Ch_P$ and $M_P$+$Ch_P$ cheeses core as being more yellowish ($p < 0.05$) than that of $M_C$+$Ch_C$ and $M_P$+$Ch_C$ cheeses. A similar effect (higher yellowness than control) was verified by the panellists after HPP treatment of *Ibores* cheeses (raw goat milk cheese treated at 50 days of ripening at 400 and 600 MPa/7 min) (Delgado *et al.*, 2013). The odours associated with general acidic smell, lactic acid, animal, and short-chain fatty acids were not significantly affected by milk pre-treatment and/or HPP cheese treatment, similarly to Molina *et al.*, (2000) and Trujillo *et al.*, (1999a), who studied cheeses from milk pre-treated at 400 MPa/15 min and 500 MPa/15 min, and were rated with a similar aroma relatively to control cheeses. The milk HPP pre-treatment induced changes in cheese texture, having the $M_P$+$Ch_C$ cheeses become softer and more unctuous at month 0, softer at month 2 and more unctuous at 5 months than $M_C$+$Ch_C$ counterparts ($p < 0.05$), characteristics very appreciated in *Serra da Estrela* cheese. Saldo *et al.*, (2000) reported that cheeses made from milk pre-treated at 50 MPa/72 h were less crumbly, more elastic, and had a higher mouth coating. Moreover, the flavour attributes (salty, acid, and bitter) were not significantly affected by HPP milk pre-treatment and/or HPP of the cheeses ($p > 0.05$). Similarly, no differences were observed for bitterness, saltiness and spicy tastes in goat cheeses made from milk pre-treated at 500 MPa/15 min (Buffa, Guamis, Pavia, & Trujillo, 2001), while for a lower pressure pre-treatment of the milk (50 MPa/72 h) the cheese was shown to be more acidic and less flavourful (Saldo *et al.*, 2000). In the present work, no differences were found for the after-taste intensity among all studied cheeses.

**Table 9.4: Mean values of sensory attributes intensities (scale from 0 to 10) of *Serra da Estrela* cheese made from control raw ewes' milk ($M_C$) and HPP pre-treated milk (MP), combined without ($M_C$+$Ch_C$ and $M_P$+$Ch_C$) and with the HPP in 60-day old ripened cheeses ($M_C$+$Ch_P$ and $M_P$+$Ch_P$) at 0, 2 and 5 months of storage.**

| Storage time (months) | 0 | | | | 2 | | | | 5 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ | $M_C$+$Ch_C$ | $M_C$+$Ch_P$ | $M_P$+$Ch_C$ | $M_P$+$Ch_P$ |
| **Rind appearance** | | | | | | | | | | | | |
| Tonality | 4.51 $^{a,b}$ | 3.65 $^{b}$ | 6.17 $^{a}$ | 4.95 $^{a,b}$ | 3.26 $^{b}$ | 5.53 $^{a}$ | 6.61 $^{a}$ | 4.97 $^{a,b}$ | 3.27 $^{b}$ | 4.74 $^{a,b}$ | 6.15 $^{a}$ | 5.56 $^{a}$ |
| Homogeneity | 2.69 $^{b}$ | 4.62 $^{a,b}$ | 6.35 $^{a}$ | 5.07 $^{a}$ | 3.21 $^{b}$ | 4.64 $^{a,b}$ | 3.75 $^{b}$ | 6.03 $^{a}$ | 4.01 $^{a}$ | 3.43 $^{a}$ | 3.78 $^{a}$ | 3.07 $^{a}$ |
| Defects | 4.34 $^{a}$ | 2.75 $^{a}$ | 3.09 $^{a}$ | 3.88 $^{a}$ | 4.31 $^{a}$ | 3.22 $^{a}$ | 3.05 $^{a}$ | 2.55 $^{a}$ | 4.13 $^{a}$ | 4.40 $^{a}$ | 4.32 $^{a}$ | 4.14 $^{a}$ |
| **Paste appearance** | | | | | | | | | | | | |
| Colour | 4.23 $^{a}$ | 2.88 $^{a}$ | 3.92 $^{a}$ | 4.02 $^{a}$ | 3.26 $^{a}$ | 3.74 $^{a}$ | 3.75 $^{a}$ | 3.29 $^{a}$ | 5.68 $^{a}$ | 5.31 $^{a}$ | 4.95 $^{a}$ | 5.74 $^{a}$ |
| Consistency | 5.93 $^{a}$ | 5.15 $^{a}$ | 4.80 $^{a}$ | 4.59 $^{a}$ | 5.59 $^{a}$ | 6.06 $^{a}$ | 3.19 $^{b}$ | 5.33 $^{a}$ | 6.12 $^{a}$ | 5.36 $^{a}$ | 5.33 $^{a}$ | 6.30 $^{a}$ |
| **Odour** | | | | | | | | | | | | |
| Lactic | 3.05 $^{a}$ | 2.20 $^{a}$ | 3.16 $^{a}$ | 2.97 $^{a}$ | 4.55 $^{a}$ | 2.64 $^{a}$ | 4.63 $^{a}$ | 3.55 $^{a}$ | 3.92 $^{a}$ | 3.72 $^{a}$ | 3.86 $^{a}$ | 4.51 $^{a}$ |
| Acid | 2.32 $^{a}$ | 2.16 $^{a}$ | 2.65 $^{a}$ | 3.42 $^{a}$ | 3.29 $^{a}$ | 3.26 $^{a}$ | 3.04 $^{a}$ | 3.57 $^{a}$ | 3.10 $^{a}$ | 3.25 $^{a}$ | 4.20 $^{a}$ | 3.72 $^{a}$ |
| Animal | 1.59 $^{a}$ | 2.74 $^{a}$ | 2.67 $^{a}$ | 2.60 $^{a}$ | 3.48 $^{a}$ | 3.42 $^{a}$ | 3.03 $^{a}$ | 3.28 $^{a}$ | 3.04 $^{a}$ | 2.81 $^{a}$ | 1.99 $^{a}$ | 2.30 $^{a}$ |
| SCFA | 2.08 $^{a}$ | 2.32 $^{a}$ | 2.27 $^{a}$ | 2.57 $^{a}$ | 2.94 $^{a}$ | 2.52 $^{a}$ | 2.73 $^{a}$ | 3.74 $^{a}$ | 2.18 $^{a}$ | 2.06 $^{a}$ | 2.58 $^{a}$ | 2.60 $^{a}$ |
| **Texture** | | | | | | | | | | | | |
| Consistency | 4.26 $^{a,b}$ | 4.98 $^{a}$ | 2.36 $^{c}$ | 3.39 $^{b,c}$ | 3.84 $^{a}$ | 4.98 $^{a}$ | 1.83 $^{b}$ | 3.71 $^{a,b}$ | 4.33 $^{a}$ | 2.56 $^{a}$ | 3.07 $^{a}$ | 4.42 $^{a}$ |
| Friability | 0.82 $^{a}$ | 1.02 $^{a}$ | 0.69 $^{a}$ | 1.05 $^{a}$ | 1.97 $^{a}$ | 2.36 $^{a}$ | 1.11 $^{a}$ | 3.16 $^{a}$ | 1.32 $^{a}$ | 1.32 $^{a}$ | 1.92 $^{a}$ | 2.41 $^{a}$ |
| Unctuosity | 3.10 $^{b}$ | 2.68 $^{b}$ | 5.11 $^{a}$ | 4.07 $^{a,b}$ | 3.32 $^{a,b}$ | 3.57 $^{a,b}$ | 5.53 $^{a}$ | 2.92 $^{b}$ | 4.00 $^{c}$ | 6.39 $^{a,b}$ | 6.64 $^{a}$ | 4.44 $^{b,c}$ |
| **Flavour** | | | | | | | | | | | | |
| Salty | 3.47 $^{a}$ | 3.91 $^{a}$ | 4.27 $^{a}$ | 3.70 $^{a}$ | 3.58 $^{a}$ | 3.83 $^{a}$ | 4.24 $^{a}$ | 3.39 $^{a}$ | 4.71 $^{a}$ | 4.12 $^{a}$ | 5.15 $^{a}$ | 4.82 $^{a}$ |
| Acid | 3.12 $^{a}$ | 3.54 $^{a}$ | 3.10 $^{a}$ | 2.85 $^{a}$ | 2.90 $^{a}$ | 3.07 $^{a}$ | 3.07 $^{a}$ | 3.36 $^{a}$ | 2.99 $^{a}$ | 3.52 $^{a}$ | 4.02 $^{a}$ | 3.18 $^{a}$ |
| Bitter | 2.26 $^{a}$ | 2.39 $^{a}$ | 3.33 $^{a}$ | 2.46 $^{a}$ | 2.17 $^{a}$ | 2.16 $^{a}$ | 2.74 $^{a}$ | 2.24 $^{a}$ | 2.46 $^{a}$ | 2.29 $^{a}$ | 3.29 $^{a}$ | 3.28 $^{a}$ |
| **After-taste** | 3.72 $^{a}$ | 4.40 $^{a}$ | 4.71 $^{a}$ | 4.14 $^{a}$ | 3.42 $^{a}$ | 3.84 $^{a}$ | 4.28 $^{a}$ | 4.07 $^{a}$ | 4.49 $^{a}$ | 4.85 $^{a}$ | 5.96 $^{a}$ | 5.52 $^{a}$ |

Different letters for the same storage time indicate statistically significant differences (Tukey test $p < 0.05$).

## 9.4. Conclusion

The present study showed the effect of raw ewes' milk HPP pre-treatment (121 MPa/30 min), the application of HPP on ripened cheeses (525 MPa/6 min), as well as the combination of both treatments, during 5 months of cheese refrigerated storage, on different microbiological, physicochemical, biochemical, textural and sensorial cheese features. In what concerns microbiota profile, cheeses made from HPP pre-treated milk were shown to have higher lactococci and lactobacilli, but similar enterococci and total aerobic bacteria viable cell numbers. On the other hand, HPP application in ripened cheese led to a decrease between 1-2 log cycles in viable cell numbers of those microbial groups. Similarly, about 1 log cycle reduction in those viable cell numbers were achieved as the effect of the combined treatments. HPP of ripened cheese led to microbial inactivation, to below the quantification level, of inoculated (as surrogate) *Staphylococcus aureus, L. innocua* and *S. enterica*. In general, moisture and protein contents were not affected by milk HPP pre-treatment and/or cheese HPP treatment. Lower pH values and higher titratable acidity were observed for cheeses made from HPP-milk and submitted to HPP. Milk HPP pre-treatment led to a significant increase of the ripening extension index, while the HPP of ripened cheese did not significantly change the WSN/TN ratio. In general, HPP milk pre-treatment, HPP of cheese and the combination of both had no effect on cheese textural properties. Sensorial analysis revealed that milk HPP pre-treatment may result in softer and more unctuous cheeses. Thus, milk HPP pre-treatment can improve cheese characteristics, while HPP in ripened cheeses can increase cheese safety, and both resulted in no or minor changes in important cheese attributes such as viable cell numbers of indigenous beneficial microflora, physicochemical, textural and sensorial attributes.

# PART VII – General conclusions and Future perspectives

# CHAPTER 10 – Conclusions

The work presented in this dissertation is the result of a concerted research effort to theoretically underpin the impact of HPP on milk and cheese microbial safety and final quality providing incremental innovation in a traditional product, where *Serra da Estrela* cheese, one of the most relevant Portuguese PDO status cheeses, was used as our model cheese. The effects of HPP were tested in a sequential setup; firstly, HPP was applied as a new method for the processing of ripened cheese in order to render *Serra da Estrela* safe while retaining desirable quality during extended storage; secondly, HPP was applied as a cold pasteurization pre-treatment to raw ewes' milk prior to cheese manufacture anticipating improved microbial control and cheese yield; finally, the combined action of HPP along the whole cheese manufacture chain – HPP pre-treated milk is used in the manufacture of *Serra da Estrela* cheese which upon ripening is HPP treated prior to extended storage – was explored using a pioneer approach.

In the first phase *Serra da Estrela* cheeses at 45 days of ripening were HPP treated at three different conditions in order to assess the combined effect of holding time and of pressure intensity. The more intense pressure treatment - P1 (600 MPa/6 min) -caused a greater impact on microbial inactivation, than the increase in holding time under pressure (P2 (450 MPa/6 min) vs P3 (450 MPa/9 min) revealed no significant differences between microbial loads). Lactobacilli and lactococci viable cell numbers were reduced in 3.2 - 3.6 and 2.7 - 3.6 log cycles, while the total aerobic, anaerobic and psychotrophic microorganisms were reduced in 1.1 - 5.3 log cycle units. *Enterobacteriaceae*, *Pseudomonas* spp., *E. coli* and yeasts and moulds were reduced to below the quantification limit.

In addition to modifying cheese microbial safety by inactivating pathogenic and contaminant microorganisms, HPP also modified cheese physicochemical and sensory characteristics. The application of HPP led to proteolysis deceleration, in comparison to control *Serra da Estrela* cheeses, with a higher impact at 600 MPa/6 min than at 450 MPa/6 min. Furthermore, it should also be highlighted that P1 cheeses maintained a stable WSN/TN index (27 – 30 %) throughout extended storage that was very close to that of the control *Serra da Estrela* cheese at 45 days of ripening (ideal sensory attributes). HPP

P1 cheeses kept the characteristic texture, although they were perceived to be harder than control cheeses from a sensorial point of view. In conclusion, this HPP treatment (600 MPa/6 min) when applied to *Serra da Estrela* cheese at 45 days of ripening may contribute to keeping the ideal ripening characteristics during longer storage periods. On the other hand, the less intense HPP treatments - (450 MPa) P2 (for 6 min) and P3 (for 9 min) - showed a milder effect on textural parameters and sensorial attributes, respectively. The P2 cheeses kept the hardness and consistency levels stable during storage, with values closer to those of control cheeses at 45 days of ripening, in agreement to what was observed for proteolytic indices. P3 cheeses revealed sensorial attributes closer to those of control cheeses throughout storage. Instrumental colour analysis revealed higher *b** values (more yellowness) in HPP cheeses' surface and core than in control cheeses, even though all became whitened due to vacuum packaging. HPP revealed no major changes in *Serra da Estrela* cheese lipid composition (triglycerides and esterified and non-esterified fatty acids) and nutritional features (atherogenicity and thrombogenicity indices), being quantified high CLA content in all cheeses.

Overall, the HPP treatment of cheese proposed and studied herein proved appropriate to control the microbiological, including safety hazards, and biochemical, textural and sensorial changes in *Serra da Estrela* cheese over extended storage. Enhancement of the fundamental knowledge comprising relationships between HPP and cheese technological and biochemical features was also accomplished enabling a rational improvement of quality and safety control of artisanal *Serra da Estrela* cheese. In fact, if minimal impact on microbial population with important metabolic activity for cheese ripening (lactobacilli, lactococci, enterococci) is to be achieved, while simultaneously inactivating contaminant and/or pathogenic microorganisms, and maintaining the artisanal cheese physicochemical and quality parameters, an intermediate pressure intensity can be the best treatment to be applied, i.e. 525 MPa/6 min.The comparative study between two packaging systems – vacuum packaging in polyamide-polyethylene plastic film and wrapping in paper without vacuum – to try and avoid whitening of cheese surface during storage of ripened *Serra da Estrela* cheese upon HPP had an important and meaningful impact for cheese dairy practices. Our study helped elucidating that the packaging system had little impact on total microbial viable cell numbers, exception for yeasts, but caused some alterations of physicochemical and sensorial parameters. Colour changes of cheese rind were related with the presence of yeasts in non-vacuum paper

wrapped cheeses. Vacuum packaging system enabled a better control of cheese proteolysis, which was reported to be closer to original control cheese values at the end of the 10 month storage period. Consequently, cheese stored under vacuum film packaging became harder than non-vacuum paper wrapped cheeses at each time point.
The most salient result of this novel approach is that we have demonstrated that the packaging system to be chosen is dependent on the storage period foreseen: paper wrap without vacuum is recommended if artisanal cheeses are to be stored for a short time period, less than 3 months; whereas for longer storage periods the vacuum packaging system in plastic film should be preferred.

The second part of this research approach reflects the role that we envisioned for HPP in artisanal cheese production and safety and quality preservation, maintaining *Serra da Estrela* cheese as our model cheese. Of utmost importance, it outlines the experimental pipeline required to use HPP throughout the cheese manufacturing process – from raw milk to ripened cheese - envisaging cheese yield increment besides effective microbial control in order to produce a microbiologically safe and stable *Serra da Estrela* cheese, maintaining unique texture and flavour attributes, leading to an extended shelf-life. To our knowledge, this is the first time that such approach has been developed using the two HPP cues (milk and cheese) in raw ewes' milk, and much more for *Serra da Estrela* cheese. Pressure intensity, holding time under pressure and time after HPP of raw ewes' milk from the specific geographical area that gives rise to the PDO certification of *Serra da Estrela* cheese were identified, within the broad screening design, as the main factors affecting *Serra da Estrela* cheese yield. The focused screening design enabled to conclude that HPP at 400 MPa as HPP pre-treatment of milk was undesirable since it caused the loss of cheesemaking properties of milk as well as the inactivation of essential microbiota; recall that, unlike other studies available in literature, our approach was pioneer in not using starters in the manufacture of this cheese upon HPP pre-treatment of milk. Optimum HPP conditions were predicted as 121 MPa for 30 min based on outputs of a response surface model (100 – 300 MPa; 5 – 30 min, 24 h before HPP, 24 h after HPP) targeting the least reduction possible of lactococci, lactobacilli, and enterococci viable cell numbers. From the validation of optimal conditions in a real artisanal dairy facility, an additional positive asset of our study, it was possible to conclude that such HPP treatment of milk increases *Serra da Estrela* cheese yield by 8.0%; led to a mild reduction of microbial load in milk, a small effect on curd and without significant differences in ripened cheese microbiota profile.

It became clear from our work that manufacture of *Serra da Estrela* cheese using HPP (121 MPa/30 min) pre-treated milk coupled to their HPP treatment upon ripening, prior to storage, produced microbial and biochemical changes. The application of HPP on ripened cheeses caused reductions similar to those reported in previous studies. Surrogate microorganisms, *Staphylococcus aureus, Listeria innocua* and *Salmonella enterica,* that were inoculated in these cheeses were reduced to below the quantification limit. The moisture and protein contents were not affected either by HPP milk pre-treatment and/or by HPP application on ripened cheeses. Proteolysis, as an essential biochemical phenomenon in *Serra da Estrela* cheese, was enhanced by HPP milk pre-treatment, while the treatment on cheese kept proteolysis comparative to the original control cheeses. Instrumental texture parameters were not affected in any of the three HPP settings. A higher proportion of volatile free fatty acids were determined for HPP treated cheeses compared to control counterparts. The higher abundance of volatile compounds is due to an improved microbiota profile where beneficial microorganisms capable of catabolic conversion of free amino acids as precursors of flavours prevail. Overall organoleptic results of HPP application both to milk and/or ripened cheese were not detrimental. Experimental cheeses made from HPP pre-treated milk were revealed to be softer and more unctuous. In general, cheeses were associated with well-accepted *Serra da Estrela* sensory and textural attributes.

In conclusion, HPP mild treatment of raw milk prior to cheese manufacture is an innovative measure that capitalizes on not requiring the addition of starters, enabling a higher curd yield and improved microbial profile important from both technological and safety points of view. HPP treatment of ripened cheese is an advanced measure to gain important features for *Serra da Estrela* cheese economy: it enables an improved microbial profile important from a safety and quality point of view, enables the maintenance of the proteolytic profile and enables improved sensory attributes in some cases, all important features from a quality point of view which will undoubtedly contribute positively to producers' and consumers' awareness of such an important cheese; furthermore, and of meaningful importance, this integrated approach may be implemented with other artisanal raw milk cheeses.

# CHAPTER 11 - Future Perspectives

The studies carried out in this PhD thesis highlight a very interesting possibility for the valorisation of a traditional dairy product using an alternative food processing technology, opening an opportunity to increase improvement of Portuguese *Serra da Estrela* cheese, namely at microbial safety level. Still, regulatory issues should be overcome if the major goal is to keep the *Serra da Estrela* cheese PDO certification. It would be of major interest to perform changes in the book of specifications of *Serra da Estrela* cheese, allowing high pressure processing, a non-thermal processing technology, as a step in cheese manufacture without loss of PDO certification. These regulatory changes could undeniably prompt *Serra da Estrela* cheese in the external markets with considerable gains for local economy, as well as a way of employment generation. This step will undoubtedly contribute positively to reach new external markets, particularly for countries with specifically higher restrictions on raw milk products importation; extension to other raw milk cheeses may also be envisioned.

At the microbial level, studies are desirable on the effect of HPP on inoculated *Yarrowia lipolytica*, an important target microorganism since it is responsible for the loss of many cheeses in dairy industry due to browning of the cheese rind.

A suitable and eco-friendly packaging system that could keep the achieved HPP advantages and avoid the changes in the colour of the rind would be useful and should be further explored. Furthermore, an eco-friendly wrapping paper with adequate oxygen permeability should also be focused upon and studied as a good option to wrap *Serra da Estrela* cheeses.

According to results obtained in Chapter 9, selected samples can be studied in more detail at proteolytic and lipolytic levels. Casein degradation can be assessed by urea-polyacrylamide gel electrophoresis and the peptide profile through reverse-phase high-performance liquid chromatography. Evolution of free amino acids and biogenic amines (chemical indicators of cheese hygienic quality and recognized as a serious health hazard for humans at significant levels) could also be monitored. The evaluation of HPP effect on *Serra da Estrela* cheese can be further detailed by microstructure analysis by scanning electron microscope (SEM), allowing to understand the effects of HPP on morphology, protein network and microbiota distribution in cheese.

To complete the present research and achieve the recognized beneficial effects of HPP on *Serra da Estrela* cheese is to achieve a certified label "*Serra da Estrela* cheese PDO – high pressure treated" and then, achieve the broad national market as well as foreign markets with different regulatory issues regarding the microbial stability of raw-like foods, without compromising the characteristic attributes of *Serra da Estrela* cheese that are so appreciated by consumers.

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# Appendices

# Appendices CHAPTER 3

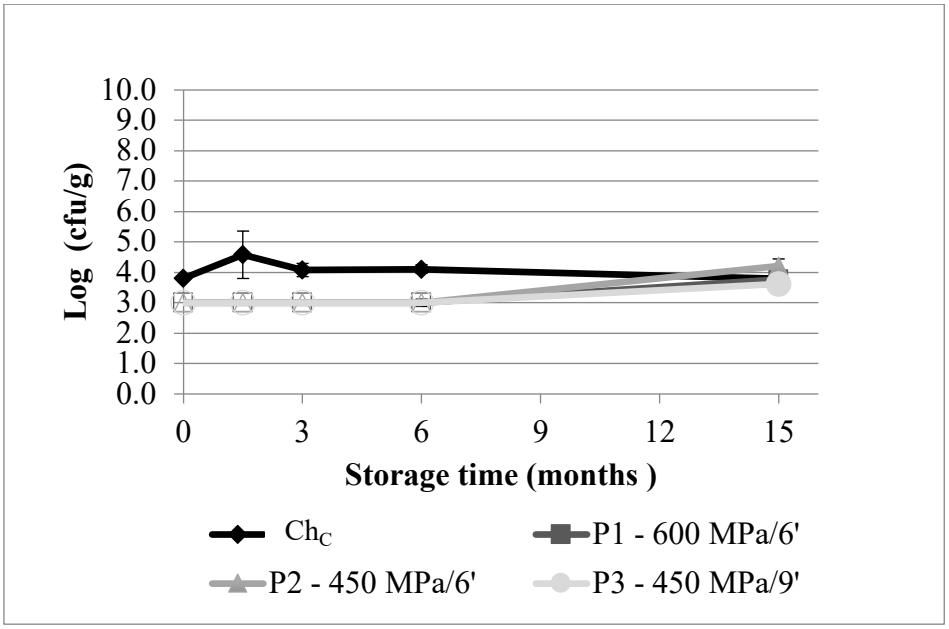


**Figure A. 3.1:** Yeasts and moulds viable cells numbers in *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage, of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3). Empty symbols represent microbial loads below the quantification limit (3 log cfu/g).

**Table A. 3.1: Colour CIE parameters measured in cheese surface and cheese core of *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage, of different HPP treatments and control cheeses ($Ch_C$).**

| | | | $Ch_C$ | | P1 - 600 MPa/6' | | P2 - 450 MPa/6' | | P3 - 450 MPa/9' | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Months | STD | | STD | | STD | | STD | |
| Cheese Surface Colour | *L** | 0 | 68.4 ± 0.57 | a,b,D | 67.60 ± 2.62 | b,D | 69.11 ± 0.75 | a,b,E | 69.61 ± 1.44 | a,D |
| | | 1.5 | 76.2 ± 1.11 | b,C | 77.00 ± 0.53 | a,b,C | 77.43 ± 1.18 | a,D | 76.60 ± 1.23 | a,b,C |
| | | 3 | 78.6 ± 1.10 | a,B | 78.78 ± 1.02 | a,B | 79.43 ± 1.23 | a,C | 79.18 ± 1.25 | a,B |
| | | 6 | 79.7 ± 1.10 | b,c,B | 81.34 ± 1.07 | a,A | 80.56 ± 0.52 | a,b,B | 78.87 ± 0.62 | c,B |
| | | 15 | 81.8 ± 1.23 | a,A | 81.59 ± 1.01 | b,A | 81.03 ± 1.15 | a,b,A | 82.32 ± 0.98 | a,b,A |
| | *a** | 0 | 0.00 ± 0.20 | a,A | -0.35 ± 0.48 | a,b,A | -0.76 ± 0.21 | b,A | -0.45 ± 0.53 | b,A |
| | | 1.5 | -0.71 ± 0.17 | a,C | -1.75 ± 0.17 | b,B | -0.84 ± 0.53 | a,A | -1.46 ± 0.16 | b,C |
| | | 3 | -0.39 ± 0.36 | a,B | -1.21 ± 0.77 | b,A,B | -0.68 ± 0.42 | a,b,A | -1.04 ± 0.41 | b,B,C |
| | | 6 | -0.37 ± 0.32 | a,B | -1.04 ± 0.35 | b,A,B | -0.32 ± 0.64 | a,A | -0.61 ± 0.19 | a,b,A,B |
| | | 15 | -0.30 ± 0.26 | a,A,B | -0.58 ± 0.50 | a,b,A | -0.56 ± 0.40 | a,b,A | -0.84 ± 0.22 | b,B |
| | *b** | 0 | 22.8 ± 0.20 | b,A | 25.74 ± 0.90 | a,A | 25.31 ± 1.10 | a,A | 25.27 ± 0.65 | a,A |
| | | 1.5 | 21.0 ± 0.17 | b,A,B | 23.66 ± 0.63 | a,B | 22.68 ± 0.37 | a,B | 23.16 ± 0.62 | a,B |
| | | 3 | 21.0 ± 0.36 | b,A,B | 23.19 ± 1.19 | a,B | 22.45 ± 1.20 | a,B,C | 22.66 ± 1.09 | a,B |
| | | 6 | 21.4 ± 0.32 | b,A,B | 23.35 ± 1.20 | a,B | 21.60 ± 1.15 | b,B,C | 22.39 ± 1.72 | a,b,B |
| | | 15 | 19.8 ± 0.26 | a,B | 19.04 ± 2.73 | a,C | 21.12 ± 2.07 | a,C | 20.97 ± 1.11 | a,C |
| Cheese Core | *L** | 0 | 85.6 ± 0.57 | b,A | 86.56 ± 1.40 | b,A | 86.83 ± 2.47 | b,A | 89.11 ± 1.70 | a,A |
| | | 1.5 | 85.1 ± 1.11 | a,b,A | 85.55 ± 3.63 | b,A | 84.75 ± 1.53 | b,B,C | 86.71 ± 1.63 | a,B |
| | | 3 | 85.8 ± 1.10 | a,A | 85.52 ± 1.01 | a,A | 85.10 ± 1.40 | a,A,B | 85.12 ± 2.60 | a,B,C |
| | | 6 | 83.5 ± 1.10 | b,B | 85.04 ± 1.58 | a,A | 85.05 ± 1.27 | a,A,B,C | 84.62 ± 0.93 | a,b,C |
| | | 15 | 83.3 ± 0.74 | a,B | 82.18 ± 0.91 | a,A | 83.03 ± 0.70 | a,C | 82.97 ± 0.85 | a,b,D |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | -1.19 ± 0.20 | a,A | -2.72 ± 0.08 | b,C | -2.65 ± 0.16 | b,C | -2.66 ± 0.11 | b,B,C |
| | 1.5 | -1.31 ± 0.17 | a,A | -2.56 ± 0.16 | b,C | -2.56 ± 0.06 | b,C | -2.76 ± 0.14 | c,C |
| ***a**** | 3 | -1.32 ± 0.36 | a,A | -2.66 ± 0.08 | b,C | -2.59 ± 0.05 | b,C | -2.59 ± 0.07 | b,B,C |
| | 6 | -1.37 ± 0.32 | a,A | -2.17 ± 0.21 | b,A,B | -2.38 ± 0.16 | c,B | -2.59 ± 0.02 | d,B |
| | 15 | -1.24 ± 0.07 | a,A | -1.73 ± 0.19 | b,C | -2.14 ± 0.07 | c,A | -2.03 ± 0.13 | c,A |
| | 0 | 17.5 ± 0.81 | b,B | 21.38 ± 0.46 | a,B | 20.94 ± 0.48 | a,C | 20.56 ± 0.41 | a,C |
| | 1.5 | 18.4 ± 1.86 | b,B | 22.12 ± 0.61 | a,B | 22.01 ± 0.65 | a,B | 22.04 ± 0.81 | a,B |
| ***b**** | 3 | 18.3 ± 1.37 | b,B | 22.86 ± 0.44 | a,A | 22.60 ± 0.52 | a,A,B | 22.69 ± 0.84 | a,A,B |
| | 6 | 21.2 ± 1.10 | b,A | 22.69 ± 0.26 | a,A | 22.71 ± 0.60 | b,A,B | 23.01 ± 0.60 | a,A,B |
| | 15 | 22.3 ± 0.34 | b,A | 23.29 ± 0.35 | a,A | 23.03 ± 0.45 | a,A | 22.60 ± 0.28 | b,A |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).

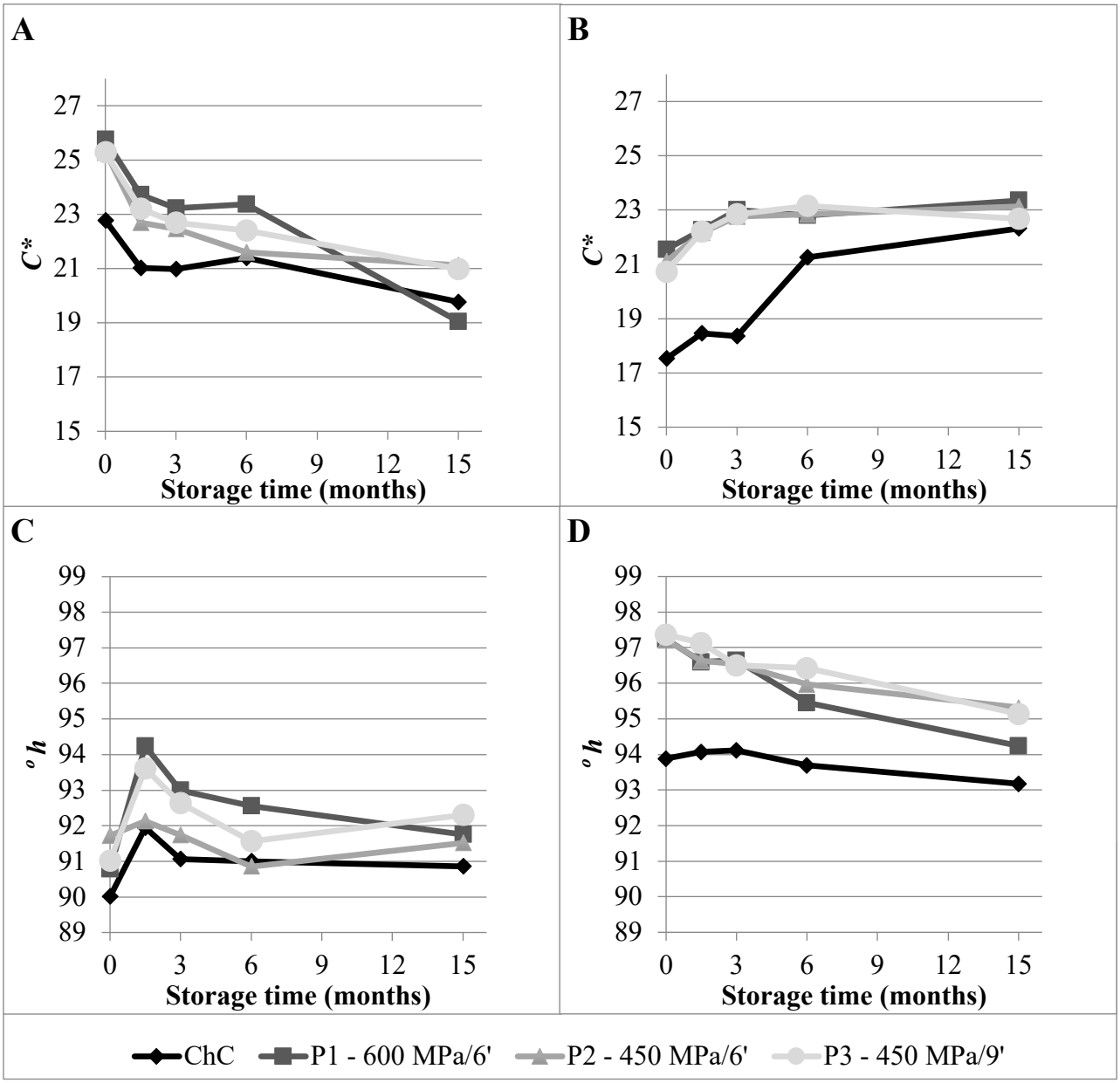


**Figure A. 3.2:** Chroma in (A) cheese surface and (B) cheese core; and hue degree in (C) cheese surface and (D) cheese core calculated comparatively to non-treated *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage of control cheeses ($Ch_C$) and HPP cheeses (P1, P2 and P3).

# Appendices CHAPTER 7

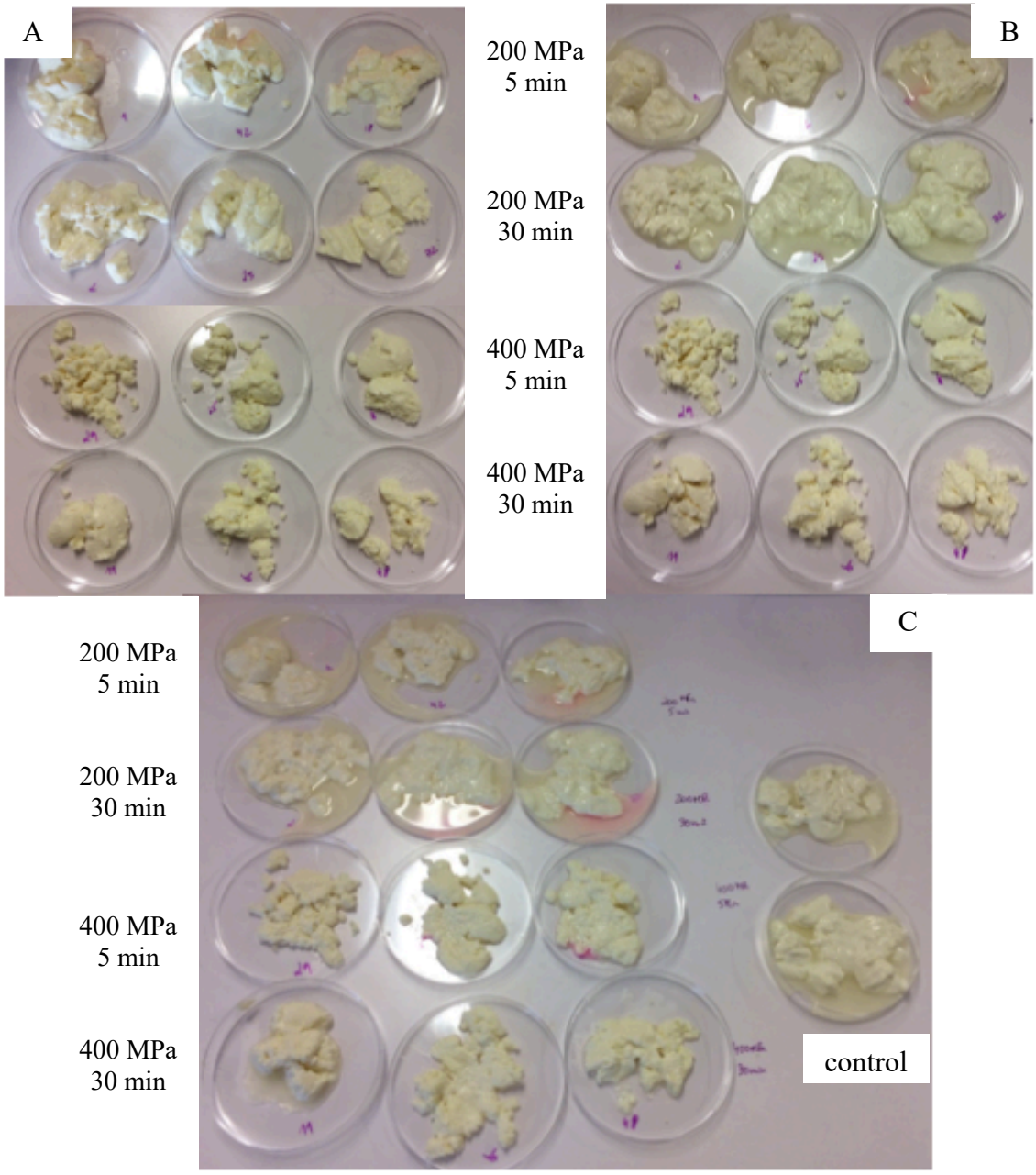


**Figure A. 7.1**: Curds obtained in the initial wide screening from HPP treated milk at different conditions: (A) after centrifugation and whey separation – 0 h; (B) after 2 h; and (C) after 24 h.

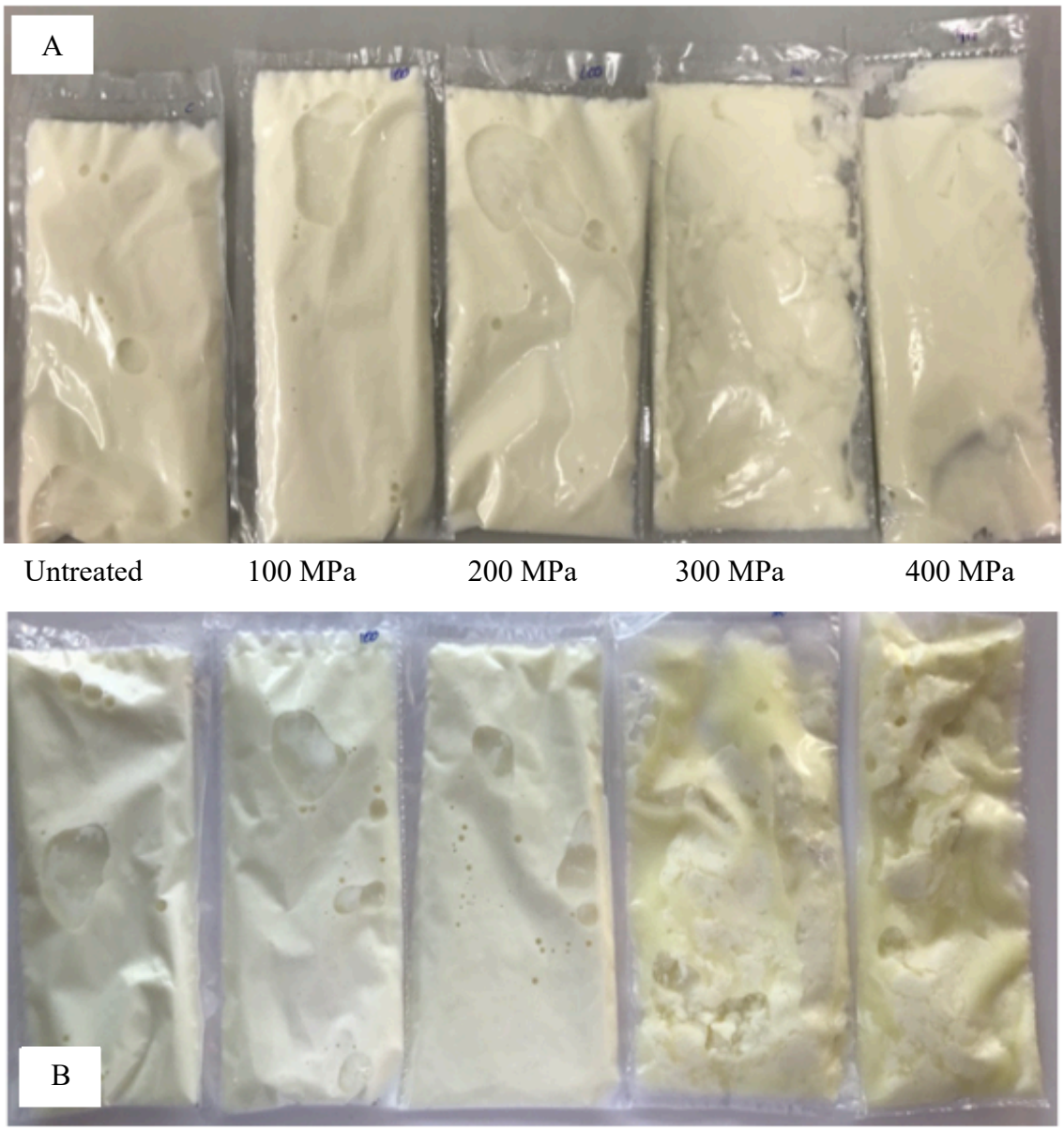


**Figure A. 7.2:** Milk aspect obtained in the focused screening: (A) 1 h after HPP at 100, 200, 300 and 400 MPa for 5 min; (B) after 24 h.

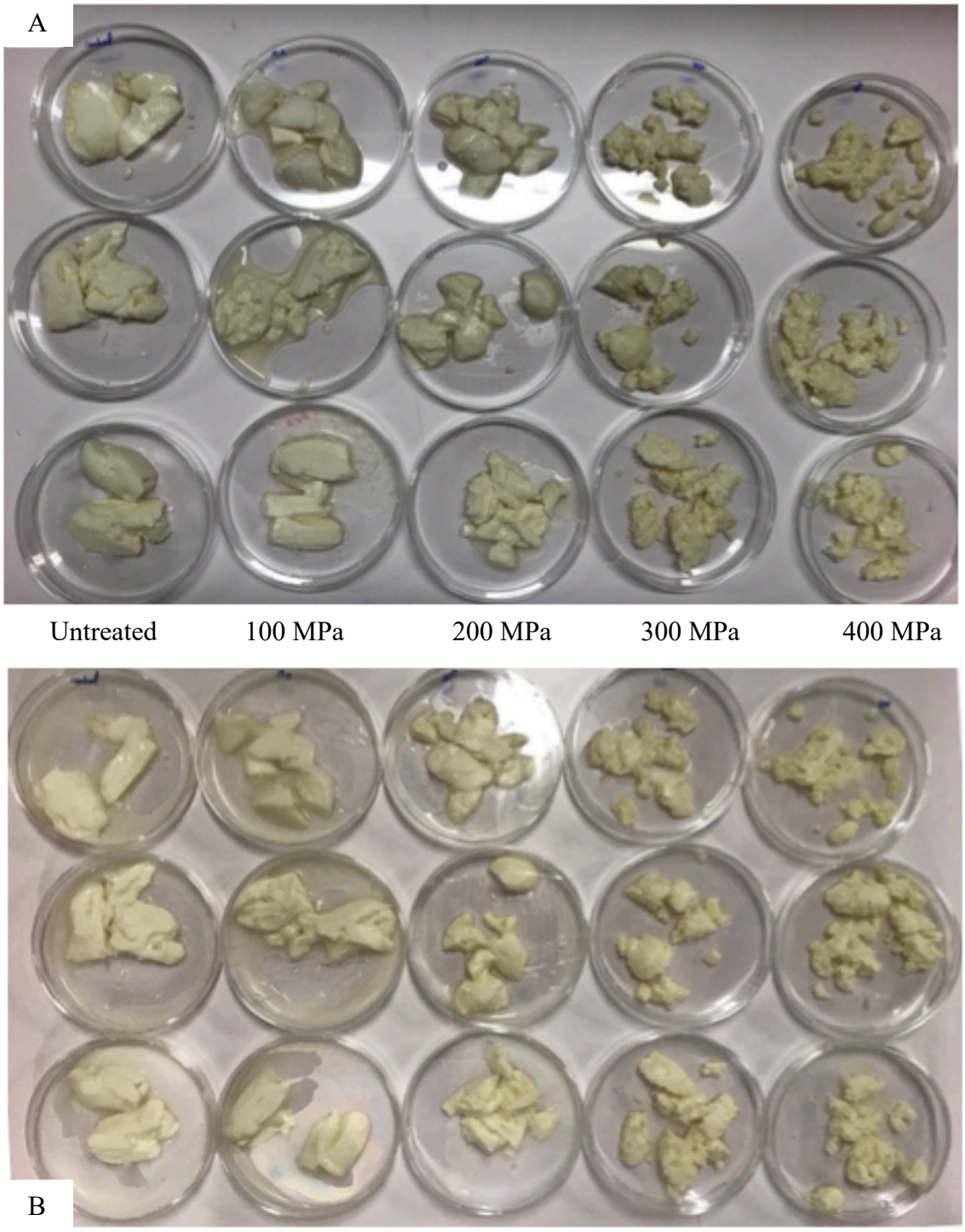


**Figure A. 7.3**: Curds obtained for the focused screening: HPP treated milk at 100, 200, 300 and 400 MPa for 5 min (A) 0 h after centrifugation and (B) after 24 h.

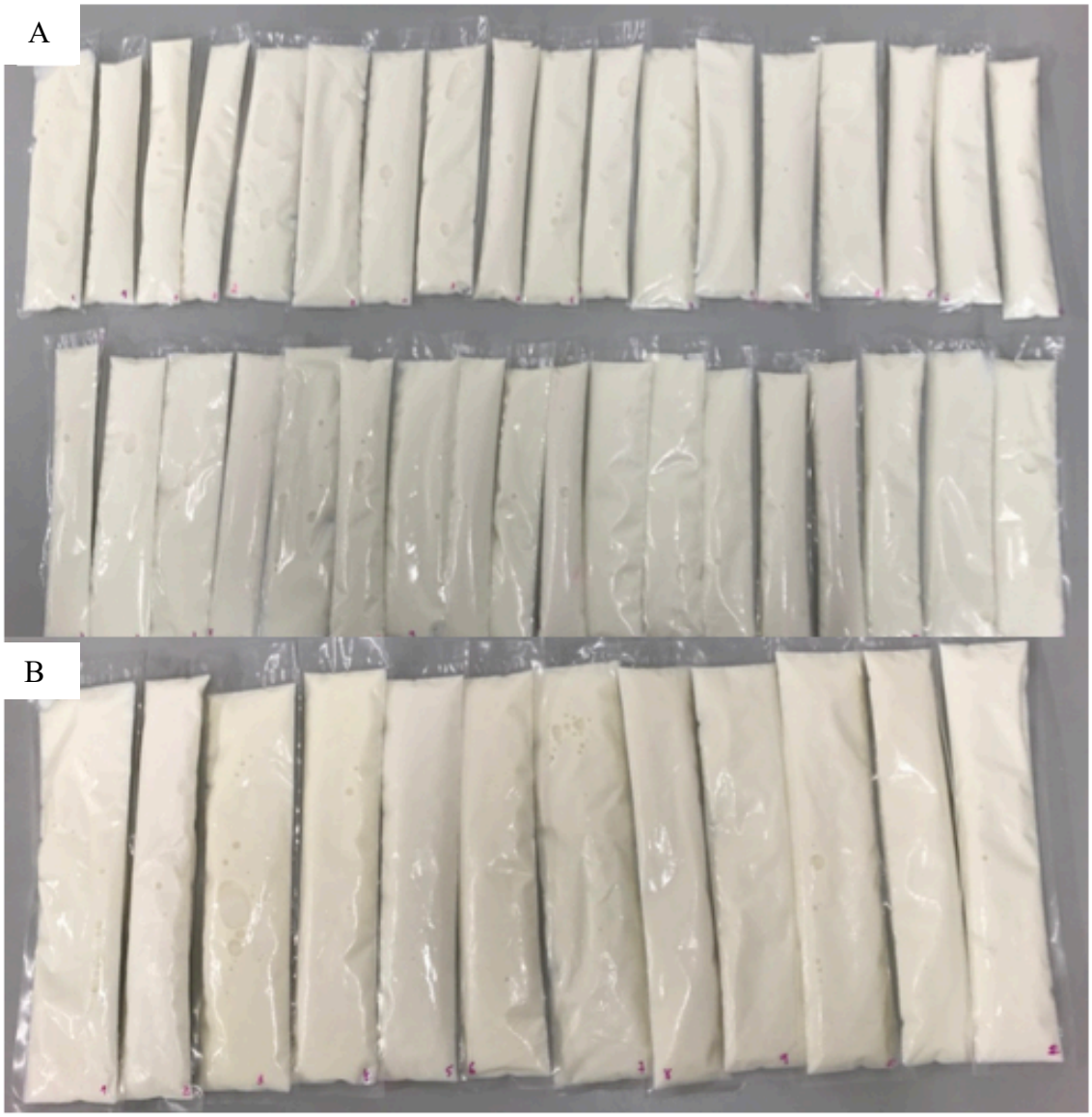


**Figure A. 7.4:** Milk aspect obtained in the design of experiment: milk bags (A) before HPP treatment and (B) immediately after HPP treatments according to central composite design.

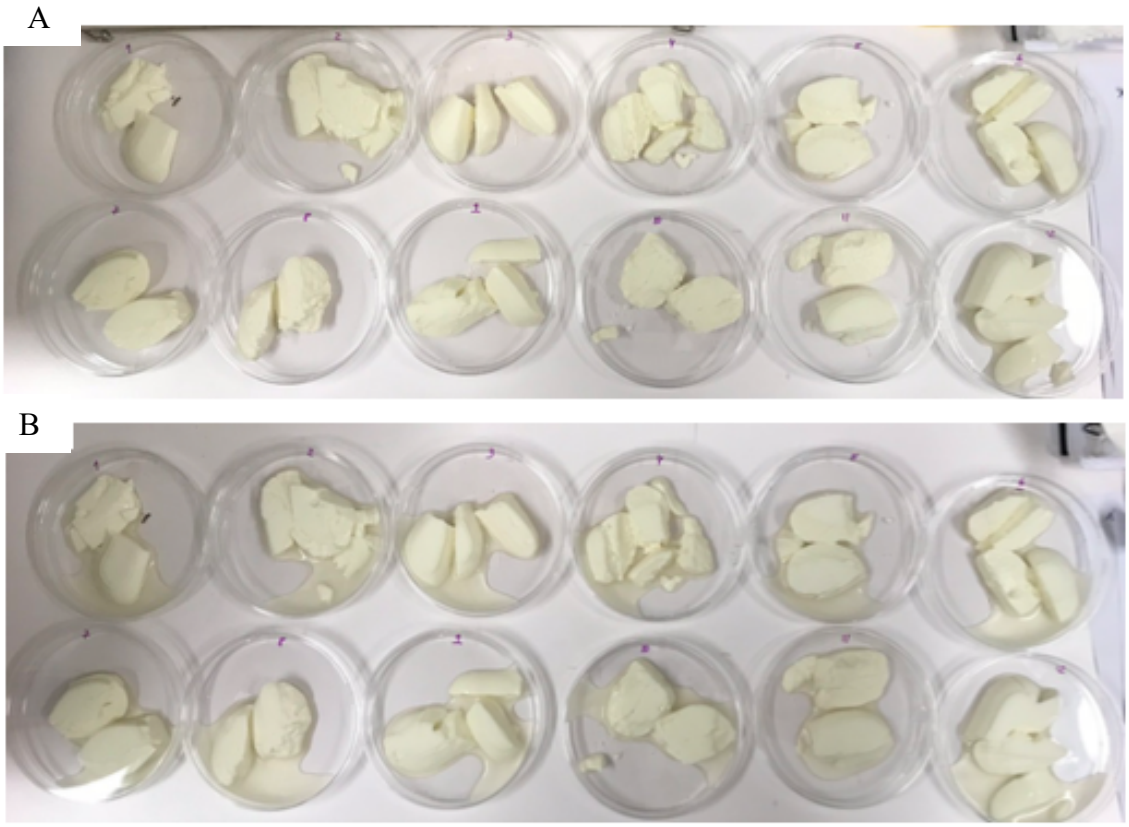


**Figure A. 7.5:** Curds obtained the design of experiment: from HPP pre-treated milk according to central composite design (A) 0 h after centrifugation and (B) after 2 h

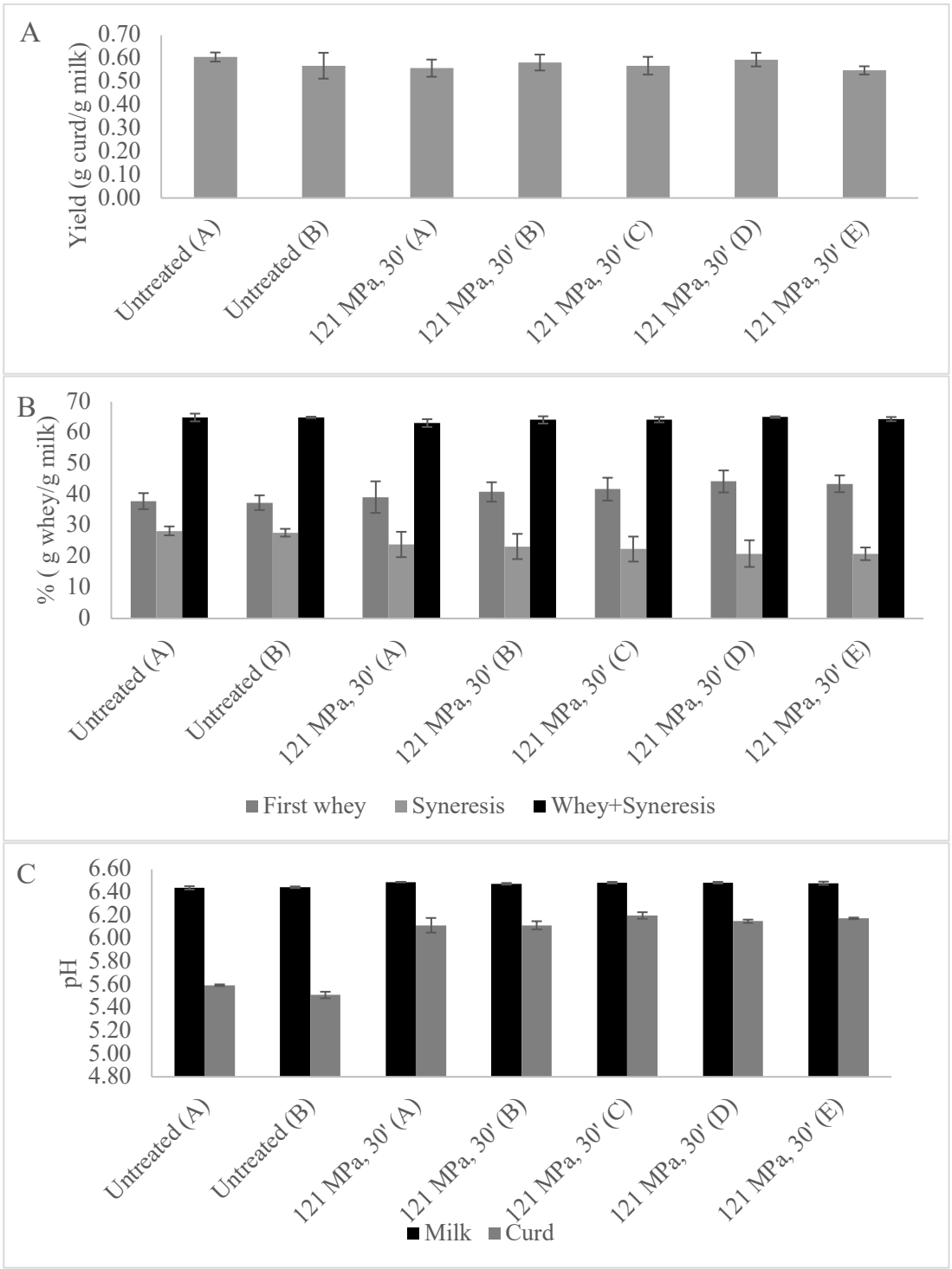


**Figure A. 7.6:** Results for model validation: (A) yield expressed in g of curd/g of milk; (B) whey release immediately after centrifugation: first whey; syneresis (24 h); whey+syneresis; (C) milk and curd pH values of HPP pre-treated milk.

# Appendices CHAPTER 10

**Table A. 10.2:** Colour CIE parameters measured in cheese surface and cheese core of *Serra da Estrela* cheese produced from control raw ewes' milk ($M_C$) and HPP pre-treated milk ($M_P$), combined without ($M_C$+$Ch_C$ and $M_P$+$Ch_C$) and with the HPP in 60-day old ripened cheeses ($M_C$+$Ch_P$ and $M_P$+$Ch_P$) at 0, 2 and 5 months of storage.

| | Storage time (months) | | $M_C$+$Ch_C$ STD | | $M_C$+$Ch_P$ STD | | $M_P$+$Ch_C$ STD | | $M_P$+$Ch_P$ STD | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cheese Surface Colour | ***L**** | 0 | 68.4 ± 2.12 | a,b,B | 67.1 ± 1.79 | b,c,B | 70.2 ± 1.21 | a,B | 65.5 ± 0.39 | c,C |
| | | 2 | 75.4 ± 1.14 | a,A | 71.9 ± 2.69 | b,c,A | 72.9 ± 3.50 | a,b,A | 68.7 ± 0.80 | c,B |
| | | 5 | 74.0 ± 1.11 | a,A | 74.6 ± 3.09 | a,A | 73.4 ± 0.99 | a,A | 72.7 ± 0.48 | a,A |
| | ***a**** | 0 | -0.98 ± 0.97 | a,A | -4.15 ± 0.24 | c,A | -2.32 ± 0.48 | b,A | -3.47 ± 0.44 | c,A |
| | | 2 | -1.89 ± 0.62 | a,A | -2.87 ± 0.21 | b,c,A | -2.48 ± 0.21 | b,A | -3.26 ± 0.32 | c,A |
| | | 5 | -2.55 ± 0.80 | b,A | -1.60 ± 0.46 | a,A | -1.47 ± 0.75 | a,A | -3.31 ± 0.35 | b,A |
| | ***b**** | 0 | 21.9 ± 0.97 | a,A | 18.0 ± 1.61 | b,B | 20.2 ± 1.02 | a,B | 20.3 ± 0.53 | a,B |
| | | 2 | 19.4 ± 0.62 | c,B | 21.2 ± 0.53 | b,A | 19.6 ± 0.95 | c,B | 23.2 ± 0.82 | a,A |
| | | 5 | 22.5 ± 0.80 | a,A | 22.3 ± 2.59 | a,A | 23.7 ± 1.14 | a,A | 22.8 ± 1.07 | a,A |
| | ***Δ E**** | 2 | 7.54 | | 5.93 | | 2.70 | | 4.40 | |
| | | 5 | 5.94 | | 9.01 | | 4.71 | | 7.65 | |
| | ***C**** | 0 | 21.9 | | 18.4 | | 20.4 | | 20.6 | |
| | | 2 | 19.5 | | 21.4 | | 19.8 | | 23.5 | |
| | | 5 | 22.7 | | 22.3 | | 23.7 | | 23.0 | |
| | ***° h*** | 0 | 92.6 | | 103.0 | | 96.5 | | 99.7 | |
| | | 2 | 95.5 | | 97.7 | | 97.2 | | 98.0 | |
| | | 5 | 96.5 | | 94.1 | | 93.6 | | 98.3 | |
| Cheese Core Colour | ***L**** | 0 | 81.6 ± 2.12 | a,A | 83.8 ± 1.62 | a,A | 82.6 ± 1.84 | a,A | 83.3 ± 1.26 | a,A |
| | | 2 | 80.5 ± 1.14 | a,A | 80.4 ± 0.97 | a,B | 80.8 ± 1.79 | a,A,B | 79.2 ± 3.25 | a,B |
| | | 5 | 79.9 ± 1.11 | a,B | 80.4 ± 2.12 | a,B | 80.2 ± 1.07 | a,B | 81.1 ± 0.91 | a,A,B |
| | ***a**** | 0 | -2.78 ± 0.97 | a,A | -4.54 ± 0.37 | b,A | -2.95 ± 0.15 | a,A | -4.92 ± 0.17 | b,A |
| | | 2 | -2.87 ± 0.62 | a,A | -5.67 ± 0.14 | b,A | -3.15 ± 0.37 | a,A | -5.70 ± 0.36 | b,A |
| | | 5 | -2.94 ± 0.80 | a,A | -5.42 ± 0.34 | b,A | -3.22 ± 0.22 | a,A | -5.80 ± 0.09 | b,A |
| | ***b**** | 0 | 15.1 ± 1.02 | a,A | 15.5 ± 1.09 | a,C | 15.3 ± 0.32 | a,B | 16.3 ± 0.12 | a,C |
| | | 2 | 15.2 ± 0.85 | b,A | 20.0 ± 1.02 | a,B | 16.0 ± 1.10 | b,A,B | 19.4 ± 1.46 | a,B |
| | | 5 | 16.3 ± 1.75 | c,A | 22.6 ± 0.59 | a,A | 17.6 ± 1.01 | b,A | 21.8 ± 0.06 | a,A |
| | ***Δ E**** | 2 | 1.03 | | 5.80 | | 1.87 | | 5.19 | |
| | | 5 | 2.00 | | 7.95 | | 3.33 | | 6.01 | |
| | ***C**** | 0 | 15.3 | | 16.2 | | 15.6 | | 17.0 | |
| | | 2 | 15.4 | | 20.8 | | 16.3 | | 20.2 | |
| | | 5 | 16.5 | | 23.3 | | 17.9 | | 22.6 | |
| | ***° h*** | 0 | 100 | | 106 | | 101 | | 107 | |
| | | 2 | 101 | | 106 | | 101 | | 106 | |
| | | 5 | 100 | | 103 | | 100 | | 105 | |

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C) in the same column indicate statistically significant differences among the same condition ($p < 0.05$).