**Universidade de Aveiro**
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Departamento de Química

**ANA RITA SANTOS INÁCIO**

# EFEITO DA ALTA-PRESSÃO NO QUEIJO SERRA DA ESTRELA

# HIGH PRESSURE EFFECT IN SERRA DA ESTRELA CHEESE

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

Dedicada aos meus pais por todas as dádivas dadas
ao longo destes 23 anos

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**Palavras-chave** Queijo da Serra, tecnologia alta-pressão, microrganismos, *L. innocua*, oxidação lipídica

**Resumo**

O queijo Serra da Estrela é um queijo tradicional Português produzido a partir de leite de ovelha crú, sal e cardo (*Cynara cardunculus*, L.). Como outros queijos, particularmente os feitos a partir de leite crú, o Queijo Serra tem na sua composição vários microrganismos.

Este trabalho teve como objetivo estudar o efeito da Alta-Pressão (AP) no Queijo Serra da Estrela - Denominação de Origem Protegida (DOP), após processamento e durante o armazenamento (100 dias a 5 °C). Queijos com 45 dias de maturação foram tratados com 400, 500 e 600 MPa durante 10, 5 e 3 minutos, respetivamente, a 4 °C. Amostras não pressurizadas foram utilizadas como controlo.

Os resultados revelaram que os microrganismos benéficos para a maturação, bactérias lácticas, foram as menos afetadas pela AP, sendo reduzidas no máximo em ~ 0,50 log UFC/g (amostras tratadas a 600 MPa foram as mais afetadas). Os microrganismos totais aeróbios mesófilos foram reduzidos em ~ 1,0 log UFC / g (amostras tratadas a 400 MPa foram as menos afetadas). No entanto, a contagem de *Enterobacteriaceae* mostrou ≥ 3,5 log reduções, valores que se mantiveram constantes durante o armazenamento; bolores e leveduras apresentaram reduções ≥ 3,6 logo após o processamento. As amostras inoculadas com *L. innocua* a 8,6 log UFC/g apresentaram reduções ≥ 4,8 log após tratamento a AP, mas passados 14 dias revelaram níveis abaixo do limite de deteção. Foram observadas pequenas alterações nos parâmetros físico-químicos (pH e acidez titulável) entre o controlo e queijo tratado durante o armazenamento. Durante esse período, a oxidação lipídica foi mais intensa no queijo não processado.

Os resultados obtidos permitem concluir que AP apresenta um bom potencial para tornar o Queijo Serra livre de microrganismos potencialmente patogénicos, sem alterações significativas nas características do queijo (azoto solúvel em água, atividade e conteúdo de água).

**Keywords** Serra Cheese, High-pressure technology, microorganisms, *L. innocua*, lipid oxidation

**Abstract**

Serra da Estrela Cheese is a traditional Portuguese cheese manufactured from raw milk, salt and cardoon (*Cynara cardunculus*, L.). As other cheeses, particularly those made from raw milk, Serra Cheese has in its composition several microorganisms.

This work aimed to study the effect of High Pressure Processing (HPP) on Serra da Estrela Cheese – Denominação de Origem Protegida (DOP) after pressure processing and during storage (100 days at 5 °C). Cheeses with 45 days of ripening were treated at 400, 500 and 600 MPa pressures during 10, 5 and 3 minutes, respectively, at 4 °C. Non-processed samples were used as controls.

The results revealed that microorganisms beneficial to cheese maturation (lactic acid bacteria; LAB) were the least affected by HPP, being reduced at maximum by ~ 0.50 Log CFU/g (samples treated at 600 MPa were the most affected). Total aerobic mesophilic microorganisms were reduced by ~ 1.0 Log CFU/g (samples treated at 400 MPa were least affected). However, *Enterobacteriaceae* counts showed ≥ 3.5 log cycle reductions, remaining unchanged during the storage; yeasts and moulds counts exhibited ≥ 3.6 log cycle reductions after process. Samples inoculated with *L. innocua* at 8.6 Log CFU/g presented ≥ 4.8 log cycle reductions after HPP, but after 14 days revealed levels below the detection limit. Small changes in physicochemical parameters (pH values and titratable acidity) were observed between control and treated cheese during storage. Throughout this period, lipid oxidation was more intense in non-processed cheese.

The results obtained allow concluding that HPP has good potential to render Serra Cheese free of potential pathogenic microorganisms, with no significant changes in cheese characteristics (water soluble nitrogen, water activity and content).

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# LIST OF ABBREVIATIONS

AOAC - Association of Official Analytical Chemists
$a_w$ - Water activity
CFU - Colony-forming unit
DOP – Denominação de Origem Protegida
FAA – Free amino acids
FFA – Free fatty acids
GC – Gas chromatography
GCMS - Gas chromatography mass spectrometry
GMP – Good manufacturing practice
HP - High pressure
HPP - High pressure processing
INRB-INIA - Instituto Nacional de Recursos Biológicos - Instituto Nacional de Investigação Agrária
LAB - Lactic acid bacteria
MDA - Malondialdehyde
MRS - Man, Rogosa and Sharpe
NP – Non processed samples
NSLAB – Non started lactic acid bacteria
OAV - Odour activity values
PA-PE - Polyamide-polyethylene
PCA - Plate count agar
PTA - Nitrogen soluble in 5% (w/v) phosphotungstic acid
RBCA - Rose-bengal chloramphenicol agar
TA – Titratable acidity
TBA - 2-thiobarbituric acid
TBARS - Thiobarbituric acid reactives substances
TCA - Nitrogen soluble in 12% (w/v) trichloroacetic acid
TMP - 1,1,3,3-tetrametoxipropano
TN - Total nitrogen content
TSA - Trypticase soy agar
TSB - Trypticase soy broth
VRBDA - Violet red bile dextrose agar
WSN - Nitrogen soluble in water

# I. INTRODUCTION

The present work is structured into seven sections. After this previous introduction, the second section consists in a comprehensive literature review and state of the art in what concerns 1) cheese in general; 2) Serra cheese manufacture, composition, microbial profile (lactic acid bacteria, yeasts, *Enterobacteriaceae,* coliforms *and Staphylococci*) and biochemical changes already studied (proteolysis, lipolysis and flavour); 3) the microbial safety of Serra cheese; and 4) the currently used non-thermal high pressure technology in cheese manufacture and the corresponding effects on microbiology, quality and cheese characteristics during the ripening and storage. Then, in section III, the objectives of this study are described. In section IV, the material and methods used during this study are detailed. Section V consists in the results obtained and the respective discussion, correlating with the available literature. This section was divided into two parts: I) microbiological analysis and II) physicochemical analysis. Section VI features the global conclusions of the previous sections. Finally, some possibilities are given to future work to be done in this research area, in Serra cheese treated by high pressure.

# II. LITERATURE REVIEW

## 1 Cheese

*"Cheese is obtained from curdled milk by removal of whey and by curd ripening in the presence of special microflora." [1]*

Cheese is known as the oldest fermented food in the world. It is believed that it first appeared in Iraqi at about 8000 years ago, during the agricultural revolution. At that time, the only way to conserve food was salting it (meat and probably some fruits and vegetables) [1]. The first cheese was made accidentally, by bacterial contamination of milk. These bacteria grew in milk and produced acid in enough quantity that decreased the pH, which led to the isoelectric precipitation of caseins [2]. Therefore, the bacterial acidification was spontaneous. However, it is thought that the rennet probably resulted from the milk storage in dried calf stomachs. At that time, they were used as containers, long before clay pots were invented [3]. Today, the main types of cheese are curdled enzymatically. This process is complemented by microbiologic acidification.

There are over than 400 different varieties of cheese known around the world. The characteristics of the cheese depend on the milk source (Table 1), the way of promoting coagulation (in some cheese starter culture is used) and the ripening conditions. Thus, cheeses with a large diversity in flavour, texture and appearance are created [4].

**Table 1. General composition in fat, protein, lactose and ash content in sheep's, goat's and cow's milk. Adapted from [5, 6].**

| | Sheep | Goat | Cow |
|---|---|---|---|
| **Fat (%)** | 6.0 to 6.8 | 3.4 to 4.0 | 3.5 to 4.5 |
| **Protein (%)** | 5.0 to 5.6 | 3.0 to 3.3 | 3.0 to 3.3 |
| **Lactose (%)** | 4.8 to 5.2 | 4.4 to 4.6 | 4.6 to 4.8 |
| **Ash (%)** | 0.90 to 1.0 | 0.65 to 0.80 | 0.65 to 0.80 |

The general process of cheese making can be divided in three steps: coagulation, curd processing and ripening (Figure 1). The raw milk is usually pasteurized and

filtered before the coagulation. In some traditional cheese making, this step is not performed, and raw milk is directly used in the coagulation stage, without being submitted to any chemical or physical treatment. The milk coagulation occurs simultaneously in two different manners:

1. Fermentation pathway

This pathway occurs due to bacteria starters, which are fermentative microorganisms that promote lactic acid fermentation by metabolizing lactose to lactic acid. This step causes a decrease of the pH, which approaches to the isoelectric point (pH=4.6) promoting the precipitation of caseins. The negative charges of micelles and the surface potential responsible for the electrostatic repulsions are neutralized [2]. Lactic bacteria can be added (depending on the type of cheese desired) or can be provided by milk native flora [4]. In this case, it occurs when raw milk is used in traditional cheese making, without pasteurization or other milk treatment. Milk contains lactic bacteria and other microorganisms from contamination (during the milking, due to the use of contaminated instruments and tools) [2].

2. Enzymatic pathway

The enzymatic pathway occurs by adding chymosin (also called rennin), which is a protease enzyme. It hydrolyzes the peptide bound Phe105-Met106 of k-casein, forming two polypeptides: para-k-casein (1-105) that is insoluble in the presence of calcium, that forms the curd along with $\alpha_{S1}$- $\alpha_{S2}$- and β-casein; and glycomacropeptide (106-169) that is soluble and is eliminated when curd is pressed. [3, 6] There are two natural origins for this enzyme: animal and vegetal. When it is originated from ruminant stomachs it is called rennet. From a vegetable origin, it is obtained from the thistle flowers of *Cynara cardunculus* L., also known as cardoon [3].

Thus, from this first step, curd and whey are obtained. The second step is the processing of curd. It is cut and stirred. The whey is drained and the curd is milled and salted. Part of this curd is sold like fresh cheese into pots (cottage and cream

cheese). The rest of the curd is poured into molds. It is pressed and the draining of additional whey takes place.

The last step of cheese manufacture is ripening. This stage can be prolonged between a few days or even years. The ripening is promoted by the action of endogen enzymes or by specific microorganisms. In this case, they release their endogen enzyme by cell lysis, leading to proteolysis and lipolysis. During this step, cheese acquires their organoleptic characteristics [7].

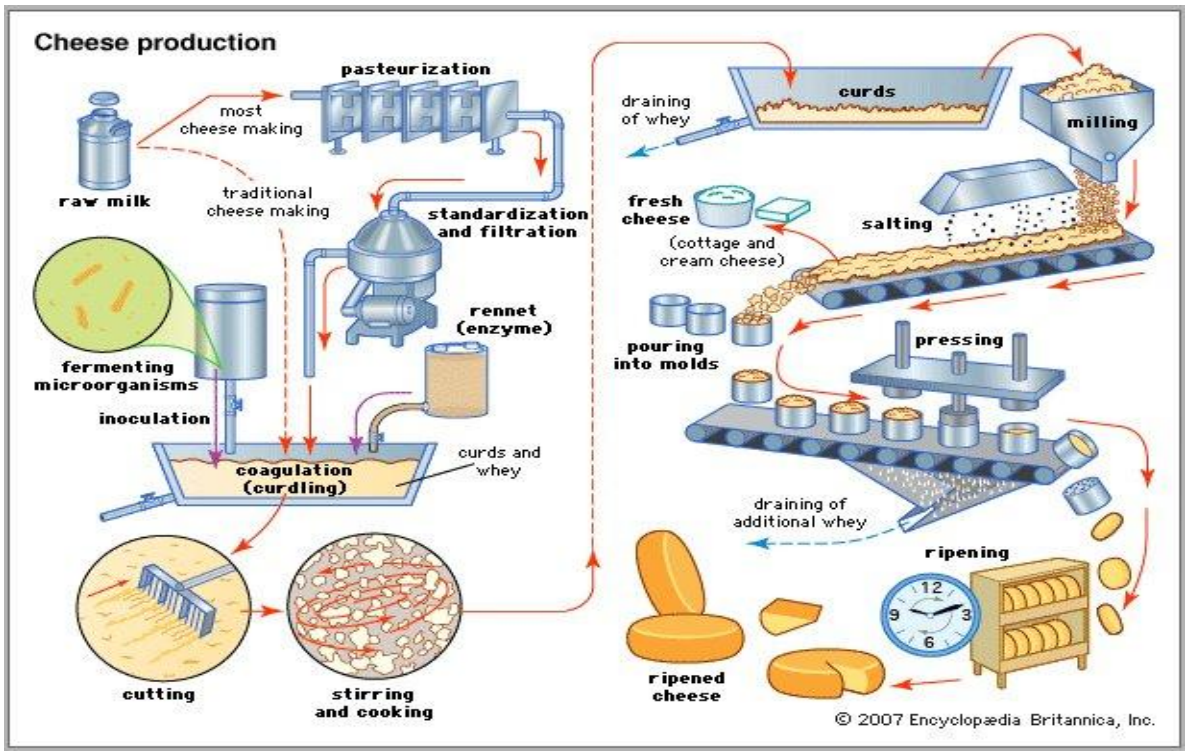


**Figure 1. Cheese production scheme.**

## 1.1 Lactic fermentation

Fermentation in milk products can involve not only lactic acid bacteria, but also other microorganisms (e. g., yeasts) [1]. Lactic acid bacteria (LAB) used in the development of fermented dairy products include *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* and *Pediococcus* genera [1, 4]. They have the ability to metabolize lactose to lactic acid and reduce the pH [4].

There are three fermentation processes: a) via glycolysis pathway, with almost

exclusive formation of lactic acid (homofermentation), b) via pentose phosphate pathway, with formation of lactic acid, acetic acid (ethanol), and possibly $CO_2$ (heterofermentation) or c) via both pathways, depending on the microorganisms involved [1]. *L. lactis* and *L. bulgaricus* are homofermentative microorganisms [1]. These bacteria have lactase activity and they are able to hydrolyze lactose to its monosaccharide units, glucose and galactose, prior to further metabolism [4]. Homofermentative lactic acid bacteria produce lactic acid from these monosaccharides, according to the reaction:

$$\text{Lactose} + 4\ \text{ADP} + 4\ H_3PO_4 \rightarrow 4\ \text{Lactic Acid} + 4\ \text{ATP} + 3H_2O$$

The lactic acid produced by LAB has a significant impact on the safety and quality of the cultured dairy products. The reduction in pH increases the shelf life and safety of the fermented dairy products through the inhibition of spoilage and pathogenic microorganisms. LAB may also contribute to the degradation of proteins and lipids through proteolytic and lipolytic reactions to further develop the unique texture and flavor characteristics [4].

# 2 Serra da Estrela Cheese

*"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." [8-10]*

The previous citation is the definition of the Serra da Estrela cheese or simply Serra Cheese. It is the product description from Serra da Estrela cheese book of specifications [8]. Those specifications were published in Diário da República in 2011 [10].

In 1994, Serra Cheese won the status for legal protection and it was designated DOP product, but it was only registered and protected in the Regulation of the European Commission in 1996 [11]. This way, the consumer is protected by the certification of the geographical origin of the cheese and by guarantee of its high quality [12]. Since 1986, there is a delimited geographic zone to produce Serra's cheese. It was described in the Decreto Regulamentar nº 31/86 in 19/08 [13]. The legally defined area of production covers some municipalities in the central-north of Portugal and it is shown in Figure 2.

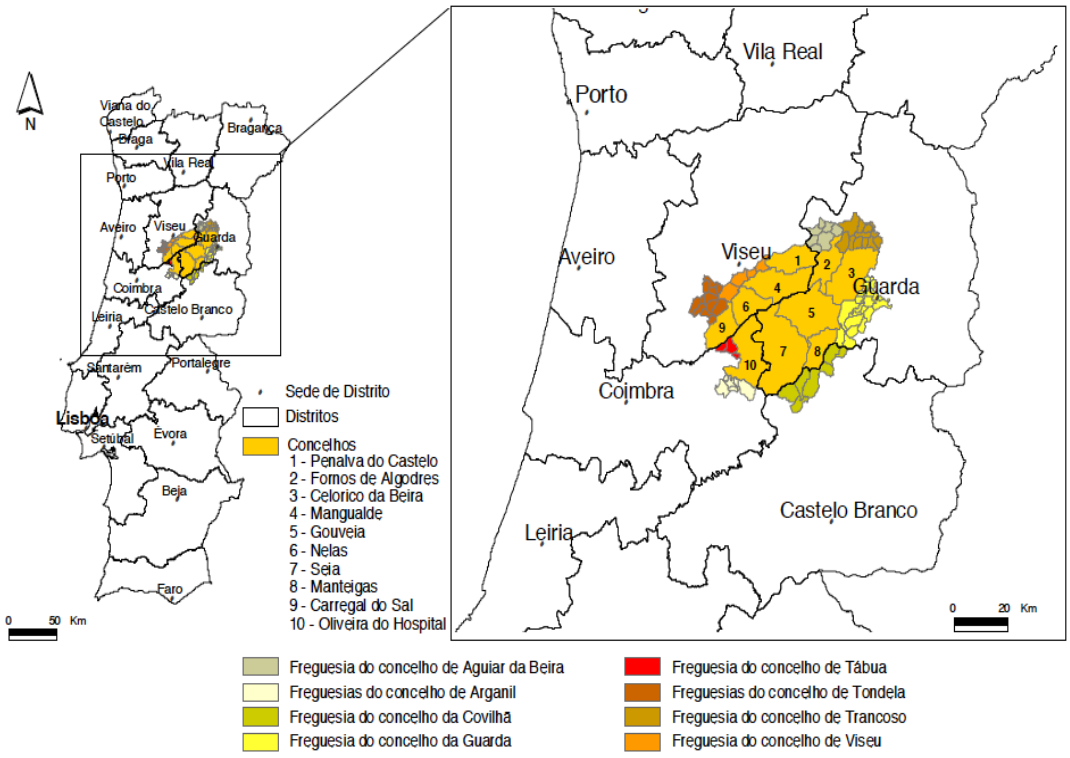


**Figure 2. Delimited geographic zone of production of the Serra da Estrela cheese [8].**

Serra Cheese is probably the most popular and the most appreciated traditional Portuguese cheese [14], due to its unique flavour and texture [15]. This cheese is manufactured from October to May (the typical lactation period of Serra da Estrela ewes) using the unpasteurized milk immediately after collection [8, 15, 16].

There are two types of Serra Cheese: Serra da Estrela Cheese and Old Serra da Estrela Cheese. The main difference between them is the ripening time: the first, between 30 to 120 days and the second, more than 120 days [17]. Serra da Estrela Cheese is characterized also by texture - closed, moderately buttery, deformable when cutting, well connected, creamy and unctuous, with few or no eyes - and sensorially as smooth, clean and slightly acidic *bouquet.* [8].

In 2011, Serra Cheese won in the appetizer category, as one of seven wonders of Portuguese gastronomy [18], due to its unique organoleptic characteristics. All these characteristics result in high values +US$30/kg or €20/Kg. The production of Serra Cheese DOP has been around 120 thousand units per year (information send by email by Estrelacoop). However, these production amounts of certified cheese correspond only to 10% of total Serra Cheese produced.

## 2.1 Bordaleira ewe's milk composition

The Bordaleira ewe's milk is white and very nutritive. The taste is smooth and slightly sweet with a characteristic flavour. The last studies about its composition are from 1997 [19, 20]. Table 2 shows the chemical composition of raw milk of the Bordaleira ewe.

**Table 2. Chemical composition of milk of the Bordaleira Serra da Estrela ewe. Adapted from [14, 19, 20].**

| Water | Fat | Protein | Lactose | Ash | Reference |
|---|---|---|---|---|---|
| | | | (%) | | |
| 80.0 ± 2.7 | 7.8 ± 1.6 | 6.0 ± 1.3 | 4.4 ± 0.5 | NA | [14] |
| 80.9 ± 1.6 | 7.4 ± 1.5 | 6.0 ± 0.9 | 4.6 ± 0.6 | 0.9 ± 0,1 | [14] |
| NA | 8.6 ± 2.0 | 5.0 ± 0.7 | NA | NA | [14] |
| 81.2 | 6.8 | 5.0 ± 0.7 | 5.1 | 0.9 | [14] |
| NA | 6.7 | 6.9 | NA | NA | [19] |
| NA | 7.0 | 7.1 | NA | NA | [20] |

NA = Not available

This milk is generally characterized by higher protein and fat on average of 6% and 8%, respectively, than other sheep's, goat's and cow's milk (Table 1).

## 2.2 Serra Cheese manufacture

The manufacturing techniques used and the environmental conditions lead to cheeses with different characteristics. Serra Cheese manufacture is resumed in Figure 3 and detailed described in Appendix A.

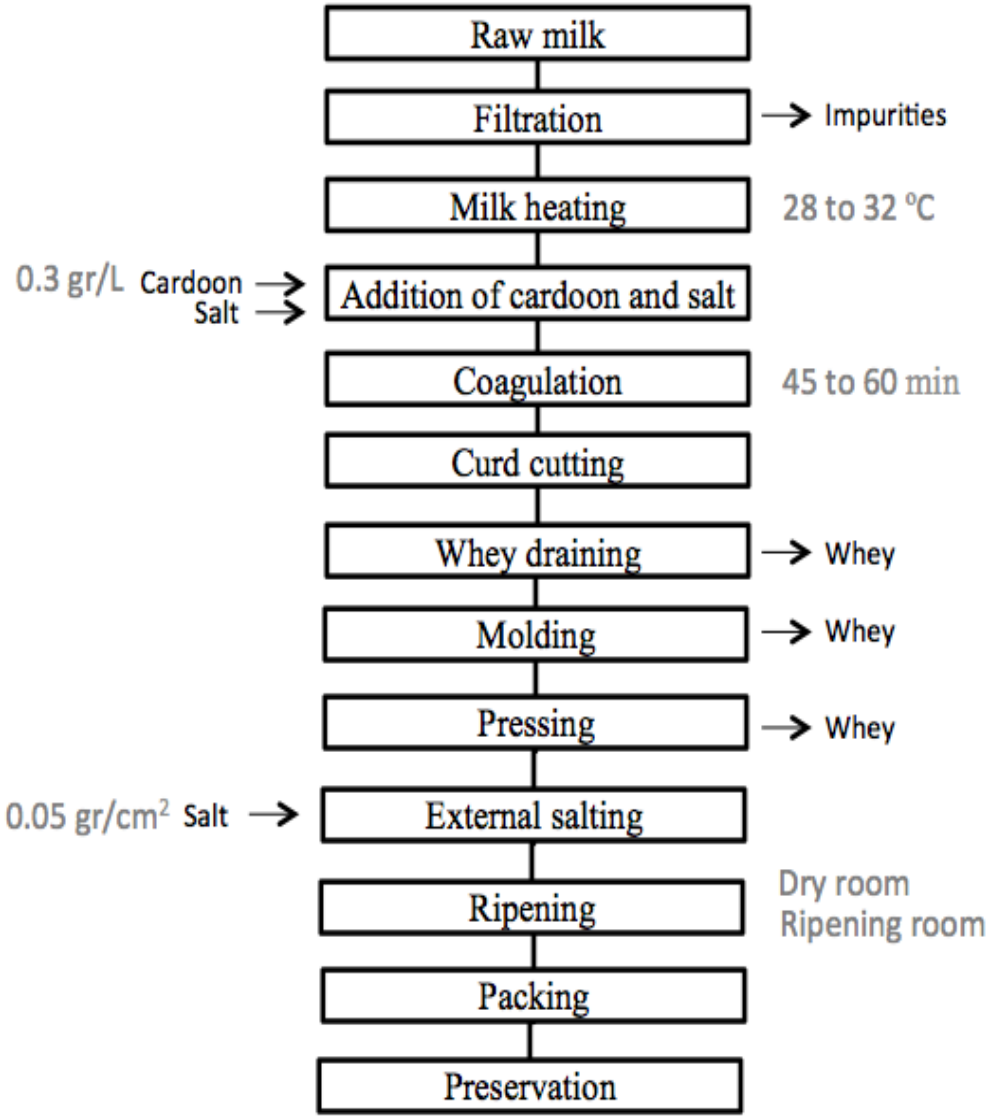


**Figure 3. Serra Cheese manufacture.**

This Portuguese cheese is made with only three ingredients: milk from Bordaleira Serra da Estrela and/or Churra Mondegueira ewes, salt and *Cynara cardunculus*, L. [8]. It is manufactured from raw ewes' milk, only filtered through a cloth (clean, fine and white) to remove impurities (like hair and dust) [8, 17].

Milk coagulation is promoted by the addition of an aqueous extract of thistle

flower, without any commercial starter culture [21]. In the traditional way, the dry cardoon flowers are macerated with salt and water; this paste is placed into a cloth with closed ends; it is submersed in the milk, agitated and squeezed [14]. After Serra cheese coagulation and pressing, the producers rub the top and bottom surfaces of the curd with salt - external salting - which contributes to the formation of the rind [8].

The ripening process of Serra Cheese occurs in two controlled environmental chambers. The first is the dry chamber (*enxugo*), where lactic fermentation starts and simultaneously the *reima* occurs [8]. *Reima* is a white-reddish viscous smear, which is important to obtain a good cheese [14]. In this phase, cheese loses humidity and allows microbial growth favourable to maturation. The second chamber is the ripening chamber itself. The conditions of ripening process in the controlled environmental chambers are listed in Table 3.

**Table 3. Environment conditions of curding chambers in the first 15 to 20 days and between 20 to 45 days of maturation.**

| | Phase | Temperature (°C) | Relative humidity (%) |
|---|---|---|---|
| **First 15 to 20 days** | Fermentation or *reima* | 6 to 12 | 85 to 95 |
| **20 to 45 days** | Maturation | 6 to 14 | 90 to 95 |

The ripening period depends on the type of cheese intended. Butter Serra Cheese ranges from 30 to 45 days [14], old Serra Cheese needs a minimal of 120 days.

#### Serra da Estrela Cheese composition

Table 4 presents the analytical indicators of Serra Cheese and Old Serra Cheese composition referred in the Serra Cheese specifications book (merely indicative values) [8].

**Table 4. Analytical indicators (in dry residue) of Serra Cheese and Old Serra Cheese composition. Adapted from [8].**

| | Protein (%) | Fat (%) | Moisture (%) | Ash (%) |
|---|---|---|---|---|
| **Serra Cheese** | 26 to 33 | 45 to 60 | 61 to 69 | 5 to 6.5 |
| **Old Serra Cheese** | 36 to 43 | > 60 | 49 to 56 | 7 to 8 |

There are some studies that reveal the chemical composition of Serra Cheese,

but they are not consistent, as shown in Table 5. This variation can be due to the absence of raw milk standardization, the lack of standardized procedures for the manufacture and the exact ripening time of the analysed cheese.

**Table 5. Chemical composition of Serra Cheese by several authors. Adapted from [14, 19, 22].**

| Moisture | Fat | Casein | Sugar | Ash | Salt |
|---|---|---|---|---|---|
| (%) | | | | | |
| 48.8 | 28.8 | 19.9 | NA[1] | 4.4 | 2.6 |
| 46.7 to 48.8 | 28.1 to 30.7 | 19.2 to 20.4 | NA | 4.1 to 4.3 | 2.2 to 2.6 |
| 31.9 | 40.1 | 22.2 | 2.2 | 3.4 | 0.9 |
| 39.4 ± 19.3 | 27.9 ± 14.4 | NA | 3.9 ± 4.0 | 5.8 ± 4.1 | 2.6 ± 2.9 |
| 34.0 to 48.8 | 30.6 ± 7.3 | NA | NA | NA | 2.9 ± 1.2 |
| NA | 23.0 to 40.0 | 1.08 to 23.0 | NA | NA | NA |
| 45.41 ± 1.55 | 25.15 ± 2.33 | 22.10 ± 0.39 | NA | NA | 3.05 ± 0.15 |
| 48.43 ± 0.36 | 53.57 ± 1.99 | NA | NA | NA | 1.41 ± 0.10 |

NA = Not available

The chemical composition changes during the ripening process. The fat and protein content slightly decreases during ripening [23]. If cheese ripening occurs under controlled environmental conditions, it will have a slightly more constant moisture content than cheeses made in the farmhouses [14]. The final moisture content is affected by relative humidity conditions, because the ripening conditions control the rate of water evaporation. With lower relative humidity, the moisture content of cheeses is quickly lost, like what happens in May [20]. On the other hand, high relative humidity reduces evaporation of water. It results in cheese with high percentage of moisture contents, high water activity ($a_w$), but it decreases the relative percentage of the remaining contents (like salt, fat, protein and residual lactose contents). High relative humidity, during the ripening process, increases the maturation index. This is observed by extensive proteolysis breakdown, because in high $a_w$ values, proteases and peptidases appear more active [20].

The salt content and the pH in the centre and in cheese surface change during the ripening (Figure 4). 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, and also probably due to water evaporation from the surface of the cheese along with the decrease of the moisture content [23, 24]. After 35 days of ripening, the average of salt-in-moisture concentration values is 4.8%, which is maintained during the ripening time [24].

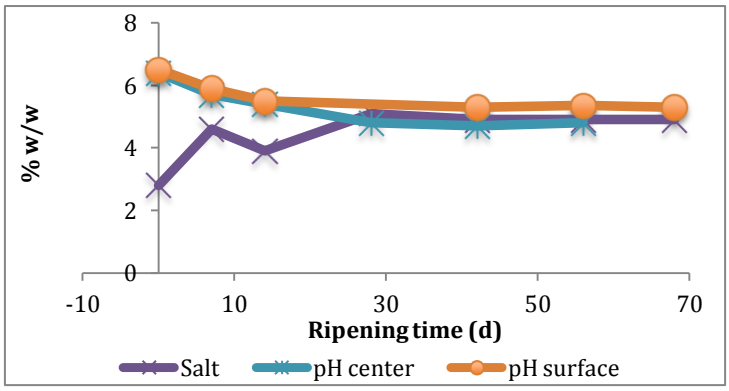


**Figure 4. Salt, pH at the center (pH center) and at the surface (pH surface) of the Serra cheese, during ripening process. Adapted from [23].**

The pH at the surface of fresh cheese manufactured with extracts of *C. cardunculus*, slightly decreases comparatively to raw milk: 6.45 and 6.69, respectively [14, 23]. The pH in the centre and in the surface, decreases considerably from the day of manufacture, reaching 5.23 at 21 day of ripening [14, 23]. This decrease is mainly due to the metabolic activity of lactic acid bacteria, which converts the lactose to lactic acid (reaching maximum numbers at 21 days of ripening) [15, 16]. The pH in the following weeks tends to stabilize. However, the pH in the centre of cheese is lower than at the surface, probably due to higher microbial activity inside, which leads to higher acidity [14]. Macedo *et al.* [24] revelled that cheese manufactured in November, February and May shows no statistically significant differences in pH values at 0, 7 and 21 day of ripening. However, at 35 days, cheese made in May presented a higher pH value (5.30) than cheese made in November and February (5.16 in both). This conclusion is in accordance with previous results of Macedo *et al* [15, 16] that showed lower numbers of lactic acid bacteria in spring. The composition and the pH of curd have a great impact in the texture, flavour and aroma of cheese [14].

The main minerals present in cheese are: sodium (Na), calcium (Ca), phosphorus (P) and potassium (K). Zinc (Zn), cooper (Cu) and magnesium (Mg) are only present in trace levels. During ripening, the concentrations of K, Ca, P, Mg and Zn decrease significantly, probably due to slower losses of these minerals via whey [25].

## 2.3 Microbial profile of Serra Cheese

Serra Cheese is made with raw ewe's milk and, since no starter is added during the production, the raw milk contains the endogenous fermentation starter and other microflora [26]. This native micro flora of milk and the adventitious flora have an important role during cheese ripening.

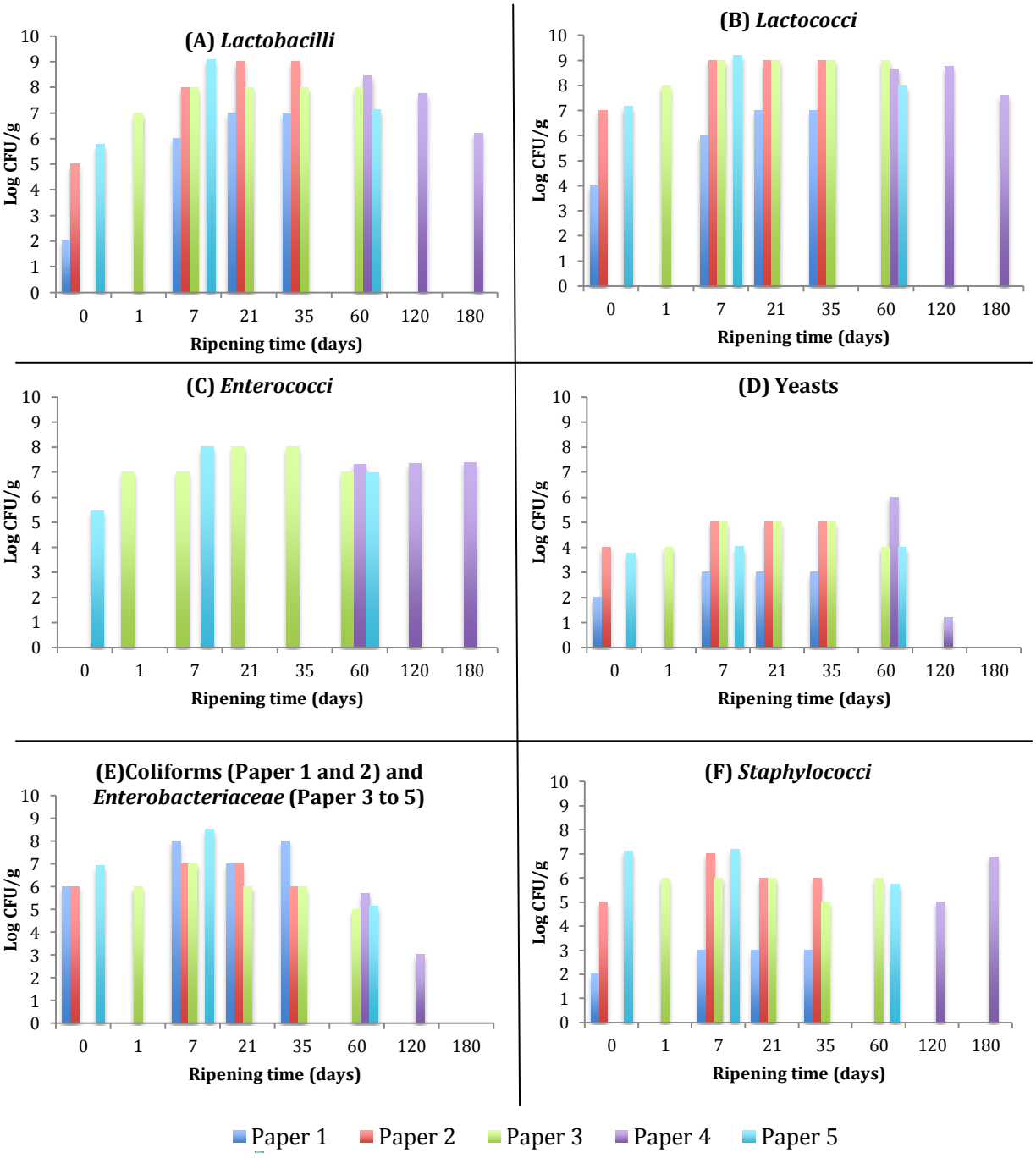


**Figure 5. Average of microbial counts of *Lactobacilli* (A), *Lactococci* (B), *Enterococci* (C), yeasts (D), coliforms and *Enterobacteriaceae* (E) and *Staphylococci* (F) during ripening by several authors. Paper 1 [16], Paper 2 [15], Paper 3 [27], Paper 4 [28] and Paper 5 [22].**

There are 5 publications [15, 16, 22, 27, 28] published between 1993 and 2003, about the microbial profile of the Serra Cheese during ripening. These publications reported great differences in the counts of some microbial groups, as shown in Figure 5.

### 2.3.1 Lactic acid bacteria

Lactic acid bacteria counts are the major part of total microflora during the ripening [28]. In general, in this microbial group are studied the *Lactococci* and *Lactobacilli* counts [16].

According to Figure 5 (A), in the first week, the counts of *Lactobacilli* increased $10^3$-fold and in the second week, 10-fold more. These values remained for one week, afterwards *Lactobacilli* slightly decreased, but are still present with $10^6$ to $10^7$ CFU/g, at the end of the ripening period [22, 28]. This constancy is due to *Lactobacilli* tolerance to dehydrated environments and high salt concentrations [29].

By analysing Figure 5, it can be concluded that *Lactococci* is the major contributor for microflora of Serra Cheese. *Lactococci* were present in high values in all studies, as shown in Figure 5 (B). The quantity profile of this microorganism was equal in publications 2 to 5 [15, 22, 27, 28] and below 100-fold in publication 1 [16]. In the first week, the counts increased 100-fold. Between 7 and 60 days, the *Lactococci* remained with $10^8$ CFU/g. Then, they decreased 10-fold and but remained at high levels ($10^7$-$10^8$ CFU/g) until the end of the ripening period [22, 27]. *Enterococci* were counted only in three publications 3 to 5 [22, 27, 28] (Figure 5 (C)) and they showed $10^7$ CFU/g in the day of manufacture. In one week, they increased 10-fold and remained constant in the next two weeks. The quantified *Enterococci* values were $10^7$ CFU/g at 120 and 180 ripening days. The most abundant LAB found in curd were *Lactococcus lactis* spp [16], which are homofermentative [4] and *Enterococcus faecium* [16].

The lactation period has a significant effect in the number of microorganisms, being higher in January to February and lower from May to June [15], indicating that lower temperature and higher relative humidity during autumn and winter, favours the LAB growth [16].

### 2.3.2 Yeasts

Inside of the cheese, yeasts tended to slowly increase in the first 7 days, from $10^4$ to $10^5$ CFU/g, and then they seem to decrease (only 10-fold), (see Figure 5 (D)). Between 60 and 180 days, the numbers of yeasts had a sharp decrease of 82%, being zero at 180 days [28]. This highlights that the highest rate of yeasts death occurred after 60 days of ripening [28].

Rind samples showed 100-fold higher values than inner cheese samples. Moreover, yeasts were found at higher levels after the second week [30]. The rind is more exposed to environmental manufacturing conditions, so it can be easily contaminated [15]. Ripening rooms, with lower temperature and higher relative humidity during autumn and winter, allow and favour the aerobic yeasts growth [16].

Some yeasts are able to synthesize proteolytic and lipolytic enzymes, which contribute to the development of aroma and flavour during the ripening in daily products [31]. The major contribution of yeasts in ripening cheese is their capacity to use lactic acid, promoting the increase of pH value, allowing the growth of bacteria which are sensitive to acid environments [16].

The yeasts that predominated in cheese with 35 days of ripening were *Leucosporidium scottail, Debaryomyces hansenii* and *Sporobolomyces roseus* [16].

### *2.3.3 Enterobacteriaceae, coliforms and Staphylococci*

The quantification of *Enterobacteriaceae,* coliforms and *Staphylococci* allows knowing the hygienic conditions under which Serra Cheese was made. Some studies presented results for coliforms, as publications 1 and 2 [15, 16], and other for *Enterobacteriaceae,* like publications 3 to 5 [22, 27, 28].

The *Staphylococci* counts in raw ewes' milk changes concerning the different seasons. In May-June there was a higher count of *Staphylococci* due to the temperature, near to the 15 °C, which is favourable to their growth [15]. In winter, there are more LAB that make cheese matrix more acid, which is unfavourable to *Staphylococci* growth [16]. Thus, higher amounts of this bacteria were found in spring and lower in winter [32]. *Staphylococci* were mainly found in the rind cheese compared to the inside, because they require oxygen to survive and grow. Moreover,

the rind is more easily contaminated due to manual washing during the ripening. The counts in the rind and inside did not decrease throughout the ripening. Normally, at 7 days, *Staphylococci* reached the maximum values, $10^3$ CFU/g [22, 27]. Others publications (1, 2 and 4) showed the same behaviour, but counted 100-fold [16] and 10-fold [27] less than in papers 3 and 5. Afterwards, they decreased slightly, only 10-fold until 35 ripening days [16]. After 180 ripening days, the viable *Staphylococci* decreased to $10^4$ CFU/g [28]. These results may show the possibility of health hazards and it was reported than 14% of the counted *Staphylococci* belonged to the *S. aureus* spp, which are clinically relevant strains [33].

There were between $10^2$ to $10^3$ CFU/ml *Enterobacteriaceae* in raw ewe's milk [15]. In cheese, in papers 1 and 2, during the first 21 ripening days, the counts of coliforms increased from $10^5$ to $10^8$ CFU/g. However, between 21 and 35 days [16] the counts of coliforms decreased 10-fold and the same occurred in the other study with *Enterobacteriaceae* [27] (see Figure 5 (E)). This decrease suggests that *Enterobacteriaceae* are somewhat controlled by competing species which will eventually prevail by the end of ripening [27].

In all publications, as shown in Figure 5, the LAB and *Enterobacteriaceae* are present in the same order of magnitude (however in paper 3 [27] were slightly lower). Thus, it revels the poor sanitary conditions during milk collection and/or cheese manufacture, which was more accented in paper 1 [16] than in paper 3 [27]. However, it is possible to verify the improvement of hygienic conditions between 1995 [16] and 2000 [27].

The most abundant and proliferative coliform found in the curd was *Hafnia alvei* [19], that is a psychrotrophic bacterium. *Escherichia coli* was found in all cases of cheese that ripens in winter. These bacteria are able to ferment lactose and are probably responsible for the formation of cheese eye-holes. The contamination of the raw milk by *E. coli* is very low when compared to other contaminations [16].

After 120 days of ripening, the number of yeasts and *Enterobacteriaceae* showed a pronounced decrease [28]. After 4 months of ripening, the number of *Enterobacteriaceae*, yeasts and *Staphylococci* declined sharply to almost negligible levels, making Serra Cheese a microbiologically safe product [28].

## 2.4 Biochemical changes

### 2.4.1 Proteolysis

Proteolysis has been considered the most important biochemical complex process in cheese ripening, because it is responsible for the development of a number of organoleptic features [34]. 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 [23]. Each one plays a specific role, but the whole set affects more the final product than each one individually [35].

Proteolysis in cheese ripening can be divided in two main processes. Primary proteolysis corresponds to the initial step of caseins breakdown, which is made by proteases retained in the curd and, to a less extent, by the indigenous milk enzymes [36], resulting in a range of small and intermediate sized peptides [37]. These peptides act as substrates [38] for secondary proteolysis, performed by proteinases and peptidases of bacteria and leading to the release of free amino acids (FAA) [37].

The enzymes that are present in the aqueous extract of cardoon 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 [8]. From the standard variety of *C. cardunculus* L., two aspartic proteinases were isolated [39] called cardosins A and B, being responsible for the clotting activity of that plant [40]. 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 (Table 6) [36]. This dual composition of the plant rennet might explain the relatively more extensive hydrolysis of caseins, than when animal rennet is used [40].

In general, $\alpha_s$-caseins are more susceptible to proteolysis than β-caseins [23]. In ovine milk cheese, the $\alpha_s$- and β-caseins are degraded in approximately 47.0% and 33.1%, respectively, by proteinases of *C. cardunculus* [23]. However, studies revelled that 82% and 76% degradation of the $\alpha_s$- and β-caseins, occurred during 35 days of ripening [39].

**Table 6. Peptide bonds cleaved by proteinases of *C. cardunculus*. Underlined are the major cleavage bonds in ovine caseinate. Adapted of [37, 41].**

| | |
|---|---|
| **κ-casein** | Phe105-Met106 and Lys116-Thr117 |
| **β-casein** | Leu6-Asn7, Glu44-Leu45, Val82-Val83, Met93-Gly94, Leu127-Thr128, Thr128-Asp129, Val140-Gln141, Thr154-Val155, Leu165-Ser166, Lys176-Ala177, Asp182-Met183, Leu190-Tyr191, Leu196-Gly197 and Arg200-Gly201 |
| **$\alpha_{s1}$-casein** | Phe23-Val24, Phe28-Pro29 and Tyr165-Thr166 |
| **$\alpha_{s2}$-casein** | Val73-Asp74, Phe88-Tyr89, Ile85- Asn86 and Trp193-Thr194 |

The proteolysis in cheese can be evaluated by proteolytic indices. For that, first it is necessary to determine 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). Then, with the experimental data generated, three proteolytic indices can be calculated: ripening extension index, WSN/TN; ripening depth index, TCA/TN; and free amino acid index, PTA/TN [35].

The WSN/TN ratio has been used to follow the aging of cheese, being proportional to proteolytic activity. This ratio was reported to increase throughout the ripening period in cheeses manufactured with plant rennet [23], being 9.5% at 1 day and 36.9% at 35 days of ripening [24].

The 12%-TCA soluble nitrogen allows quantifying small peptides containing between 2 and 20 amino acid residues and FAA [23]. These peptides basically result from secondary proteolysis, by cleavage by the enzymes produced by the starter cultures and released thereby upon lysis. However, in Serra Cheese manufacture no starter cultures are added, thus, relatively low levels of TCA/TN are obtained [36], 5.5% at 35 days of ripening [24]. The TCA/TN ratio has also been used in order to evaluate the action of lactic acid bacteria in the formation of soluble nitrogen compounds in cheese [24].

The PTA/TN ratio represents the free amino acid index, the smallest peptides (that contain less than 6 amino acids residues, with a molecular weight lower than 600 Da) and FAA [24], that are the final products of proteolysis [35]. In Serra Cheese, this value is particularly low, 1.24% [24], which reveals that cardoon has little activity against peptides [24]. The PTA/TN ratio can decrease from the 14 to 21 days, by microbial consumption of the FAA that are available in the aqueous phase during

lactic acid bacteria stage of exponential growth [23]. Ovine cheese manufactured with plant rennet has a lower PTA/TN ratio than cheese made with animal rennet. This is due to proteinases of cardoon that cleave into high-molecular weight peptides, which were not extensively broken down to low-molecular weight peptides and FAA [23].

### 2.4.2 Lipolysis

Serra Cheese is known for its high fat content, which is mainly constituted by triglycerides [2]. Triglycerides can be hydrolysed by lipases into glycerol and three fatty acids. There are some sources of lipases that can be found in native milk, as contaminant in plant rennet, or produced by microorganisms with growth activity in the first ripening week [19, 42]. In cheese, LAB revealed low lipotytic activity [2], but *Lactobacilli* [14] and *Lactococcus* spp. [14] have shown to produce lipases. Psychrotrophic bacteria [19, 43] have a great lipolytic activity and their exocellular lipases are active at pH 5.0 [2] but only in refrigerated milk [12]. *Enterobacteriaceae* are known to produce lipases that breakdown milk fat [28, 44]. Some yeasts also have lipases [2, 19, 28].

Macedo *et al.* [42] evaluated the extension of the lipolysis in Serra Cheese. The results revealed that lipolysis changes during ripening time, being more intense in the first week (the study only analysed 35 ripening days). Seasons also have an impact in lipolysis, which is more intensive in spring than in autumn or winter [42]. The extension of lipolysis seems to be affected by relative humidity, because cheeses with higher moisture content (and lower salt content) lead to higher fat acidity [20].

Gas chromatography (GC) enables the separation of all triglycerides in milk and in cheese, and it was possible to find compounds with carbon number/molecular weight between C24 and C56. Cheese with 42 ripening days showed lower value in triglycerides with C24 to C38, when compared to milk values [45]. Lipolysis is dependent of the number of carbons of fatty acids, being predominant in the low molecular weight fatty acids that occupy preferentially the Sn3 position of the triglyceride molecule [2].

In ewe's milk samples were found 26 different FFA, however in cheese samples were found 39 [45]. The results of the total FFA content are shown in Table 7.

**Table 7. Average of total FFA and ratio of Σ C4-C10/Σ C11-C20 in milk and cheese during 0, 21 and 42 ripening days. Adapted from [45].**

| | Total FFA | | Ratio* |
|---|---|---|---|
| | $mg/kg_{product}$ | $mg/100\ g_{fat}$ | |
| **Milk** | 214.9 | 268.6 | 0.29 |
| **0 days** | 625.6 | 625.6 | 0.28 |
| **21 days** | 1294 | 646.9 | 0.28 |
| **42 days** | 1012 | 226.9 | 0.24 |

*Ratio = Σ C4-C10/Σ C11-C20 of FFA produced from lipolytic activity.

During the first 21 days, the concentration of fatty acids increased from 625.6 to 1294 mg/kg. Then, fatty acid content slightly decreased to 1012 mg/kg at 42 days [45].

As far as the ratio between the sum of short volatile fatty acids (C4-C10) and the sum of the medium and long chain fatty acids (C11-C20) from lipolytic activity is concern, it was smaller after 42 days of cheese ripening (0.24) than in milk (0.29). So, the FFA with C4-10 are more extensively transformed (volatilization, esterification, bacterial catabolism) [45]. These results are in accordance with those of other authors [46] who found higher concentration of long-chair fatty acids in all stages of ripening in ovine cheese.

The FFA composition of ewe's milk is similar to other kinds of milk used in cheese manufacture [45], like cow's and caprines' milk, but ovine and caprine milk have twice the content of FFA than cow [46]. However, the final FFA composition in Serra Cheese, with 35 ripening days, is lower than other cheeses [45].

These results can be justified by several reasons. Lower temperatures do not favour the lipolytic activity [45] and most cheese makers kept the cheese under 12 °C [8]. The short ripening period of Serra Cheese (30-45 days) is another reason for lower lipotytic activity. In addition, Serra Cheese is manufactured only with the addition of plant rennet, which has a low lipolytic activity. Other cheeses ripen during more days and are also made with the addition of other type of rennet with higher lipotytic activity [45].

FFA are precursors of many other compounds like alcohols, esters, aldehydes, ketones, and lactones [47].

#### 2.4.2.1 Lipid oxidation

Lipid oxidation is a major factor affecting the quality of processed dairy products, especially during long periods of storage [48, 49]. It is the major cause of quality deterioration during this period [49, 50], especially concerning lipids that contain polyunsaturated fatty acids (PUFAs) [51].

Lipid oxidation comprises a complex chain of reactions that firstly yields to the production of peroxides (primary products), that give rise to secondary oxidation products [52]. Different pathways for lipid oxidation have been described: autoxidation (radical mechanism), photooxidation (singlet oxygen-mediated mechanism) or enzymatic oxidation (catalyzed by lipooxygenases) [52].

Quantification of primary lipid peroxidation products (hydroperoxides) is difficult due to the unstable and reactive nature of these compounds [50]. Hydroperoxides are further oxidized (as shown in Figure 6), turning into secondary oxidation products, which include aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers [51, 52]. Most of these secondary oxidation products produce undesirable sensorial effects [50], known as rancid off-flavour [49]. Among the secondary oxidation products, non-volatile compounds can be found, such as malondialdehyde (MDA) as main representative, being the most commonly used aldehyde as oxidation marker [52]. It is mainly formed from linolenic acid oxidation and it is not generated by the oxidation of other lipids, which means that this analysis corresponds only to a minor amount of the secondary oxidation products, which spoiled its role as a lipid oxidation marker that is usually assumed for this compound [52].

The most common method to determine MDA in food is the spectrophotometric measurement of MDA with 2-thiobarbituric acid (TBA) [51]. The reaction occurs by “attack” of the monoenolic form of MDA to the active methylene groups of TBA at low pH and high temperature, producing the a chromophore, which is a complex formed by TBA-MDA that gives a maximum absorbance at 532 nm [52]. However, TBA is not selective to MDA; it also reacts with many other compounds (such as: other aldehydes, carbohydrates, amino acids and nucleic acids), interfering in the TBA assay, and consequently leading to variability in the results [52]. For that reason, this method was called TBA reactive substances method (TBARS).

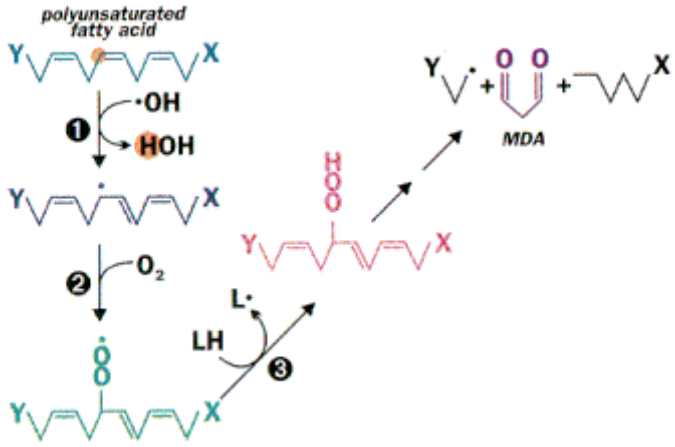


**Figure 6. Formation of MDA during peroxidation of PUFAs. Adapted from [53].**

### 2.4.3 Flavour

Cheese is not only distinguished by its physical feature but also by its flavour, which depends on the milk used, the manufacturing methods and the conditions and duration of the ripening phase [45]. Cheese manufactured with raw milk acquires more intense flavour than cheese made with pasteurized milk, due to the presence of high levels of native lactic acid bacteria [44, 54]. The aroma compound and the flavour profile of Serra Cheese result mainly from the microorganism and enzymes, thus being an enzymatic process with several and interdependent reactions during ripening [55]. However, there is low knowledge about the reactions that lead to flavour in Serra Cheese matrix [28].

The major families of volatile compounds in Serra cheese found by gas chromatography mass spectrometry (GCMS) were ketones, pyrazines, alcohols, aldehydes, phenolic compounds, ethyl esters and FFA during 180 days of ripening [44], as shown in Table 8. The volatile compounds in Serra Cheese with 42 ripening days, that are present in low concentration in the order of ppm or mg/Kg, present high relevance in flavour profile and are: ethanol, methanol, acid acetic, 2,3-butanediol (diacetyl) and esters. Acetic acid was associated with a positive flavour [56]. Diacetyl has great contribution for the typical flavour, "butter-cream texture" in cheese with soft consistency [57]. Esters and ethanol were associated with fruity flavours [47].

**Table 8. Compounds identified in volatile fraction of Serra cheese by GCMS during 180 ripening days. Adapted from [44].**

| | |
|---|---|
| **Ethyl esters** | Ethyl: butanoate, pentanoate, hexanoate, octanoate, decanoate and dodecanoate |
| **Ketones** | 2-Heptanone; 2-nonanoe and 2-undecanone |
| **Phenolic compounds** | 2,4-Dimethylbenzene; 1,2,4-trimethylbenzene and benzoic acid |
| **Alcohols** | 1-Decanethiol; 2-phenyethanol; phenol, 4-methyl phenol; benzyl alcohol and 4-methyl-4-nonanol |
| **Pyrazines** | 2,5-Dimethylpyrazine; trimethylpyrazine and diethylpyrazine |
| **Aldehydes** | 2-Hydroxy-4-methylbenzaldehyde; 2,4-Decadienal; 2,4-octadienal; 2,4-nonadienal; 3,7-dimethyl 7-octenal; 2,4-dodecadienal and octadecanal |
| **Sulfur derivatives** | Dimethyldisulfide |

Fox *et al.* [58] study the effect of skim milk in flavour profile and proved that the milk fat is essential for flavour development. FFA contribute direct or indirectly to the flavour, being the precursor for the formation of other compounds [46]; however FFA did not seem to contribute to "off flavours" in Serra Cheese [45].

The organic acids found with major odour activity values (OAV)[1] were isovaleric, capric and butyric acids present in 232, 113 and 88 mg/kg, respectively [44]. Caproic and caprylic acids were also found in high concentration, 210 and 100 mg/kg at 180 ripening days [28]. The high concentration of long-chain fatty acids in Serra Cheese [45] do not contribute to aroma due to low relative volatility, thus being practically odourless [44].

[1] OAV is the concentration of compound/odour threshold for all short-chain FFA

# 3 Microbial Safety of Serra Cheese

As already described, Serra Cheese is produced with raw milk, which during the milking and cheese manufacture is subject to several ways of contamination. This way, Serra Cheese has a great amount of indigenous microflora as described in section 2.3 Microbial profile of Serra Cheese.

One study realized by INRB-INIA (Instituto Nacional de Recursos Biológicos - Instituto Nacional de Investigação Agrária) found *L. monocytogenes* in 39% of 91 randomly gathered cheeses from several commercial surfaces [59]. Moreover, many outbreaks of listeriosis around the world were registered during the last 20 years, due to consumption of cheese contaminated with *L. monocytogenes* [60, 61]. Thus, this pathogen can be present and requires strict control due to the risk that *Listeria* represents to the public health.

Besides the possible presence of some pathogenic charge, Serra Cheese, like other cheeses, can allow deteriorative microorganisms growth during ripening and cheese storage. They promote chemical and physical changes, which will result in unpleasant flavour and odours and also changes in consistency, colour and appearance of cheese. In addition, these microorganisms release intra and extracellular enzymes that deteriorate the quality of cheese. Thus, it is necessary to control the growth of this microorganisms to decrease the deterioration of cheese [4]. The deteriorative microorganisms present in cheese are mainly filamentous fungi and moulds.

A new food processing technology, that could reduce at the same time the risk of the presence of *Listeria monocytogenes* and also deteriorative microorganisms in Serra Cheese, will improve the microbiological quality and safety of this traditional product.

# 4 High hydrostatic pressure technology

*"High pressure offers unique advantages over traditional thermal treatments." [62]*

Pasteurization is the thermal treatment most used in food preservation [62]. This technology reduces microbial levels, however, it causes undesired effects in food, such as: lost of flavour, colour, texture, smell and nutritional value, that leads to the loss of final product quality [63]. Consumers prefer more natural, preservative-free, shelf-stable, safety and tastier food that created the need of developing improved food processing technologies [64].

Since 1970, there is 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 [62].

HPP offers unique advantages over traditional thermal treatments. This technology produces microbiologically safe products and causes negligible impairment in sensory properties and in nutritional quality of foods [65-67]. There are many advantages of HPP for preservation of food, such as: elimination or reduction of heating, avoiding thermal degradation of some components in products; retention of flavour, colour and nutritional value; uniform and instantaneous transmission throughout food products and reduced or no need of chemical additives addition [63, 64]. HPP is a key to maintain the quality attributes of processed food, while improving shelf life and convenience.

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 [65]. Nowadays, there are vessels with a volume up to 420 L and machines operating at pressures in the rage 100 to 1000 MPa [62, 64].

A basic principle governing the effect of HPP in foods is the isostatic principle, being the pressure applied in HPP instantaneously and uniformly transmitted throughout the food, regardless of size, shape, and composition [67]. Thus, food is treated by uniform pressure from every direction and when pressure is released, the food returns to original shape.

## 4.1 Application of HPP in cheese manufacture

There are many areas of interest of HPP application in manufacture of cheese [66]. HPP was tested in milk and it was reported the inactivation of microorganisms, the reduction of rennet coagulation time and the increase of the yield of cheese [68, 69]. According with Huppertz *et al.* and O'Reilly *et al*. [62, 69, 70], the thermal pasteurization of milk can be replaced for HPP in cheese manufacture. HPP was also directly applied after pressing the curd or during the ripening and it influences the cheese preservation and the ripening process [67], as explained further ahead.

### 4.1.1 Application of HPP for microorganism inactivation in cheese manufactured from raw milk

Treatment of cheese with pressure between 200 to 800 MPa, showed the ability to inactivate or reduce LAB, pathogenic and spoilage microorganisms. This technology has been applied in cheese manufactured from raw milk, to improve its microbiological quality [71], increasing cheese safety and shelf life [66]. There are some publications of HPP in ovine cheese manufactured from raw milk, but none in Serra Cheese (Table 14– Appendix B).

Microbial inactivation by HPP was influenced by microbial characteristics, process conditions and product parameters [72]. As far as microbial characteristics are concerned, yeasts and moulds are microorganisms that are more sensitive to HPP than bacterium. Among bacterium, Gram-positive bacteria are more pressure-resistant than Gram-negative bacteria [67]. Concerning the process conditions, microbial inactivation increases by increasing the treatment intensity and by increasing the 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) [73].

#### *4.1.1.1 Effect of HPP in inactivation of pathogenic and spoilage microorganisms*

In order to achieve preservation, HPP was used in cheese to inactivate or reduce pathogenic strains such as, *Staphylococcus aureus* [69, 74], *Listeria monocytogenes* [68, 75-80], *Escherichia coli* [68, 69, 81, 82], as well as spoilage microorganisms (such as *Staphylococcus* spp. *Enterococcus* spp.), coliforms [83], yeasts and moulds [84].

*S. aureus* CECT 976 was studied by Arqués *et al.* [74], showing more than 5.3 log reductions after 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 HPP at 300 and 400 MPa for 10 min [83]. Alonso *et al.* [85] at 60 ripening days showed 0.7 and 1.3 log reductions at 400 and 500 MPa, 10 min at 10 °C of *Staphylococci* in raw ovine milk cheese.

HPP was applied in raw cow cheese inoculated with *Listeria monocytogenes* Scott A. The HPP (500 MPa, 5min at 10 °C) was applied at 2 and 50 days of ripening, showing 5.02 log reductions and complete inactivation (>6.34 log reductions), respectively [86]. *Listeria monocytogenes* ATTC 19115 inoculated in raw ewe milk cheese was reduced in 4.9 logs after HPP at 600 MPa during 10 min at 25 °C [87].

In Cheddar raw milk cheese, *E. coli* K-12 was completely inactivated (>6.5 log reductions), after HPP at 350 MPa, 3 min at 50 °C [88]. Rodríguez *et al.* [82] studied the effect of HPP 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).

*Enterococci* were pressure treated at 300 and 400 MPa, (10 min at 10 °C) in La Serena cheese (raw milk cheese), being reduced in 2.05 and 2.68 when pressured on day 2, and 1.37 and 1.98 on day 50 [83]. In the same publication, using the same HPP conditions, coliforms were studied, 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, when applied on day 50 of ripening.

Yeasts are not associated to food-borne diseases, however they are responsible for cheese spoilage [84]. HPP above 300 MPa during 5 min in fresh curd cheese caused complete inactivation (> 4 log reductions) of spoilage yeasts [84].

According with these studies, the barotolerance of spoilage and pathogenic bacteria in cheese, follow the order: *S. aureus* > *L. monocytogenes* > *E. coli* > yeasts and moulds [89].

#### *4.1.1.2 Effect of HPP in beneficial microorganisms*

HPP does not affect only pathogenic and spoilage microorganisms, but also affects mesophilic and termophilic LAB. Termophilic LAB in raw ewe cheese were reduced in 0.46 [90], 1.9 and 2.7 log reductions [85] after HPP at 300, 400 and 500 MPa, respectively, during 10 min at 10 °C. Mesophilic LAB in the same cheese with the same HPP, were reduced in 0.68 [90], 2.1 and 4.2 log reductions [85].

According to these studies, HPP cheese above 500 MPa cause a great reduction of beneficial microorganisms present in cheese.

### 4.1.2 Effect of HPP in quality of cheese during ripening and storage

HPP can affect some biochemical characteristics, leading to their change during the ripening and storage; it is necessary to understand the effect of this technology on proteolysis, lipolysis (Table 15 – Appendix B), physicochemical proprieties, on rheological properties and on sensorial properties. However there are few studies concerning the effects of HPP during cheese storage.

#### *4.1.2.1 Influence of HPP on proteolysis*

The effect of HPP on proteolysis during ripening was studied in 4 cheese types made with ewe's milk: La Serena (raw milk cheese) [71], Spanish ewe's milk cheese (pasteurized milk cheese) [91-93] and Hispánico cheese (raw milk cheese but made with ewe and cow milks) [94] and during storage on Irish blue-veined (raw milk cheese) at 4 °C [95] and on Hispánico cheese at -24 °C [85, 90].

After 50 days of ripening, La Serena cheese 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 [71].

Irish blue-veined cheese with 42-days-old was exposed to HPP at 400 to 600

MPa for 20 min at 20 °C, which accelerated primary and secondary proteolysis [95]. The breakdown of β- and $\alpha_{s2}$-casein was accelerated and the PTA/TN index increased after 28 days of storage (10.8% in HPP cheese and 9.0 % in control cheese) [95].

Hispánico cheese made with a mixture of raw cows' and ewes' milk (80:20), was treated after 15 days of ripening at 300 [90] and 400-500 MPa [85] (5 min at 10 °C) and the treatments accelerated the hydrolysis of casein and increased the FAAs levels, after 45 days of storage.

Ewe's cheese was pressured after 15 days of ripening at 500 MPa (10 min at 18°C), and at 75 days of ripening were observed lower FAA contents than control cheeses [96].

There are other studies, that verified the influence of HPP in proteolysis during storage, in other cheese varieties, mainly in Cheddar [97, 98], Camembert [99], Gouda [99, 100], Edam [101] and Garrotxa cheese [102].

All studies considered, it can be concluded that HPP treatments could accelerate or arrest proteolysis in cheese ripening. The results obtained depend on the cheese variety and the intensity of treatment. In general, HPP between 300 and 500 MPa showed: acceleration of casein breakdown, increase of the total FAA content and increase of the SN/TN index.

HPP inactivates enzymes and microorganisms, but also activate some enzymes, that may be released through the lysis of starter cells [96] and may destabilize casein micelles [70]. According to this, HPP enhances proteolysis due to weakening of hydrophobic interactions, which might led to an increased exposure of the susceptible bonds allowing their cleavage by proteolytic enzymes [71].

#### *4.1.2.2 Influence of HPP on lipolysis*

There are few studies about the effect of HPP in lipolysis during the ripening in cheese manufactured with ewe milk [93, 103].

In ewes' milk cheese pressure-treated at 400 to 500 MPa on day 15 of ripening for 10 min at 12 °C, it was reported a lowest concentration on FFA than in control cheese, both evaluated after 45 days of ripening [93].

Hispánico cheese with 15 days of ripening was treated at 300 MPa (10 minutes at 10 °C) by Alonso *et al.* [90] and showed lower FFA concentration than control cheese at 45 days of ripening, 1259.65 and 1205.49 mg/kg, respectively. In another study by Alonso *et al.* [85], the same type of cheese was treated at 400 MPa (10 minutes at 10 °C) after 15 days of ripening and showed higher FFA concentration than control cheese at 45 days of storage, 813.9 and 754.4 mg/kg, respectively.

In general, cheeses treated at 300-500 MPa showed a lower rate of lipolysis during storage.

### 4.1.3 Effect of HPP in cheese characteristics

Serra Cheese has peculiar characteristics, physicochemical and sensorial, which may be changed, when processed by high pressure (Table 16 – Appendix B). Thus, other studies made with ewe's cheese can be indicative of possible changes that can occur after pressurization.

#### *4.1.3.1 Effect on physicochemical proprieties*

Studies demonstrated that protein, fat, ash, moisture and nutrient content do not change with HPP [104-106]. Nevertheless, HPP modify the pH values, the water retention capacity, the salt distribution and the colour of the cheese.

Several cheeses showed a higher pH after HPP, such as: La Serena [71, 86], ewes' milk cheese [91, 92, 107], Cheddar [104], Edam [101], Garrotxa [102, 108], Gouda [99, 100], and Manchego cheese [106]. HPP reduces LAB, decreasing the capacity to produce lactic acid (as result of damage of glycolytic enzymes).

HPP treatments also influence the water retention and salt distribution in cheese matrix. Ewe's cheese treated at 200 – 500 MPa on day 1 of ripening showed lower moisture content than control cheese, since to HPP promoted water expulsion from cheese [107]. But at 15 days of ripening, control cheese and pressured cheese showed identical moisture contents [107].

As far as salt distribution is concerned, HPP improves solute diffusion in the cheese matrix. In ewes' milk cheese a better salt diffusion was observed after being

treated at 300 MPa for 10 min at 12 °C, on day 1 or 15 of ripening. Ewe's processed cheese showed high level of salt content in medium and interior of cheese than control cheese [92].

Colour of cheese is another parameter affected by HPP. The effect depends on the pressure intensity, treatment temperature and duration. Ewe's milk cheese treated at 300 MPa on day 1 showed significantly lower *L-values* (visual lightness) and higher *b-values* (yellowness to blueness), than control cheeses and cheese pressured at 15 days of ripening [92]. So, HPP treatment resulted in more yellowness of cheese.

#### *4.1.3.2 Effect on rheological properties*

Rheological properties influence texture, eating quality, and physical behaviour and are dependent on composition, microstructure, macrostructure, and physicochemical state of cheese components [109].

In La Serena cheese rheological proprieties after pressure treatments were studied. Cheese pressure-treated at 300 or 400 MPa for 10 min at 10 °C on day 2 of ripening showed higher fracturability, hardness, and elasticity than control cheese and cheese treated at 50 ripening days, at 60days. HPP at 50 days did not influence the texture after 10 days of storage after the treatment. Trained panellists revealed that HPP on day 2 had a negative effect on texture preference and taste quality. However, when pressure was on 50 days applied, it did not affect texture preference and taste quality [71].

In ewe's milk cheese, after pressure treatment at 200 to 500 MPa for 10 min at 12 °C, applied on day 15 of ripening, it was reported that the results were analogous to control cheese [107]. Firmness in ewe's cheese treated at moderate pressures, 200 to 300 MPa, was improved. However, cheese processed at high pressure, 500 MPa, revealed high deformability and low fracturability and rigidity. Juan *et al.* [92, 107] attributed these results to higher water retention capacity, higher pH value, cheese matrix modifications as weakening of intermolecular structure, and a more homogeneous microstructure in HPP-treated cheese (reduce the area of fracture), which contributed to higher disposition to deformation.

Hispánico cheese treated at 300 [90], 400 and 500 MPa [85] showed lower fracturability, hardness and elasticity after 60 days of storage.

In general, HPP promote the decrease of the fracturability, the crumbly, the hardness and the increase of the elasticity and the disposition to deformation, due to more homogeneous microstructure, more water retention and weakening of intermolecular structure.

#### *4.1.3.3 Effect on sensorial properties*

One problem of the traditional methods for food processing is the heating, which promotes changes that affect the sensorial characteristics. HPP retains the sensory quality characteristics of fresh food products, if the treatment conditions are not intense and not applied in an early ripening stage. When early applied, causes a great reduction in microorganism charge, which result in a decreased amount of most volatile compounds, thus affecting aroma [110].

Volatile compounds, odour and aroma were evaluated in La Serena cheese treated at 300 and 400 MPa (10 min at 10 °C) on day 50 after manufacture. After 10 days, HPP was showed not to influence the volatile compounds profile and sensorial characteristics of the cheese [111].

Ewe's milk was processed at 200 MPa (10 min at 12 °C) on day 15 of ripening, and the volatile profile was similar to control cheese [91]. In another study, Ewe's milk cheese was pressured at 300 MPa on day 1 and 15 being analysed by panellists at 30 and 90 days of ripening. The treated cheese on day 1 received the lowest taste, aroma and odour quality scores than control cheese [92], they preferred the treated cheese at 15 ripening days [92, 107].

Hispánico cheese with 15-days-old was studied by Alonso *et al.* [85] and did not show significant differences in flavour intensity and quality for 45 days of storage, after it be pressured at 400 and 500 MPa (10 min at 10 °C).

# III. OBJECTIVES

The aim of this work was to study the effect of HPP on Serra Cheese after the processing and during storage on:

- Indigenous microflora: total microflora, lactic acid bacteria, yeasts and moulds and *Enterobacteriaceae*;
- Inoculated *Listeria innocua* as surrogate for *Listeria monocytogenes*;
- Physicochemical changes: water content, water activity, pH-values, titratable acidity, proteolysis and lipid oxidation.

The rationale behind this work is to see the possibility to render Serra cheese free of potential pathogen by HPP, with no changes on quality.

# IV. MATERIAL AND METHODS

## 1 Cheese manufacture and sampling

One batch of Serra da Estrela cheese was manufactured according to traditional procedures [14] (Appendix A) in December in a dairy of the DOP region, Oliveira do Hospital, Portugal.

The sampling was performed by choosing four cheeses at 45 days of ripening. Fractions from each cheese were obtained after removal of the rind. The innermost and the intermediate layers of the three cheeses were handed and homogenized aseptically and divided in two groups. The first group was divided into several samples for microbiological and physicochemical analysis after treatment and during storage. The second group was inoculated with *Listeria innocua* NCTC 10528 and divided in samples. All samples were placed into polyamide-polyethylene (PA-PE) bags, previously irradiated with UV light for 15 min, and vacuum-sealed at 85%.

## 2 Culture and inoculum preparation

*Listeria innocua* (10528, National Collection of Type Cultures, UK; NCTC) was used to inoculate the second group of cheese samples. The strain was obtained cryopreserved from the Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Porto and was revived in Trypticase Soy Broth (TSB; Liofilchem, Italy) and incubated overnight at 37 ± 1 °C, 170 rpm in VWR Incubating Orbital Shaker. The broth culture was checked by plating into Trypticase Soy Agar (TSA; Liofilchem). *L. inoccua* was stored on TSA petri dishes at 4 °C. One colony isolated from TSA plate was picked and inoculated in 50 mL of TSB, incubated at 37 ± 1 °C, 170 rpm, overnight.

Two hundred microliters of the previous broth were transferred to 200 mL of fresh TSB in 250 mL Erlenmeyer flask, and left 10 to 12 h at 37 ± 1 °C, 170 rpm in order to obtain cells in stationary phase of growth. The growth of bacteria was followed hourly by optical density (at 600 nm). At the same time, decimal dilutions were prepared in Ringer's solution (Merck, Germany) and were plated in TSA. After 48 ± 2 h at 37 ± 1 °C the colonies formed were counted.

# 3 Inoculation procedures in cheese

Under aseptic conditions, the cellular suspension was centrifuged at 2500 xg for 20 min at room temperature. The harvested cells were washed twice with 1400 μL of Ringer's solution after removal of the same volume of supernatant. It was used 5.7 mL of cell suspension to inoculate 100 g of the second group of cheese samples.

# 4 High Pressure Processing

The high pressure processing (HPP) was carried out using a hydrostatic press (High pressure system, Model U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature and a mixture of propylene glycol and water (60:40) was used as pressurizing fluid.

Cheese samples were treated at three pressure levels with different duration, at 400, 500 and 600 MPa for 10, 5 and 3 min, respectively. The initial temperature of the pressure vessel was set to 4 °C. For each pressure treatment, cheese samples previously divided in bags were inserted into a small flexible plastic bag that was also vacuum-sealed, this procedure was repeated for all samples. Samples with inoculated *L. innocua* were treated with the same procedure.

**Table 9. Nomenclature attributed to non-processed samples and pressurized samples for Group I and Group II of sampling; and the aim of study.**

| | **HPP Conditions** | | | |
|---|---|---|---|---|
| | **Pressure (MPa)** | **Duration (min)** | **Nomenclature** | To study the effect of HPP after process and during the storage on: |
| **Samples Group I** | - | - | NP | General microbiology and physicochemical parameters |
| | 400 | 10 | P400/10 | |
| | 500 | 5 | P500/5 | |
| | 600 | 3 | P600/3 | |
| **Samples Group II** | - | - | L+NP | Inoculated *Listeria innocua* |
| | 400 | 10 | L+P400/10 | |
| | 500 | 5 | L+P500/5 | |
| | 600 | 3 | L+P600/3 | |

After HPP, cheese samples were stored at 5 °C until 14, 42, 70 and 100 days, then at −80 °C until further use for physicochemical analysis. The unpressurized cheese samples were used as a control and were called non-processed cheese (NP). The nomenclature used to distinguish the samples is shown in Table 9.

# 5 Microbiological analysis

The first group of samples was analysed for counts of aerobic mesophilic microorganisms, mesophilic acid lactic bacteria, *Enterobacteriaceae* and yeasts and moulds. From the second group of samples were analysed the viable counts of *Listeria*.

## 5.1 Sample preparation and dilution

One gram of each cheese sample was homogenized with 9 mL of Ringer's solution for 4 min in a Stomacher 80 Biomaster. Decimal dilutions were prepared in Ringer's solution. Duplicates of each sample were plated twice on appropriated media.

## 5.2 Count of total aerobic mesophilic microorganisms

Total aerobic mesophilic counts were determined in plate count agar (PCA; Merck), following the standard method NP 4405 [112] / ISO 4833: 2003 [113], being the pour-plated method used with 1.0 mL of diluted solution sample. The plates were incubated aerobically at 30 ± 1 °C for 72 ± 3 h and the yellow colonies formed were counted.

## 5.3 Count of total anaerobic mesophilic lactic acid bacteria (LAB)

Mesophilic LAB were grown anaerobically using Man, Rogosa and Sharpe (MRS; Merck) medium, which allow cultivation of *Lactobacillus* spp. The diluted solution sample was plated in double layer, using 1.0 mL to pour-plated in the first layer. The anaerobic conditions were created through anaerobic jars (Merck) with Merck Anaerocult® A (Merck), these conditions were confirmed by Microbiologie

Anaerotest® (Merck). The plates were incubated at 30 ± 1 °C for 5 days and the yellowish-white colonies formed were counted, according to ISO 15214:1998 [114].

## 5.4 Count of Enterobacteriaceae

*Enterobacteriaceae* counts were done in violet red bile dextrose agar (VRBDA; Merck), by pour-plated method, being incubated for 24 h at 37 ± 1 °C aerobically and counted the red-pink colonies formed, according to the described in standard method NP 4137:1991/ ISO 21528: 2004 [115].

## 5.5 Count of yeasts and moulds

Yeasts and moulds were enumerated on rose-bengal chloramphenicol agar (RBCA; Merck) medium, according to the standard method NP 3277-1 [116]. The spread-plate method with 200 µL per sample using five plates (having duplicate of sample) with serial dilutions. RBCA plates were incubated at 25 ± 1 °C for 5 days, being counted pink colonies of yeasts and moulds as filamentous colonies, with various shades of pink on the reverse.

## 5.6 Count of *Listeria innocua*

The viable counts of *L. innocua* were determined as the number of characteristic colonies on plates of PALCAM agar selective agar base (Liofilchem), with selective supplement for PALCAM (Liofilchem). One hundred microliters were spread on the surface of the medium (by pour-plated method). The plates were incubated at 37 ± 1 °C for 48 ± 1 h, being the grey-green colonies surrounded by a black zone counted. This methodology is according ISO 11290-1:1998 [117].

## 5.7 Microbial counts

Petri dishes containing 30-300 colony forming units (CFU) were selected for counting, according to ISO 4833:2003 [113]. In RBCA and PALCAM media were

considered counting realized in plates containing 15 – 150 colonies. The microbial counts were calculated following the equation (1):

$$N = \frac{\sum Characteristic\ colonies}{V[(n_1 + 0.1 \times n_2) \times d]} \quad \textbf{(Equation 1)}$$

being:

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

$V - Sample\ volume\ (mL)$

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

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

$d - 1^{st}\ dilution$

The results were converted into logarithmic decimals of the number of CFU per g of sample, and values below the limit of quantification were considered < 2.0 log CFU/g.

# 6 Physicochemical analysis

## 6.1 Determination of moisture content – oven method

In silver paper was weighed 1 g of cheese in triplicate per sample and dried to a constant weight (ca 72 h) at 105 °C using a drying equipment.

## 6.2 Determination of water activity ($a_w$)

The sample was placed in the cuvette of the Novasina – *Lab*Swift-$a_w$ analyser (Switzerland). Direct reading was performed in the equipment after the value had stabilized (± 45 minutes) at 20 °C. Each sample was read twice.

## 6.3 Determination of pH value and titratable acidity (TA)

Solution preparation: Five grams of cheese were added to 52.5 mL of water at 40 °C and homogenized in a Stomacher at high-speed during 120 s. The solution was

filtered through Whatman grade No. 41 and cooled to room temperature, according to AOAC 920.124.

Determination of pH value: Using the previous solution, the pH value was measured with a pH meter (Crison – Titromatic 1S), which was calibrated with pH 4.0 and 7.0 buffer.

Determination of titratable acidity (TA): Ten mL, representing 1.0 g sample, were titrated with standard NaOH 0.1 M, using automatic titration (Crison – Titromatic 1S) with set point 8.9. The acidity was expressed as lactic acid, being 1 ml of 0.1M NaOH = 0.0090 g lactic acid. The TA was quantified in duplicate per sample and in triplicate per analysis, according to AOAC 920.124.

## 6.4 Proteolysis analysis

### 6.4.1 Determination of water soluble nitrogen (WSN)

Bradford reagent: 100 mg of Coomassie Blue G250 were dissolved in 50 mL of 95% ethanol. This solution was then mixed with 100 mL of 85% phosphoric acid and made up until 1 L with distilled water. The reagent was filtered through Whatman no. 1 filter paper and then stored in an amber bottle at room temperature. The reagent was filtered every time before used to remove the precipitated formed during storage [118].

Protein standards: Bovine serum albumin (BSA) at a concentration of 2 mg/mL was prepared in distilled water and used as stock solution. From this, protein standards ranging from 0.01–1.6 mg/ml were prepared in distilled water. The protein standards were stored at –20 °C and calibration curve was performed every time that samples were quantified. The standard curve obtained was fitted by the equation (2), as shown in Figure 22 in Appendix C.

$$\text{Abs}(540) = 2.29\text{x}10^{-1} \times \text{CWSN} + 1.50\text{x}10^{-2} \quad R^2 = 0.995 \qquad \textbf{(Equation 2)}$$

Extraction of cheese: The water soluble nitrogen extracts of cheese were prepared following [24]. Five grams of cheese sample were homogenized in 10.0 mL of distilled water at room temperature for 10 min using a Stomacher at high-speed. The resulting slurry was then held at 40 °C for 1 h with stirring. The insoluble material was then separated by centrifugation at 3000 xg for 30 min at 4 °C in a refrigerated centrifuge. The supernatant was filtered through glass wool in a Pasteur pipette to remove residual suspended fat. This method for extraction was performed in duplicate per sample. The nitrogen content was determined by Bradford method.

WSN quantification: The WSN was determined by Bradford method, using a microplate spectrophotometer Thermo Scientific – Multiskan Go with Brand plate of 96 wells. To each well 250 µL of Bradford reagent previously filtered and 5 µL of extract of water soluble nitrogen of cheese were added. The plate was shaken for 30 s. Then the absorbance at 595 nm of the samples was measured between 5 min and 1 h after mixing. The blank was prepared by adding 5 µL of distilled water. The standards were measured following the same procedure. WSN value resulted from six quantifications in the microplate (duplicate of sample and triplicate of analysis) and was expressed in g per 100 g of cheese.

### 6.4.2 Determination of nitrogen soluble in 12% trichloroacetic acid (TCA)

Extraction of cheese: The 12% trichloroacetic acid extracts of cheese were prepared following [24]. Two mL of a 48% (w/v) aqueous solution of TCA were added to 6 mL of the water-soluble nitrogen fraction. The mixture was allowed to stand for 30 min at room temperature and then filtered through Whatman no. 42 filter paper. The nitrogen content was determined but due to the low content of protein the sample was then concentrated.

Nitrogen concentration the TCA fractions: Four mL of 12% trichloroacetic acid extracts of cheese were evaporated in speed vacuum for 4 hours, and the remained

volume was determined. The nitrogen content was determined by the Bradford method. The analysis were run in duplicate of samples and in triplicate of analysis.

## 6.5 Lipid oxidation analysis

### 6.5.1 Determination of malondialdehyde (MDA)

MDA standard: 1,1,3,3-tetrametoxipropano (TMP) at a concentration of 10 μM in 7.5% TCA was prepared and used as stock solution. From this, MDA standards ranging from 0.1–10 μM were prepared in 7.5% TCA. The standard curve obtained has the equation (3), as shown in Figure 23 in Appendix C.

$$\text{Abs}(532) = 2.27\text{x}10^{-1} \times \text{CMDA} + 1.17\text{x}10^{-2} \quad R^2 = 0.996 \quad \textbf{(Equation 3)}$$

MDA extraction: Five grams of cheese sample were homogenized in 10.0 mL of 7.5% TCA at room temperature for 240 s using a Stomacher at high-speed. The resulting slurry was then centrifuged at 3600 xg for 20 min at 4 °C. The supernatant was filtered using Whatman no.1. The clear filtrate was used for the TBA reaction [119]. The MDA extraction was followed twice per sample.

MDA determination: One milliliter of cheese MDA extract and 1 mL of TBA reagent (46mM in 99% glacial acetic acid) were mixed in a test tube and heated in boiling water for 40 min. The reaction mixture was cooled, 300 μL were pipetted in triplicate for each well of a microplate and the absorbance was measured at 532 nm. The blank was prepared by adding 1 mL of extracting solution to 1 mL of TBA reagent. The standards were measured following the same protocols [51]. MDA value resulted from six quantifications in microplate (duplicate of sample and triplicate of analysis) and was expressed in μg per g of cheese.

## 7 Statistical analysis

Differences in microbial counts and in each physicochemical parameter caused by HPP and between samples stored at different times, were assessed at a 0.05 level of significance by analysis of variance (ANOVA), followed by a multiple comparisons test (Tuckey's HSD). The data are expressed as "mean ± standard deviation", and the standard deviation was always < 10 %.

## 8 Kinetic analysis

Decreases of CFU counting per gram by storage time and lipid oxidation were subjected to reaction kinetic analysis, and for NP, P400/10, P500/5 and P600/3 microbial counts and MDA concentration variation along time.

For microbiology, the rate constant was determined from a first-order kinetic (Equation 4), were Ln N represents the counts during the storage and Ln $N_0$ the initial value.

$$\mathrm{Ln\,N} = -\mathrm{k} \times \mathrm{t} + \mathrm{Ln\,N_0} \qquad \textbf{(Equation 4)}$$

For lipid oxidation, the rate constant was determined from a zero-order kinetic (Equation 5), were CMDA represents the concentration of MDA during the storage and $\mathrm{CMDA_0}$ the initial value.

$$\mathrm{CMDA} = -\mathrm{k} \times \mathrm{t} + \mathrm{CMDA_0} \qquad \textbf{(Equation 5)}$$

# V. Results and Discussion

## Part I: Microbiological analysis

### 1.1 *Listeria innocua* growth

Prior to the experiment with inoculated *L. inoccua*, it was necessary to grow *L. inoccua*. To do this, a growth curve for this microorganism was performed with cell growth being measured, using two complementary ways: (1) by optical density (OD) readings at 600 nm and (2) by CFU counting. The results are shown in Figure 7.

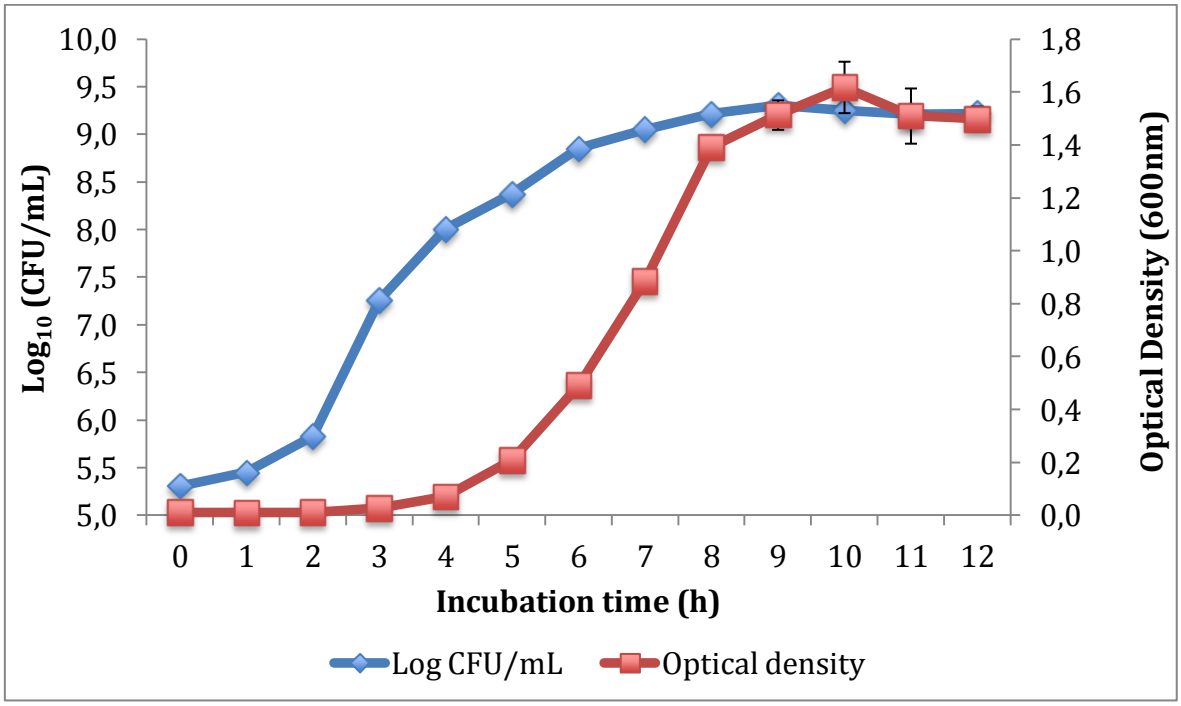


**Figure 7. Growth curve of *L. innocua* by optical density (OD600nm) and CFU counting (expressed in $log_{10}$ CFU/mL).**

As it was expected, *L. innocua* showed 3 phases of growth during 12 hour of incubation at 37 ± 1 °C. Analysing the Figure 7, it is clear that in the first 2 h, cells remained in lag phase. The OD did not change during this phase and the colonies counted were 0.0110 ± 0.010, 0.0095 ± 0.006 and 0.0110 ± 0.001 OD (0, 1 and 2 h, respectively).

The exponential phase was verified between 3 and 8 h. Plate counting results were 7.26 ± 0.014 and 9.22 ± 0.054 log CFU/mL at 3 and 8h, respectively, which directly correspond to 0.027 ± 0.003 and 1.39 ± 0.010 OD measurements. From OD 0.700, samples were appropriately diluted using TSB medium.

Between 9 and 12 h, bacterial cells reached the stationary phase. During this period, the counted colonies remained unchanged, among 9.31 and 9.22 log CFU/mL, which corresponded to 1.510-1.618 of OD. Concerning all, to inoculate *L. innocua* in cheese, it was necessary to grow this microorganism during 10 to 12 h, until it reaches the stationary phase. Between 3 and 12h, it is possible to directly relate the log of CFU counts with the log OD measured, as shown in Figure 8, with the following equation (6):

$$\text{Log}\, CFU\ /mL = 1.06 \times Log\ OD + 9.08 \quad R^2 = 0.984 \qquad \textbf{(Equation 6)}$$

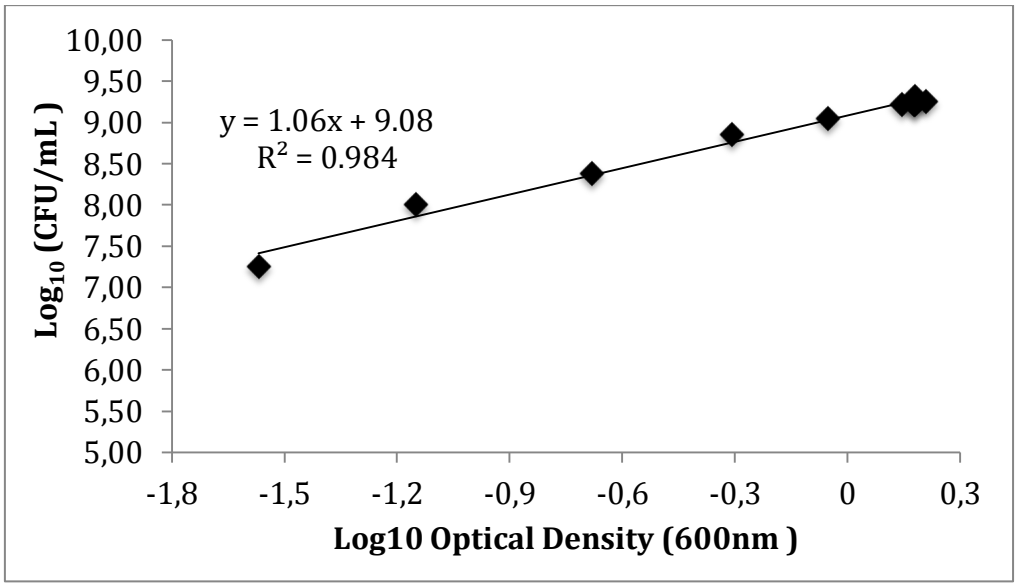


**Figure 8. Direct relation between $\log_{10}$ CFU/ml and log optical density at 600nm to *L. innocua* growth among 3 and 12h.**

## 1.2 Microbial quantification in Serra Cheese

There are several microorganisms in Serra Cheese that can be endogenous and/or adventitious. In this study, it was characterized the total aerobic mesophilic

flora, the mesophilic LAB, the E*nterobacteriaceae*, yeasts and moulds, and *L. innocua* after HPP and during the storage for 100 days.

### 1.2.1 Total aerobic mesophilic microflora

The counts of total aerobic mesophilic microorganisms were performed at 0, 14, 42, 70 and 100 days of storage at 5 °C, as shown in Figure 9.

The total microflora in NP cheese remained unchanged during 42 days of storage at 9.04 ± 0.122 log CFU/g, with an increase to 10.0 at 70 days (which was significantly different compared to the all NP samples, $p<0.001$) and decreased to 8.74 ± 0.067 log CFU/g at 100 days. Macedo *et al.* (1996) [15] counted the total viable microorganisms at 35 days of ripening of Serra cheese produced in October-November and reported 9 log CFU/g [15], which comprises the 9.04 log CFU/g quantified in this study at 45 ripening days, equivalent to 0 days of storage.

HPP induced low cycle reductions, causing 0.47, 1.06 and 1.20 log cycle reductions on cheese P400/10, P500/5 and P600/3, respectively, on day 0, being counted 8.57, 7.98 and 7.85 log CFU/g, respectively. The results obtained were similar to those of Juan *et al.* (2008) [92], having HPP caused 1.82 log cycle reductions in the total microflora (being the initial counts of 9.14 log CFU/g). However, these authors treated a semihard ewe milk cheese, manufactured from pasteurized milk, at 300 MPa at 15 days of ripening [92].

Analysing Figure 9, for each time of storage, it was possible to verify one profile: the total aerobic mesophilic counts in HPP samples were below the counts in NP samples. However, no significant differences were verified between NP and P500/5 at 14 days (9.04 ± 0.122 and 8.65 ± 0.072 log CFU/g) and between NP and P400/10 at 42 days (9.03 ± 0.065 and 8.94 ± 0.090 log CFU/g) ($p>0.05$), which seem to indicate a recovery of the total mesophilic bacterial counts. Similar results were shown by Juan *et al.* (2008) [92], in spite of reductions caused through HPP, treated cheese also reached similar counts to untreated cheese (7.95 and 7.9 log CFU/g, respectively), at 90 days of ripening (at 12 °C and 85% relative humidity).

Between processed samples, it was verified one pattern: the total microflora counts decreased with the increase of the pressure applied. However no significant

differences were verified between all treated samples at 14 and 70 days of storage ($p>0.05$).

In general, cheese treated at 600 MPa (P600/3) showed more differences in counts relatively to NP cheese, and it was considered the most severe treatment for total microflora; on the other hand, the total microflora counting numbers in treated cheese at 400 MPa (P400/10) were the least affected. The same conclusion was found by Voigt *et al.* (2010) [95], which verified that cheese treated at 600 MPa appeared significantly different to the control and the cheese pressurized at 400 MPa, (4.26; 6.04 and 5.39 log CFU/g, respectively), after 28 days of storage. They treated mature blue-viened cheese (6-12 weeks) at 400 and 600 MPa at 20 °C for 10 min, and then cheese samples were stored at 4 °C [95].

The counts of total aerobic mesophilic microorganisms decreased in all conditions at 100 days, showing no significant differences ($p>0.05$) among the same samples counted at 0 days of storage.

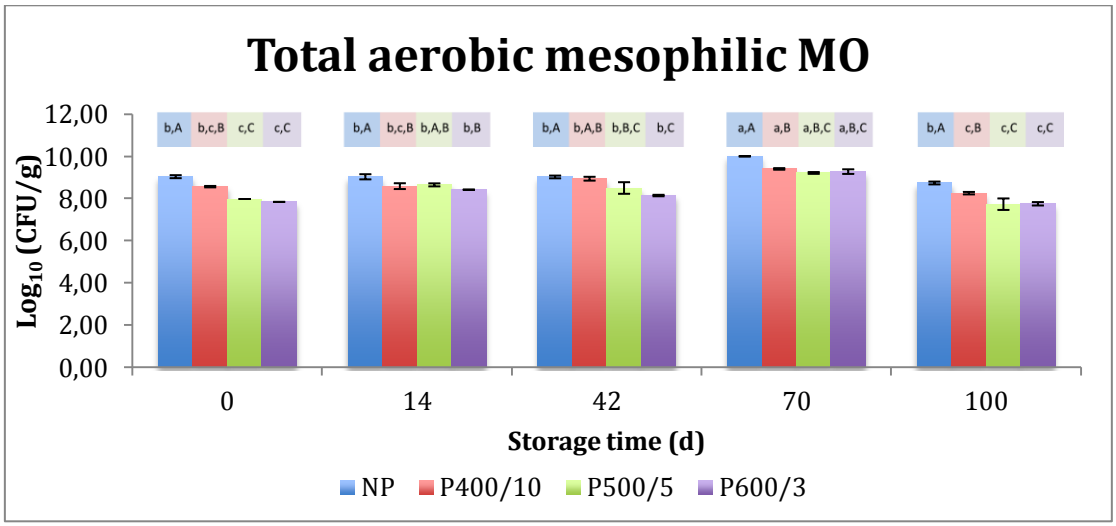


**Figure 9. Counting of total aerobic mesophilic microorganism after 0, 14, 42, 70 and 100 days of storage in NP cheese and in HPP cheese. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

### 1.2.2 Mesophilic LAB

The lactic acid bacteria in cheese are an important population due to its metabolic activity during the ripening. In other to understand its role in the cheese characteristics this microbial group was studied in Serra Cheese after HPP and during storage.

At 0 days of storage (equivalent to 45 days of ripening), it was counted 8.84 ± 0.291 log CFU/g of mesophilic LAB, which increased to 9.02 ± 0.165 at 14 days of storage, then decreased slightly, reaching 8.36 ± 0.077 log CFU/g at 100 days of storage, having no significantly differences during this period in NP samples ($p>0.05$), as shown in Figure 10. These counting results were closer from those that had been quantified in Serra cheese: 9 log CFU/g at 35 days of ripening by Macedo *et al.* (1996) [15]; 8.30 log CFU/g at 60, 90 and 120 ripening days by Tavaria *et al.* (2006) [21]; 8.18 log CFU/g at 60, 8.38 at 90 and 8.02 log CFU/g at 180 days in Serra cheese by Dahl *et al.* (2000) [28].

HPP caused low cycle reductions in LAB counts, being quantified 0.42 in P400/10, 0.26 in P500/5 and 0.82 in P600/3 samples, which resulted in 8.43, 8.53 and 8.02 log CFU/g, respectively at 0 days of storage. Low cycle reductions in LAB microflora had been reported by Juan *et al.* (2008) [92], having HPP caused 0.95 log reductions (being the initial counts of 7.58 log CFU/g). They treated at 300 MPa for 10 min at 15 days of ripening, a semihard ewe milk cheese (manufactured from pasteurized milk) [92].

For each time of storage, analysing Figure 10, it was possible to verify one profile: the LAB counts in HPP samples were below the counts in NP samples. There were significant differences between NP and all treated samples at 14 ($p<0.01$) and 70 days of storage ($p<0.001$), which showed 9.02 ± 0.165 and 8.44 ± 0.019 log CFU/g in NP samples and 8.31 ± 0.001, 8.21 ± 0.092 and 7.80 ± 0.029 log CFU/g (at 14 days); and 7.50 ± 0.025, 7.43 ± 0.031 and 7.14 ± 0.016 log CFU/g (at 70 storage days) for P400/10, P500/5 and P600/3, respectively. However, no significant differences occurred between NP and P400/10 samples at 0, 42, and 100 days of storage ($p>0.05$).

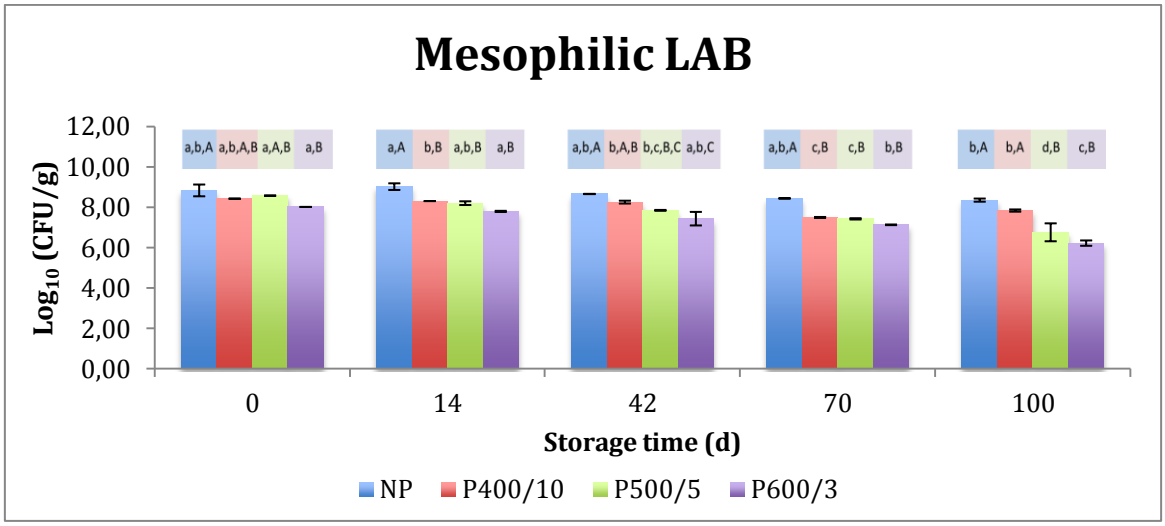


**Figure 10. Counting of mesophilic lactic acid bacteria during 0, 14, 42, 70 and 100 days of storage. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

Between processed samples, it was verified one pattern: the LAB counts revealed higher decrease in samples treated with higher pressure. This effect is more evident by analysing the decrease during the storage, which followed a first order kinetics, with rate constant (*k*) that was determined using least squares linear regression from a semilogarithmic plot (Figure 11).

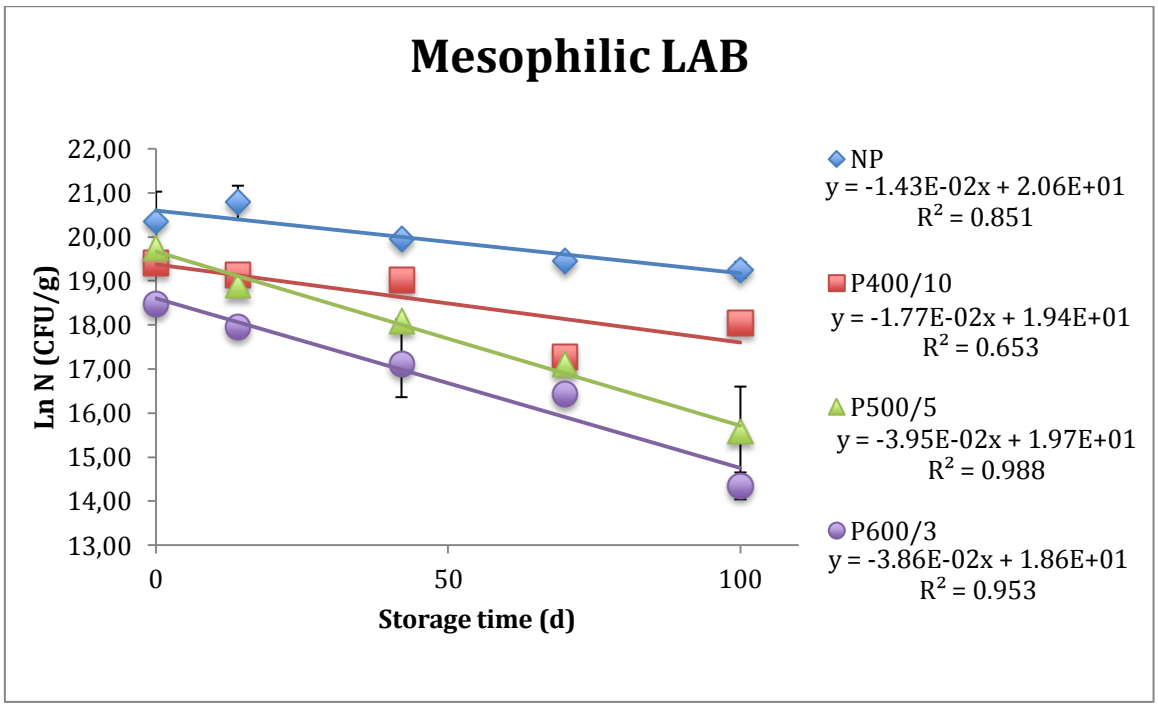


**Figure 11. Linear decrease the Ln LAB counts in non-processed cheese and in pressurized cheese, which storage during 100 days.**

The determined rates were $1.43x10^{-2}$ for NP, $1.77x10^{-2}$ for P400/10, $3.95x10^{-2}$ for P500/5 and $3.86x10^{-2}$ $d^{-1}$ for P600/3 samples, being the higher rate constant quantified in samples treated at higher pressure. At 100 days, it was verified that treatment at 600 MPa was the most severe for LAB counting, having significant differences ($p<0.001$) compared to NP samples, 6.23 ± 0.132 and 8.36 ± 0.077 log CFU/g, respectively. On the other hand, the treatment at 400 MPa caused less effect in LAB counts, being counted 7.84 ± 0.055 log CFU/g, having no significant differences with NP samples at 100 days ($p>0.05$). These results were according with the publication of Juan *et al.* (2008) [92], where no significant differences were shown between control and treated cheese (8.02 and 7.95 log CFU/g) at 300 MPa (10 min), after 90 days of ripening. These authors pressurized a semihard ewe milk cheese (manufactured from pasteurized milk), at 15 days of ripening [92]. Other authors, Voigt *et al.* (2010) [95], verified that cheese treated at 600 MPa revealed significant differences in LAB counts to the control and the cheese pressurized at 400 MPa, (2.10; 5.40 and 5.41 log CFU/g, respectively), after 28 days of storage at 4 °C. They treated mature blue-viened cheese (6-12 weeks) at 400 and 600 MPa at 20 °C for 10 min [95]. Concerning all, the results obtained in this work were according with those of the literature.

### *1.2.3 Enterobacteriaceae*

The counting of *Enterobacteriaceae* includes all members of the coliform group (including *E. coli*), comprising foodborne pathogens *Salmonella, Shigella*, and *Yersinia*. It was shown to be an alternative to coliform counts because it is more inclusive considering pathogenic bacteria. The *Enterobacteriaceae* counts may be superior to coliforms because they have collectively greater resistance to the environment conditions than the coliforms, and, this way, they can be used as indicators of sanitation good manufacturing practice (GMPs) [120]. *Enterobacteriaceae* counts were analysed after HPP and during storage period.

In NP cheese with 45 day of ripening (0 days of storage), it was counted 5.46 ± 0.054 log CFU/g. This quantification is in accordance with Tavaria *et al.* (2000) [27], which quantified ≈ 6 log CFU/g of *Enterobacteriaceae* in Serra cheese made in Oliveira

do Hospital at 35 ripening days and ≈ 5 log CFU/g at 60 ripening days. Previously, Macedo *et al.* (1995) [16] enumerated ≈ 6.7 log CFU/g of coliforms in Serra cheese with 35 days of ripening, manufactured from ewe's lactation of autumn. The same authors obtained ≈ 6 log CFU/g of coliforms in cheese with the same ripening period but produced in October-November in 1994 [15].

Analysing Figure 12, it is possible to verify a great decrease of *Enterobacteriaceae* counts in control cheese during storage. After 14 days, it was registered 4.56 ± 0.001 log CFU/g, at 42 days 2.51 ± 0.023 and at 70 and 100 days below the detection limit (considered < 2.0 log CFU/g). This behaviour describes a first order kinetics, as shown in the inner graph in Figure 12, with the following equation (7):

$$\text{Ln N}\,(CFU/g) = -1.63 \times 10^{-1} \times Time + 1.27 \times 10^{1} \quad R^2 = 0.999 \quad \textbf{(Equation 7)}$$

The decrease of *Enterobacteriaceae* counts during the ripening period had already been reported by Tavaria *et al.* (2000) [27], which refer 4.56 log CFU/g at 60 days of ripening. Dahl *et al.* (2000) [28] have extended the studies during a longer ripening time and counted 5.70 log CFU/g at 60 ripening days, 6.10 at 90 days, 4.19 at 120 days, 3.02 at 150 days and zero at 180 days.

This microbial group is usually associated with poor sanitary conditions, but it was observed a decrease in the cellular counts in [27, 28] during the ripening period and as shown in this study, in Figure 12, during the storage, which suggests that *Enterobacteriaceae* are somewhat controlled by competing species that will eventually prevail by the end of ripening and during the storage [27].

HPP caused a great impact in the counting of this microbial group, which was shown by the counting numbers that were below the limit of quantification, being registered more than 3.46 log reductions for P400/10, P500/5 and P600/3 samples after treatment and during 100 days of storage. A similar effect was obtained in La Serena cheese (manufactured with ewe's raw milk), by Arqués *et al.* (2006) [83]. They verified 4.85 and > 5 log reductions in coliforms counts on day 60, being treated at 50 days of ripening at 400 and 500 MPa (for 10min at 10 °C) respectively [83].

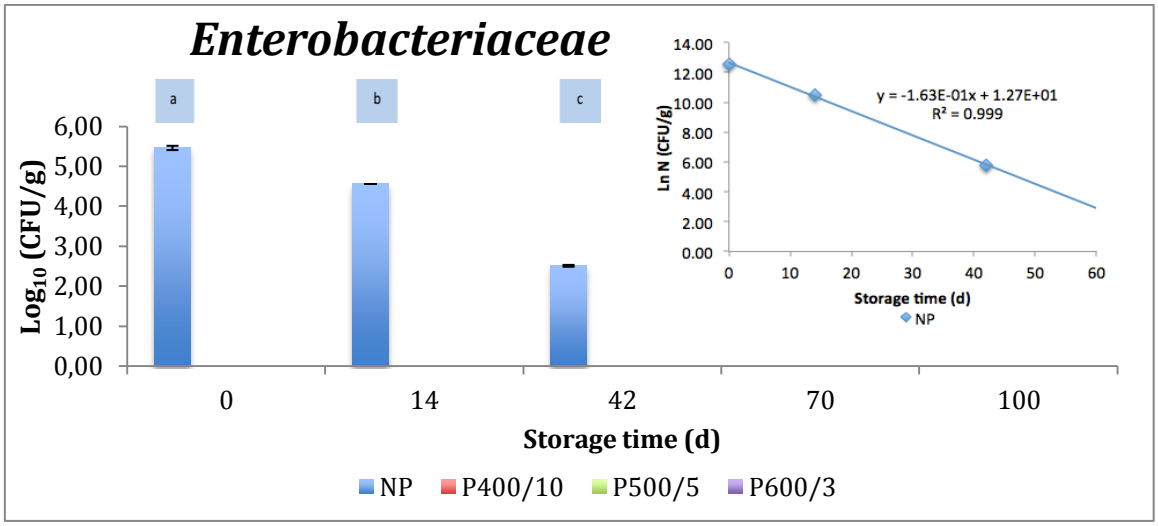


**Figure 12. *Enterobacteriaceae* counts in NP samples and treated samples (P400/10, P500/5 and P600/3); treated samples were below the limit of quantification (bars not shown). Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters). The inner graph shows the linear decrease of Ln *Enterobacteriaceae* counts in NP cheese at 5 °C during 42 days.**

### 1.2.4 Yeasts and moulds

The number of yeasts and moulds are affected by the ripening period and lactation period [15]. Rind samples had shown more counting of yeast than interior samples, possibly because the rind is easily contaminated from the environment [15]. However, in this study, it was studied the yeasts and moulds counts of the interior mass of Serra cheese.

At 0 days of storage (45 days of ripening) in NP cheese, it was counted 5.58 ± 0.371 log CFU/g of yeasts and moulds. During the storage, this microbial group slightly increased until 6.59 ± 0.006 log CFU/g at 70 days of storage, and then tended to stabilize, at 100 days of storage (6.52 ± 0.028 log CFU/g), as shown in Figure 13.

The literature referred different average values of the yeasts counts. At 35 days of ripening it was registered ≈ 3 log CFU/g (in the center of Serra cheese produced in Autumn) by Macedo *et al.* (1995) [16], but 5 log CFU/g (in the interior of Serra cheese manufactured in October-November) by Macedo *et al.* (1996) [15]. Other study realized by Tavaria *et al.* (2000) [27], revealed ≈ 4 log CFU/g at 35 days and ≈ 6 log CFU/g at 60 days of ripening (they studied the cheese interior manufactured in 1997). More recently, the inner Serra cheese was studied by Dahl *et al.* (2000) [28], which quantified 4.91 log CFU/g at 60 days of ripening however

increasing the counting for 6.53 log CFU/g at 150 days of ripening. Identical behaviour was reported by Tavaria *et al.* (2006) [21]. They quantified ≈ 4.3 log CFU/g at 60 days and ≈ 6.4 at 120 ripening days.

Through the analysis of the Figure 13, it was possible to verify that HPP caused a high cycle reductions in all treated samples, being registered more than 3.58 reductions at 0 days of storage. Moreover, during the 100 days of storage, the counts in all treated samples were below the limit of quantification (< 2.0 log CFU/g). Yeasts and moulds are the microorganisms most sensitive to HP treatments [67]. Daryaei *et al.* (2008) [84] reported that pressure treatments ≥ 300 MPa applied for 5 min to fresh lactic curd cheese (manufactured from pasteurized bovine milk) controlled the outgrowth of yeasts, below the limit of quantification, during 4 weeks of storage at 4 °C. However, Voigt *et al.* (2000) [95], described only 0.15 and 3.07 log cycle reductions in yeast counts (having 6.37 log CFU/g in control cheese) on mature blue-veined cheese treated at 400 and 500 MPa (at 20 °C for 10 min), respectively, after 28 days of storage at 4 °C.

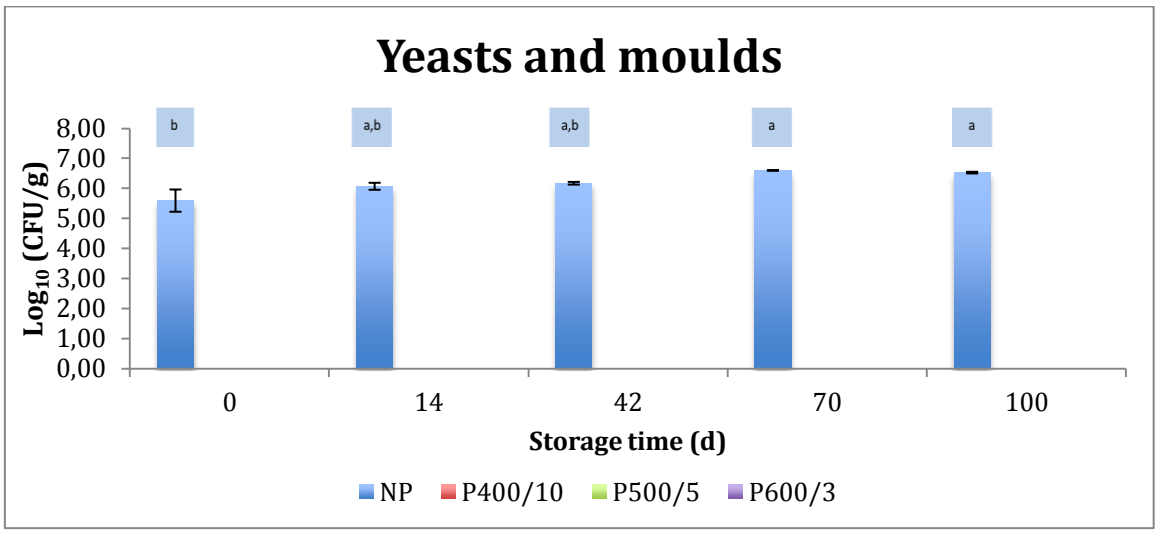


**Figure 13. Yeasts and moulds counts in NP samples and treated samples (P400/10, P500/5 and P600/3) during 100 days storage; treated samples were below the limit of quantification (bars not shown). Different letters denote significant differences (p < 0.05) between samples at the same conditions (non-capital letters).**

### *1.2.5 Listeria innocua*

*Listeria innocua* was inoculated in Serra cheese as a surrogate of *Listeria monocytogenes*. This pathogen has caused several outbreaks associated with cheese contamination [60, 61]. Moreover, the USA Food and Drug Administration (FDA), at 6 of May 2013, prohibited the interstate sale or distribution of unpasteurized milk products for human consumption [121]. FDA alerted the consumers that raw milk products are never guaranteed to be "pathogen-free". Thus, HPP can be a useful technology for improvement of the safety of Serra cheese.

In this study, *L. innocua* was inoculated in the cheese, to see if HPP can significantly reduce the counts of *Listeria*, if Serra cheese was accidently contaminated by *L. monocytogenes* (using *L. innocua* as surrogate). This purpose may open the possibility to export Serra cheese to other countries, like USA, with the label "Pathogen-free" or "Listeria-free" or "Cold-pasteurized Product".

*L. innocua* was inoculated in the second group of cheese samples at 8.58 ± 0.056 log CFU/g. Then, some samples were pressurized (L+P400/10, L+P500/5 and L+P600/3) and one was not processed (L+NP), and all were stored at 5 °C.

In L+NP samples, the *L. innocua* counts decreased during the storage, showing a first order kinetic with a rate constant of $1.02 \times 10^{-1}\ d^{-1}$ (Figure 14), being counted 7.03 ± 0.023 at 14 days and 3.62 ± 0.152 CFU/g after 100 days of storage, showing significant differences in *Listeria* counting during these period ($p<0.001$).

HPP samples showed no significant differences between them, once the treatment caused 4.79 in L+P400/10, 4.85 in L+P500/5 and 4.84 log cycles reductions in L+P600/3 samples, at 0 days of storage, which correspond to 3.80 ± 0.015 in L+P400/10, 3.73 ± 0.083 in L+P500/5 and 3.74 ± 0.039 log CFU/g in L+P600/3 samples. The same treatments had been applied in fresh cheese inoculated with *L. innocua* (ATCC 51742, ATCC 33090, and SEA 15C10) at 7.20 log CFU/mL by Hnosko *et al.* (2012) [75]. These authors reported 1.96 (on HPP at 400 MPa for 10 min), 3.04 (on HPP at 500 MPa for 5 min) and 5.72 log reductions (on HPP at 600 MPa for 3 min).

Analysing Figure 14, it is possible to verify that the *L. innocua* inoculated was below the limit of quantification (< 2 log CFU/g) from 14 days of storage onwards for all treated samples, and it remained like this during 100 days of storage. Thus, at 14

days of storage, > 5.03 log cycle reductions were verified in pressurized samples. These results can be due to the effect of the storage in *L. innocua* counts or possibly due to some cell injuried by damage caused by HHP that did not allow recovery until 14 days.

A similar behaviour was reported by Arqués *et al.* (2005) [86], with complete inactivation (being the initial count of 5.66 log CFU/g) of *L. monocytogenes Scott* A after 10 days of storage. They inoculated this pathogen at 50 ripening days in cheese made from raw milk and it was treated at 500 MPa (5 min at 10 °C). In other study, by López-Pedemonte *et al.* (2007) [68], a model washed-curd cheese was inoculated *L. monocytogenes* (strains NCTC 11994 and Scott A) at 7.5 log CFU/g, then treated at 400 and 500 MPa (10min at 5 °C). Both strains showed > 5 log reductions storage at 8 °C for 30 days [68].

Concerning all, HPP treatments showed to reduce > 5 log cycles reductions in Serra Cheese, as it was already reported in the literature.

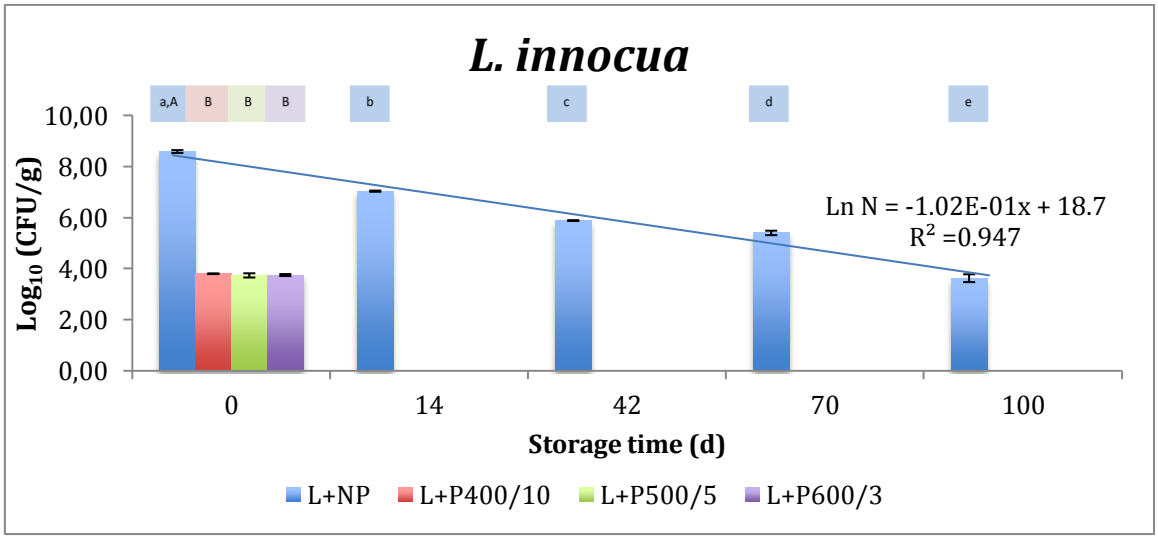


**Figure 14. *L. innocua* counts in L+NP samples and treated samples (L+P400/10, L+P500/5 and L+P600/3); from 14 days of storage treated samples were below the limit of quantification (bars not shown). Straight-line equation was calculated on linear X-axis, which is not the case of this Figure, and so, the straight-line segment is only intended to give the reader the visual information of linear trend. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

# Part II: Physicochemical analysis

## 2.1 Effect of HPP in moisture content

The moisture or water content in Serra cheese is variable with the time (ripening and storage), and the zone of the gathered sample in analysis [15].

The moisture content in Serra cheese was significantly different ($p<0.001$) between 0 days of storage and all other storage time, in all conditions, as shown in Figure 15. The water content quantified at 0 days (45 days of ripening) was between 54.71 ± 1.38 and 58.45 ± 2.80 %, showing no significant differences between the four samples at different conditions (NP and three HP treatments) ($p>0.05$); however higher moisture content values were quantified in P400/10 and P500/5 HP treated samples. The determinations at zero time were repeated after 100 days of samples storage at -80 °C and the moisture contents obtained were among 54.84 and 59.72%, being registered higher values for treated samples.

The typical behaviour is the decrease of moisture content during ripening. Macedo *et al.* (1996) [15] quantified ≈ 59% at 0 days of ripening, decreasing until 42.5% at 35 days, in the center of the cheese produced in October-November [15]. The same behaviour was reported by Sousa *et al.* (1997) [23], being recorded ≈ 47% (w/w) at 45 and 60 ripening days. Other studies revealed 47.35 at 45 days of ripening [20]; 49.79% at 60 ripening days [19]. More recently, Macedo *et al.* (2004) [22] verified a mean of 49.0, 48.3 and 48.0 at 28, 42 and 63 ripening days, respectively. Concerning all, the water content at 45 ripening days, equivalent to zero days of storage, should be among 47 and 50%. Nevertheless, it this study, the cheese showed to have more moisture content at 0 days of storage.

Analysing Figure 15, it is possible to observe that from 14 days of storage, for certain conditions (NP, P400/10, P500/5 and P600/3), there were no significant differences between samples, considering different storage time ($p<0.05$), except for P600/3 at 42, 70 and 100 days of storage ($p<0.05$) (showing, however, no significant differences between those 3 quantifications).

In general, in each period of storage, no significant differences were annotated between four samples ($p>0.05$), with the exception of P600/3 at 70 days and

P400/10 at 100 days of storage ($p<0.05$). From 14 days of storage, the water content ranged between 41.23 ± 2.93 and 44.90 ± 0.554 % in NP samples, between 42.81 ± 0.222 and 46.25 ± 1.40 in P400/10, between 42.12 ± 0.484 and 45.06 ± 0.205 % in P500/5 and between 40.83 ± 1.73 and 44.96 ± 0.575 % in P600/3 samples.

Publications reported that the moisture content decreased after HPP, but increased during the ripening when comparing to control cheese [71, 92, 122]. This way, HPP revealed that treated cheeses had better water retention properties than control cheese during the ripening [92, 107], which could be explained by the fact that HP treatments causes changes in the cheese protein network, forming new structures that better retain the water in cheese [122]. HPP treated cheeses showed no more than 1% [71, 92, 122] of water content in relation to control samples. Nonetheless, in this study, some treated samples showed more than 1% of moisture content when compared with non-processed samples, such as P400/10 at 100 days, P500/5 at 0, 14 days and P600/3 at 14 days of storage.

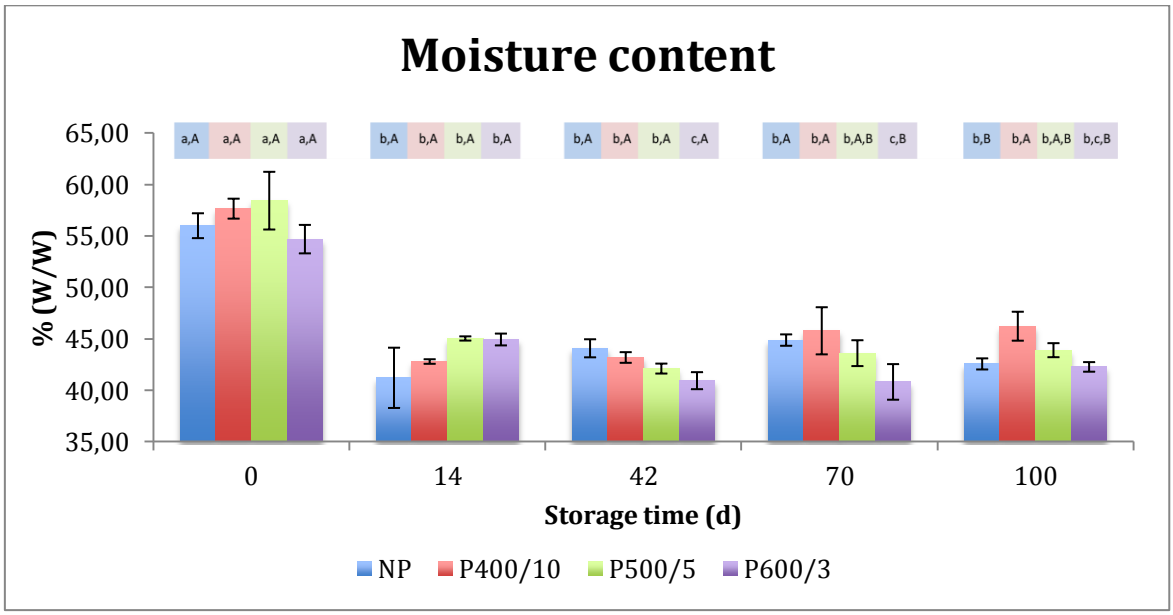


**Figure 15. Moisture content in Serra cheese: non-processed and treated samples at 0, 14, 42, 70 and 100 days storage at 5 °C. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

## 2.2 Effect of HPP on water activity – $a_w$

The water activity is an important parameter in food preservation, because it can inhibit microbial growth, being the $a_w$ equal to 0.90 the lower limit for bacterial growth (in general) and some yeasts are inhibited to growth [123].

Analysing Table 10, it is possible to observe one pattern in water activity in Serra cheese: in all conditions the $a_w$ measured at 0 days of storage was a little higher than the $a_w$ measured during the storage. However, no significant differences were verified in the $a_w$ between all samples at 0 days ($p>0.05$), which changed between 0.949 and 0.953. These values were above the 0.94, which was determinate by Macedo *et al.* (1997) [20], at 45 days of ripening of Serra cheese.

In general, starting from 14 days, no significant differences were verified in $a_w$ measured during 100 days ($p>0.05$), between the four samples in each time of storage (with exception for P500/5 at 14 days); and between samples at the same condition, at different storage time (exception to NP and P500/5 at 42 days and P500/5 at 14 days). During this period the $a_w$ ranged between 0.940 and 0.948.

These results showed that HPP caused no changes in $a_w$ in Serra cheese.

**Table 10. Water activity measured in non-processed samples and treated samples at 0, 14, 42, 70 and 100 days of storage at 5 °C. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

| | NP | | P400/10 | | P500/5 | | P600/3 | |
|---|---|---|---|---|---|---|---|---|
| Storage time (d) | $a_w$ | STD | $a_w$ | STD | $a_w$ | STD | $a_w$ | STD |
| 0 | 0.950 ± | 7.07E-04 $^{a,A}$ | 0.953 ± | 7.07E-04 $^{a,A}$ | 0.949 ± | 7.07E-04 $^{a,A}$ | 0.949 ± | 7.07E-04 $^{a,A}$ |
| 14 | 0.942 ± | 2.12E-03 $^{b,B}$ | 0.944 ± | 0.00E+00 $^{b,B}$ | 0.941 ± | 1.41E-03 $^{a,A}$ | 0.947 ± | 7.07E-04 $^{a,A,B}$ |
| 42 | 0.948 ± | 2.12E-03 $^{a,A}$ | 0.945 ± | 0.00E+00 $^{b,A}$ | 0.948 ± | 7.07E-04 $^{a,b,A}$ | 0.946 ± | 1.41E-03 $^{a,A}$ |
| 70 | 0.943 ± | 2.83E-03 $^{a,b,A}$ | 0.941 ± | 7.07E-04 $^{b,A}$ | 0.944 ± | 7.07E-04 $^{b,A}$ | 0.944 ± | 0.00E+00 $^{a,A}$ |
| 100 | 0.945 ± | 2.12E-03 $^{a,b,A}$ | 0.940 ± | 2.12E-03 $^{b,A}$ | 0.942 ± | 1.41E-03 $^{b,A}$ | 0.945 ± | 5.66E-03 $^{a,A}$ |

## 2.3 Effect of HPP on pH-values and on titratable acidity

The pH variations in Serra cheese are mainly due to the formation of lactic acid, mainly due to the metabolism of LAB [16]. This study was completed by determination of titratable acidity, which was expressed in grams of lactic acid per 100 grams of cheese. Both chemical parameters were measured in samples of the

interior of Serra cheese with 45 days of storage, which were stored during 100 days at 5 °C.

The pH of Serra cheese at 45 days of ripening (0 days of storage) was 5.24 ± 0.06, which slightly increased during 70 days of storage to 5.41 ± 0.03, then decreased to 5.33 ± 0.06 at 100 days. The typical behaviour is the decrease of the pH during the first 28-42 ripening days [15, 22, 23], that can be associated with the degradation of residual lactose in lactic acid [107], lactic fermentation [15], by LAB leading to pH-values between 4.75 and 5.40 at 35 days in the center of the cheese [15, 23], depending on the lactation season. Macedo *et al.* (2004) [22] reported the decrease of the pH in Serra cheese (manufactured in November) during the ripening, reaching 5.24 at 42 days (being 6.37 at 0 days of ripening), and increasing to 5.50 at 63 days of ripening. Macedo *et al.* (1997) [24], revealed the same behaviour, shown in average 5.23 at 35 days of ripening in Serra cheese. Thus, the pH-values measured in NP samples in this study were according with the literature. However, other studies reported higher pH values; for example Macedo *et al.* (1997) [19] revealed 5.55 at 60 days of ripening.

Analysing Figure 16, it is possibly to verify one profile, the pH-values of NP samples were below the pressurized samples during 70 days of storage and increased slightly during this period in all samples. Only at 100 days the pH-values were practically equal in all samples, ranging between 5.30 ± 0.05 and 5.33 ± 0.06, having no significant differences between them ($p>0.05$). The highest differences between NP and treated samples were registered at 42 days of ripening, with 5.38 ± 0.06 and among 5.44 ± 0.05 and 5.49 ± 0.08, respectively ($p<0.05$).

The effect of HPP in pH-values of cheese have been reported in several publications (in different kinds of cheese) [71, 91, 92, 99-101, 104, 106, 108], which showed higher values for treated samples. Ewe milk cheese treated at 15 days of storage at 300 MPa (for 10 min) reported 4.91 of pH in HPP samples and 4.80 in control cheese at 60 days of ripening [122]. However, Garde *et al.* [71] treated at 300 and 400 for 10 min at 50 days of ripening La Serena cheese (manufactured from raw ewe's milk) and revealed no significant differences in pH value after 10 days of treatment.

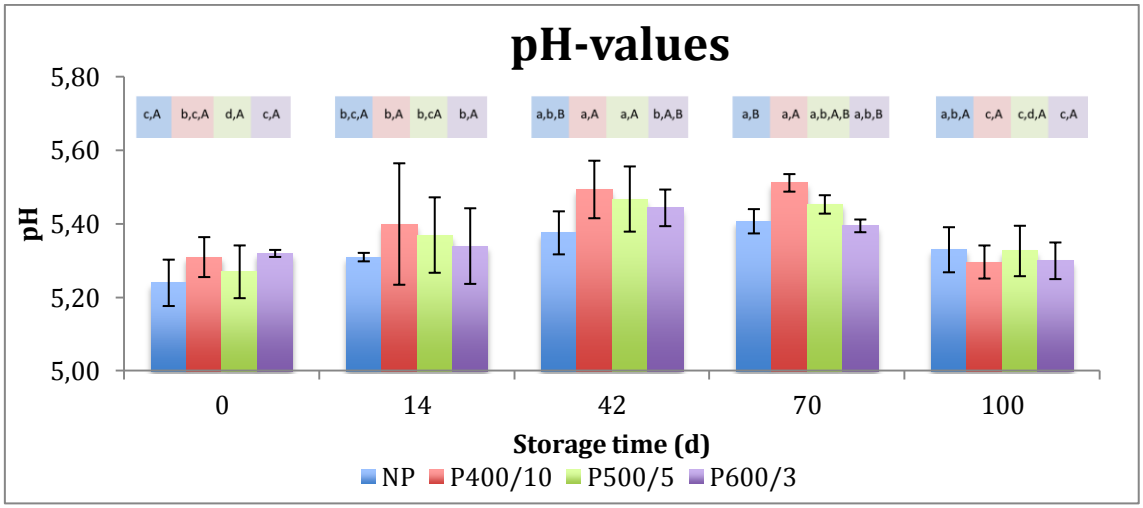


**Figure 16. Mean of pH-values measured in cheese samples non-processed (NP) and HPP samples (P400/10, P500/5 and P600/3) at 0, 14, 42, 70 and 100 days of storage. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

The titratable acidity measured, showed in Table 11, shows one pattern: in each time of storage, NP samples revealed generally higher titratable acidity than treated samples.

These behaviours can be correlated with LAB counts, which were in NP samples higher than HPP. In HPP samples, the LAB counts were below than those of NP samples, and decreased during the storage, which might explain the increasing of the pH-values, maybe due to inactivation of LAB and their enzymes [71], leading to the decrease of lactic acid formation (cheese acidification), which was according with the lower titratable acidities measured in treated cheese samples.

**Table 11. Mean of titratable acidity expressed in grams of lactic acid per 100 grams of cheese in NP and treated samples storage during 100 days. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

| | NP | P400/10 | P500/5 | P600/3 |
|---|---|---|---|---|
| Storage time (d) | $g_{lactic\ acid}$/100 g STD | $g_{lactic\ acid}$/100 g STD | $g_{lactic\ acid}$/100 g STD | $g_{lactic\ acid}$/100 g STD |
| 0 | 0.133 ± 7.98E-03 $^{c,A}$ | 0.119 ± 3.90E-03 $^{c,A}$ | 0.128 ± 4.39E-03 $^{b,A}$ | 0.131 ± 5.63E-03 $^{a,b,A}$ |
| 14 | 0.146 ± 1.87E-02 $^{b,c,A}$ | 0.135 ± 7.07E-03 $^{b,c,A,B}$ | 0.134 ± 1.29E-02 $^{a,b,A,B}$ | 0.128 ± 9.57E-03 $^{b,B}$ |
| 42 | 0.145 ± 7.37E-03 $^{b,c,A}$ | 0.140 ± 7.72E-04 $^{a,b,A}$ | 0.140 ± 5.55E-03 $^{a,A}$ | 0.149 ± 8.77E-03 $^{a,A}$ |
| 70 | 0.150 ± 6.24E-03 $^{b,A}$ | 0.143 ± 3.17E-03 $^{a,b,A,B}$ | 0.140 ± 6.13E-03 $^{a,A,B}$ | 0.129 ± 3.34E-03 $^{b,B}$ |
| 100 | 0.184 ± 8.09E-03 $^{a,A}$ | 0.154 ± 1.02E-02 $^{a,B}$ | 0.145 ± 1.07E-02 $^{a,B}$ | 0.142 ± 1.02E-02 $^{a,b,B}$ |

In general, the titratable acidity increased during the storage, being more evident in NP samples which showed 0.133 at 0 days and 0.184 $g_{lactic\ acid}$/100g at 100 days of storage.

Between treated samples, in general, P400/10 samples showed higher pH-values and P600/3 showed lower pH-values. However, at 0 and 100 days of storage, it was measured the same pH value, 5.31 ± 0.05 and 5.30 ± 0.05, respectively in P400/10 and P600/3. Juan *et al.* (2007) [107] reported similar behaviour to that showed in Figure 16, in which the pH increased more in samples treated with higher pressure. These authors described 5.21 to samples treated at 300 MPa, 5.14 at 400 MPa and 5.16 for 500 MPa, being the control 5.09 at 60 ripening days. This study was also realized in ewe milk cheese, treated for 10 min at 12 °C at 15 days of ripening. The HPP samples behaviour showed in this study were according to the literature.

## 2.4 Proteolysis analysis

Several authors [22, 24, 71, 92, 122, 124] reported that proteolysis is the most important set of biochemical events taking place during the ripening of cheese, due to its major impact on texture and flavour development. During proteolysis, proteins are degraded to polypeptides (primary products) and subsequently to secondary products such as small and medium-size peptides and eventually free amino acids. Primary proteolysis is believed to cause softening of the cheese texture early during ripening, via disruption of its three-dimensional protein matrix. The secondary proteolysis generates peptides that are small enough to be detected by the human taste receptors [124].

The proteolytic activity is proportional to the ratio between nitrogen soluble in water (WSN) per total nitrogen (TN), which indicates the ripening extension index. The ripening depth index can be obtained by the ratio between nitrogen soluble in 12% (w/v) in trichloroacetic acid (TCA) and TN. It was attempted to determinate TCA by Bradford method; however the samples showed to be below the limit of quantification. These samples were concentrate by evaporation to increase the concentration, but they become viscous and dense, leading to deposition on spectrophotometry plate, which did not allow a correct quantification.

### 2.4.1 Effect of HPP in Water soluble nitrogen content (WSN)

The WSN content allowed to determinate the water soluble polypeptides at 0, 14, 42, 70 and 100 days of storage, and the results are shown in Figure 17.

Non-processed cheese showed 0.207 ± 0.021 g/100g at 0 time of storage, but it increased for 0.338 ± 0.023 at 42 days; then it slightly increased for 0.385 ± 0.003 g/100g at 100 days. It was not possibly to compare with the literature, because into publications WSN is expressed in g per g of TN [22-24, 124].

HPP samples at zero days showed 0.212 ± 0.013, 0.221 ± 0.010 and 0.221 ± 0.023 g/100g, for P400/3, P500/5 and P600/3, respectively. At 14 days, least differences between the four conditions were registered, showing no significant differences between them ($p > 0.05$).

Analysing Figure 17, it can be observed that: i) the WSN increase in all conditions during the storage; and ii) in each period of storage, NP and treated samples showed identical WSN content, having no significant differences between them ($p>0.05$). In treated samples, the WSN content ranged between 0.323 ± 0.015 and 0.351 ± 0.021 g/100g (being higher in P400/10) at 42 days, and from 0.381 ± 0.003 to 0.384 ± 0.019 g/100g at 100 days of storage.

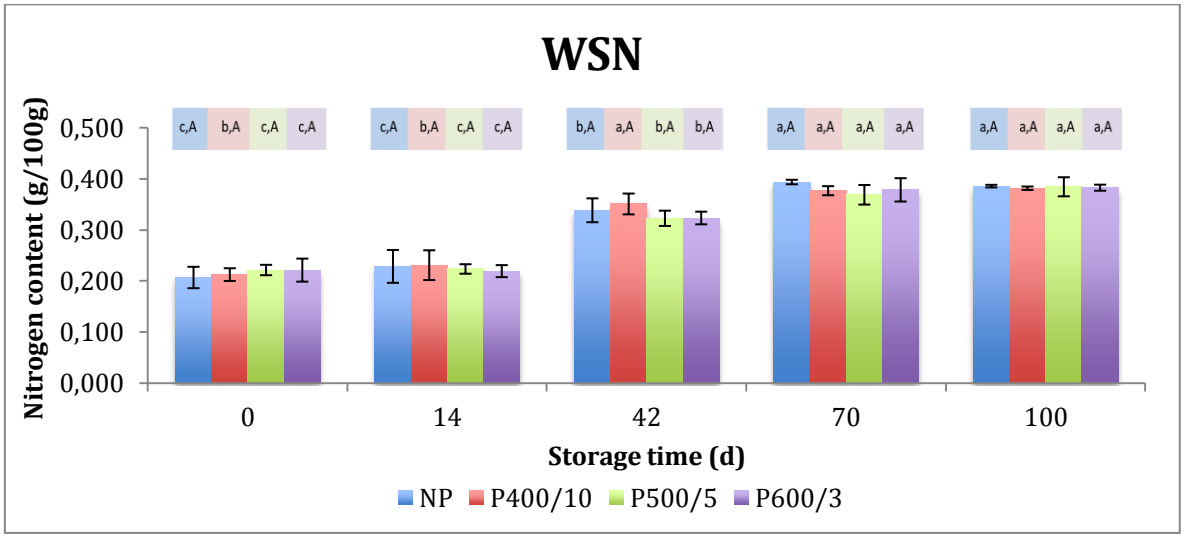


**Figure 17. Water soluble nitrogen content in g per 100g of cheese at 0, 14, 42, 70 and 100 days of storage at 5 °C in non-processed and treated samples. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

Proteolysis was studied in cheese HPP treated, but these publications reported the results in WSN/TN [95, 122]. In ewe's milk cheese no significant differences were

found in WSN/TN between control and treated cheese (with 15 d at 300 MPa for 10 min) at 60 and 90 ripening days [92]. However, lower proteolysis values were reported in La Serena cheese treated at 300 MPa (10 min on day 50) at 60 days, whereas, the same cheese, treated at 600 MPa exhibited similar proteolysis level on day 60 than control cheese [71].

## 2.5 Lipid oxidation analysis

Serra cheese has between 45 and 60% of lipid content [14], which can oxidize, causing deterioration of the fat content. Polyunsaturated fatty acids (PUFA) are susceptible to oxidation during food manipulation, processing and storage [125]. In Serra cheese, it was recorded 51.2 mg/kg at 21 days of ripening and 40.3 mg/kg at 44 days in total of linolenic acid [45].

The unsaturated fatty acids can be oxidized to form odourless, tasteless and colourless hydroperoxides [50]. These are further decomposed to flavourful secondary oxidation products, which are mainly aldehydes, such as malondialdehyde (MDA) [49]. It is used in assessment of lipid oxidation.

### 2.5.1 Effect of HPP in malondialdehyde content (MDA)

The lipid oxidation in Serra cheese, non-processed and HP treated, were determined through quantification the MDA content, as shown in Figure 18.

By analysing Figure 18, it is clear that the general behaviour was the increase of the MDA content in all conditions until 70 days of storage, and then the decreased at 100 days. The determinations of MDA content in cheese at 100 days of storage were repeated after 3 days of samples storage at -80 °C and similar values were obtained.

It is possible to verify that non-processed samples showed higher MDA content than treated samples, with exception of 0 days. The quantification immediately after the treatment revealed higher MDA values on treated samples, between 0.0177 ± 0.004 and 0.0213 ± 0.001 μg/g but 0.0174 ± 0.001 μg/g in NP cheese; thus, HP caused the increase of MDA after the treatment.

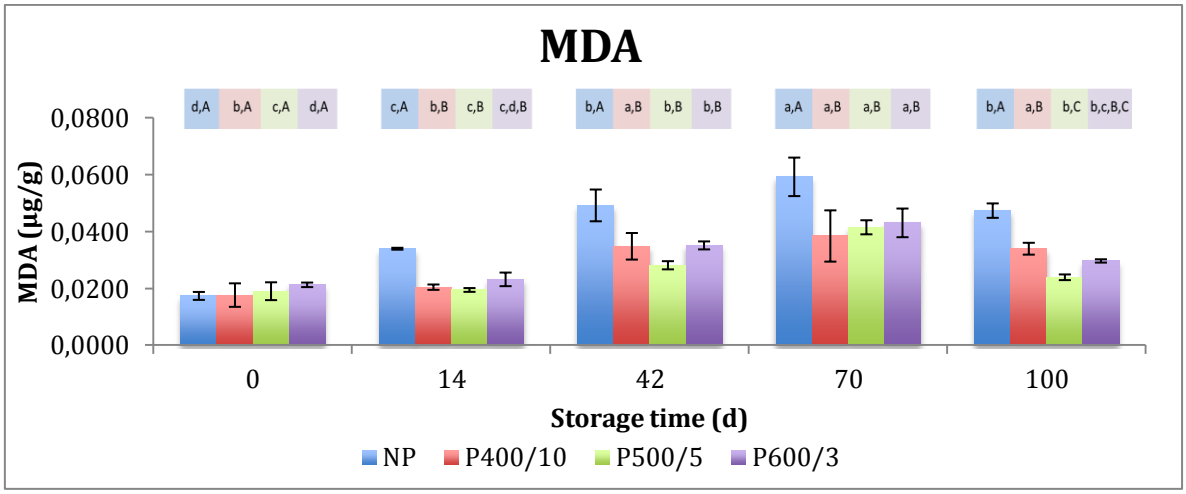


**Figure 18. Malondialdehyde content in Serra cheese non-processed and HP treated at 0, 14, 42, 70 and 100 days of storage at 5 °C. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

Until 70 days, between treated samples, higher MDA concentrations were found in samples P600/3, but no significant differences ($p>0.05$) were recorded in each storage period between samples treated at 3 HP conditions. During this period, lipid oxidation followed a zero order kinetics, with a rate constant between $3.22x10^{-4}$ and $3.29x10^{-4}$ $d^{-1}$ for treated samples, being the lower rate found for cheese treated at 400 MPa; and $5.72x10^{-4}$ $d^{-1}$ for non-processed samples, as shown in Figure 19. Nevertheless, significant differences ($p<0.001$) were registered between non-processed and treated samples in each storage time. These results show that the HPP have positive effect in reducing lipid oxidation, since the rate constants determined were about half of those obtained for NP cheese.

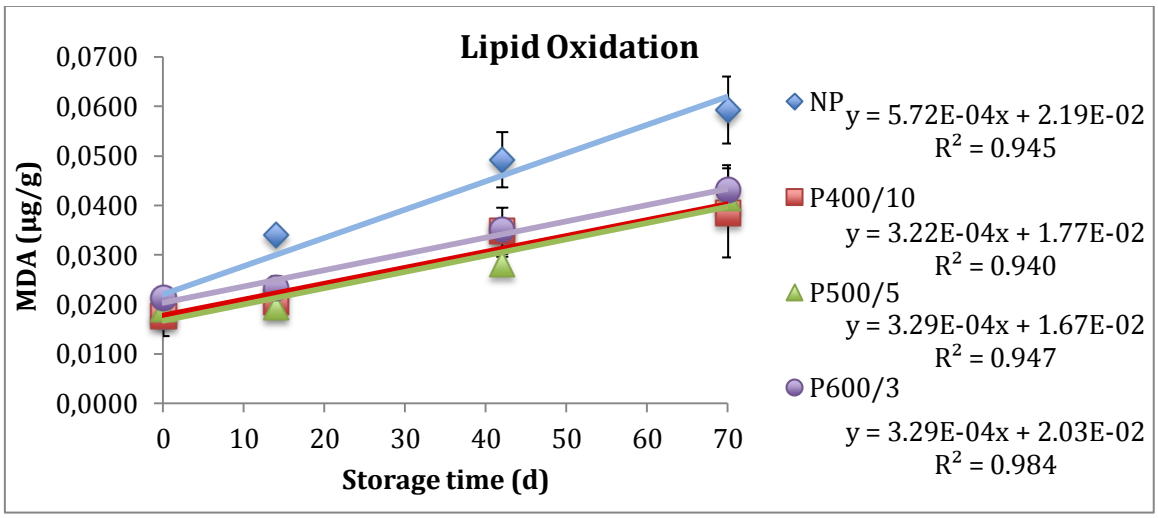


**Figure 19. Lipid oxidation of NP and treated samples during 70 days of storage, following the zero order kinetics with the respective equations.**

Lipid oxidation was not studied in Serra cheese, but it was already determined in raw goat milk cheese with higher content of linoleic acid, 257.2 μg/g. In this case, the lipid oxidation also increased, with 0.04, 0.05 and 0.08 μg/g at 1, 60 and 90 ripening days [126]. In Gouda cheese (with 30.26% of total lipids) were recorded 0.25 μg/g, much higher than in Serra cheese, which can be due to the cheese storage at 4 °C for an unknown, but probably prolonged, period of time [51].

# VI. CONCLUSIONS

This study showed good results for Serra cheese preservation: HPP inactivated deteriorative and a pathogenic surrogate microorganism and low effects were verified in microorganisms beneficial to cheese maturation. Lactic acid bacteria were the least affected by HPP, being reduced at maximum by ~ 0.5 Log CFU/g. This microbial group decreased during the storage, following a first order kinetics, with a rate constant of $1.43x10^{-2}$ $d^{-1}$ for non-processed samples and between $1.77x10^{-2}$ and $3.86x10^{-2}$ $d^{-1}$ for treated samples (the higher rate was found for cheese treated at 600 MPa). Total aerobic mesophilic microorganisms were reduced by ~ 1.0 Log CFU/g (samples treated at 400 MPa were the least affected). In general, in each period of storage, the counts of total mesophilic in HPP samples were below the counts in non-processed samples. *Enterobacteriaceae* counts showed ≥ 3.5 log cycle reductions in HPP samples, which was below the detection limits, remaining unchanged during the storage, while non-processed cheese showed a first order decrease throughout storage, with a rate constant of $7.06x10^{-2}$ $d^{-1}$. Yeasts and moulds counts exhibited ≥ 3.6 log cycle reductions after HPP. In NP samples remained constant at ≈ 6 log CFU/g. Samples inoculated with *L. innocua* at 8.58 log CFU/g presented ≥ 4.8 log cycle reductions after HPP, and levels below the detection limits after 14 days. Non-processed samples showed a first order kinetics decrease of *L. innocua* with a rate constant of $1.02x10^{-1}$ $d^{-1}$.

Physicochemical parameters revealed small changes, in general, having no significant differences between non-processed and treated cheese during the storage. Concerning the water content and the water activity, no significant differences were verified between treated and NP samples during the storage. The pH-values and titratable acidity were different in non-processed and processed samples. Treated samples showed high pH-values and low titratable acidity, possibly due to less LAB counts. The water soluble nitrogen increased during the storage but no significant differences were verified between treated and non-processed samples. The lipid oxidation increased faster in non-processed cheese with a zero order kinetic constant of $5.72x10^{-4}$ $d^{-1}$, and revealed to be less intense in treated samples P400/10 and P500/5 with rate constant of $3.29x10^{-4}$ $d^{-1}$.

The obtained results allowed to conclude that HPP has a good potential to render Serra Cheese free of microbial pathogens, with no significant changes in LAB and in cheese characteristics.

# VII. FUTURE WORK

Considering all the results obtained in the present work, it is clear that some aspects would benefit from further investigation in order to clarify other interesting parameters of the effect of HPP in Serra cheese. At the microbial level, it could be studied the effect of HPP in *Staphylococci* counts and in inoculated *Yarrowia lipolytica*, which causes browning of the rind and can lead to the loss of many cheeses.

The proteolysis can be explored throughout the determination of total nitrogen content (TN), nitrogen soluble in 12% (w/v) trichloroacetic acid (TCA), nitrogen soluble in 5% (w/v) phosphotungstic acid (PTA). The free amino acids could also be studied after HPP.

The lipolysis study can be completed by determination of free fatty acids. The identification and quantification of all fatty acids, by gas chromatography can be an interesting study.

It is important to know if the volatile compounds release is affected by HPP, which can be studied by Solid Phase Micro Extration (SPME).

The effect of HPP in cheese rheology should be studied, revealing the fracturability, firmness, cohesiveness, adhesiveness, springiness, gumminess, and chewiness of the cheese.

It can be interesting to realize microscopic analysis in processed cheese samples to know if HP causes alterations in microbial distribution and cheese structure.

To complete the present research, it would be of major interest to perform sensorial analysis but this would require higher amounts of cheese.

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# APPENDICES

## Appendix A – Serra Cheese Manufacture

**Serra Cheese manufacture**

The Serra Cheese manufacture can be divided in milk handling, milk coagulation, working the curd and draining the whey, pressing, external salting, ripening, packing and preservation.

**Milk handling**

The shepherds bring the raw pure milk in larger containers to the cheese-making area and kept it warm for 30-60 minutes [14]. The milk is filtered through a cloth (clean, fine and white) to remove impurities (like hair and dust). The milk is poured in the cloth fixed in the edge of the coagulation vat. The milk is heated up to 28 – 32 ° C.

The most dairies add the salt in the total milk. Some dairies add the salt to the cheese surface during the draining of whey.

**Milk coagulation**

Milk reaches the desired temperature for coagulation at 28 °C (some cheese makers evaluate this via fingertips). Milk coagulation is promoted by adding of thistle flower (*Cynara cardunculus*, L.) [8]. This can be added by three methods. In the first, the dry cardoon flower is added to the warm milk, mixed for a few minutes and filtrated through a fine and clean cloth. In the second, the dry cardoon flowers are macerated with a little amount of water and salt; these results in a paste that is diluted in water to extract the enzymes, that are filtrated through a fine and clean cloth; the extract is added directly in the milk. In the third, the dry cardoon flowers are macerated with salt and water; this paste is placed into a cloth with closed ends; it is submersed in the milk, agitated and squeezed. The last method is the most commonly used [14].

After 45 to 60 minutes coagulation ends, which can be confirmed by analyzing the consistency of the gel or curd, though of the delicate agitation of the pan [8].

The best Serra Cheese is made with 0.3 g of thistle flower per litre of milk, with a coagulation temperature of 28 °C [19]. Higher temperatures lead to losses in the softness of the final cheese [14]. However, lower temperature coagulation leads to vulnerable three-dimensional casein networks that retain less water, which causes to the loss of proteins and enzymes during curd cutting [30].

However, the average coagulation temperature (in 14 farmhouses) and the coagulation time changes throughout three distinct periods: January to February, March to April, and May to June, as shown in Table 12. These different periods are related to different weather, pasture, and handling conditions [127].

**Table 12. Mean coagulation temperature and coagulation time throughout three periods.**

| | January to February | March to April | May to June | Reference |
|---|---|---|---|---|
| **Coagulation temperature (average in °C)** | 28.2 | 30.0 | 30.9 | [128] |
| **Coagulation time (min)** | 28 to 57 | 25 to 63 | 25 to 54 | [127] |

The coagulation time varies possibly due to the different amounts of cardoon used and the native acidity of the milk, which is caused by the fact that raw milk is not always used after milking, allowing microbial growth which decreases the pH [14].

Some authors [129, 130] defend that rennet concentration influences the rheological properties of the coagulum. Coagulation with lower and higher contents of rennet leads to slower coagulation, or faster, respectively. In 1997, Serra Cheese was made with 0.3 and 0.5g/L of cardoon, this study demonstrated no significant differences in the chemical, biochemical, textural and sensorial characteristics in Serra Cheese [20].

## ***Cynara cardunculus* L. characteristics**

In Portugal, the manufacture of traditional cheeses from raw ovine milk is obtained from *Cynara cardunculus* L or thistle flowers or simply cardoon. This flower grows wild and abundantly in the dry, rocky, and uncultivated areas of the southern and north-eastern parts of Portugal during all summer. This thistle variety is prickly and produces large heads and purple flowers, Figure 20 (A) [131].

The mature plants are collected, then the flowers are dried in the shade and in the open air [14]. When dry, they are stored in a cloth or plastic bag in a dry place and sold at local markets, shown in Figure 20 (B) [22, 131]. Thought ion-exchange chromatography, Faro *et al.* [131] concluded that the drying process increase the enzyme heterogeneity, because, it were found four different peaks from the protease isolated from dry flowers, in contrast he obtained only one peak for the fresh flower.

Thistle flower is known to be highly proteolytic [20]. Numerous studies have shown that the maximum proteolytic activity is associated with the stylet proteases [14, 131]. The enzymes extracted from the thistle flower have the maximum activity at pH 5.1 [24].

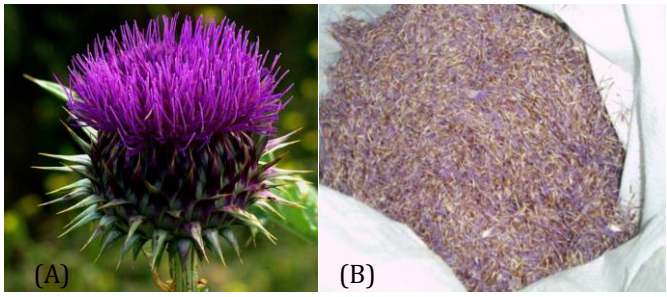


**Figure 20. (A) The *Cynara cardunculus* L. flower. (B) Dry thistle flower.**

## Working the curd and draining the whey

In this step, the curd is worked. It is manually cut with bare hand (obtaining very small pieces) or with the help of a knife, cup, or kitchen spoon. The curd pieces obtained are irregular in size and shape [132]. They are poured in white cloths (like a cloth bag with both ends together in the hand), afterwards they are tighten to drain the whey and put in *cinchos,* Figure 21 (A).


**Figure 21. A) Typical *cincho*. B) Typical *francela*.**

*Cinchos* are perforated cheese metal moulds with adjustable diameter, which allow to obtain cheeses with 13 to 20 cm of diameter and 8 to 10 cm of height. There are plastic moulds with defined diameter. Though the manual pressing and successive tightening, the whey is softly drain form the curd by the *francela,* Figure 21 (B)*. Francela* is like a round-sloped table with a straight extension and a gap to draining the whey onto an open vessel. Afterwards, the collected whey is heated to precipitate the soluble proteins that origin the cottage cheese [8, 14].

During the draining, the curd cannot cool down because it will make the operation harder. The hands are the main workers. Cold hand are better for make cheese, which is contrary to popular belief [133].

In order to the final cheese to have a plane, compacted and uniform surface, the cheese marker pricks the top of the cheese with the fingers, between 3 to 4 cm thick to desiccate more, and them squeezes it very well with their palms [8].

Working the curd and draining the whey can last of 1 hour per cheese.

The cutting method used can influence the microbial level. The microorganisms tend to grow and develop in colonies along curd junctions [26].

**Pressing**

Afterwards, the curd is pressed with a weight of 4 to 5 kg on top of each unit, for about 6 hours. In modern dairy producers, cheeses are kept 4 hours under pressure in industrial machines [8].

Pressing the curd is an important step because it avoids the development of extensive cracks in the Serra Cheese [134].

**External Salting**

Salting is made with kitchen salt. In the major cheese makers, the salting process is made before the cardoon flower is added, during the milk handing in proportion of 30g per litre of milk [14].

Other cheese producers prefer to rubs the top and bottom surfaces of the curd with salt after taking them *cinchos.* This salting process contributes to the formation of the rind. By diffusion the salt enters the cheese matrix. The salt gradient, decreases

from surface to the centre, the moisture gradient increase in the opposite direction, which leads to a thicker rind when high salting levels are used [30].

There are normally 6 [132] to 24 hours [127] intervals between consecutive rubbings. In average 0.5 to 0.9 g/cm$^2$ of salt is used on the top surface of cheese and 0.5 to 0.6 g/cm$^2$ on the bottom surface [132].

Rubbing the cheese surface is practiced in fewer producers or in producers that respect more the tradition. The dairywomen do not weigh the salt, they put it instinctively, which reveals the knowledge of generations [8].

The best cheese is made with salt added to fresh cheese surface in quantity of the 0.05 g/cm$^2$ [19].

This step has impact in the cheese. The salt has effects in: the control of microbial growth and activity, the control of extracellular enzymatic activities, the control the curd syneresis and the control of moisture (particularly on the surface) [135, 136].

**Ripening**

In this step, the sides of cheeses are involved in a white, clean and dry cloth (linen or cotton). This band cloth is necessary to avoid the loss of the cylindrical shape of cheese [14]. Cheese is now ready to ripe.

In artisanal cheese producers (more family production or the small scale production), the ripening process occurs in two rooms, the first is the dry room (enxugo) and the other is the ripening room itself.

The cheese is made in the following day and placed in shelves in the dry room. In old houses, there were wooden cabinets previously covered with cloth, for this specific purpose. Nowadays nobody uses them, due to the difficulty of cleaning [8].

Usually, in the dry room there is not controlled temperature and humidity. The humidity is also near saturation (95%).

The lactic fermentation starts and simultaneously the *reima* occurs. In this phase, cheese loses its humidity, which concentrates in cheese surface and allows microbial growth favourable to maturation. *Reima* is a white-reddish viscous smear, important to obtain a good cheese. [128, 132] Between the 3$^{rd}$ and 4$^{th}$ day in the dry room, the dairy producers pass their hands in still malformed and fresh ring, to

homogenize the cheese exterior. In the 9th and 10th day, when cheese is fairly dry, it is washed with warm water, whey, or plain raw milk for the first time.

Cheeses are kept in the dry room for only 10 to 15 days. After this they go to the maturation room [8].

Due to tradition and generation knowledge, women cheese makers, control the opening of doors and windows in the ripening rooms, to create temperature and aeration necessary conditions, for the successful maturation of cheese. The typical cheese makers' house is made in granite with a proper (characteristic/peculiar) microclimate that influences the final characteristics of Serra Cheese.

In the maturation room, cheeses are kept in shelves. They are turn over and washed daily, but less frequently throughout of the ripening process [8], for example once a week, depending on the quantity of viscous smears [14]. The band cloth is adjusted if necessary. In this room, the humidity is about 85% [8].

In farmhouses, the temperature in the ripening rooms ranges between 4 and 30 °C [128, 132] and the relative humidity ranges from 42 and 98% [132] and are directly dependent on the exterior weather conditions [14]. The best cheese ripening conditions are 10°C and 90 to 95% of relative humidity [137]. It is also possible with 80 to 90% relative humidity, to obtain good yields of high quality cheese. If temperature and relative humidity is maintained during ripening a better cheese is obtained, contrary to what happens when this parameters fluctuate [127, 128].

During the winter, the natural ripening conditions are 8 to 11°C and 96% of relative humidity [127]. Cheeses made during March to April in farmhouses, are less buttery than cheeses made with winter conditions [127]. Thus, the best cheeses are produced between December and March [138].

Innovation was important in semi artisanal dairies or large-scale cheese producers with the introduction of the ripening with controlled environmental chambers. The conditions of ripening process in controlled environmental chambers are expressed in Table 13.

**Table 13. Environment conditions of curding chambers in the first 15 to 20 days and between 20 to 45 days of maturation.**

| | Phase | Temperature (°C) | Relative humidity (%) |
|---|---|---|---|
| **First 15 to 20 days** | Fermentation or *reima* | 6 to 12 | 85 to 95 |
| **20 to 45 days** | Maturation | 6 to 14 | 90 to 95 |

In the fermentation phase, cheeses are washed and turned daily. During the maturation, the wash and turn frequency depends on the rind exterior appearance, maintaining its clean and smooth appearance [8].

The conditions for Old Serra da Estrela cheese are identical to shown in Table 13.

The producers face a problem, the formation of cracks on the rind, which leads to the product's loss of value, selling them at a lower price. In 1997, this occurrence was studied and it concluded that this problem could be reduced by increasing the ripening temperature or by increasing the ripening relative humidity (with less impact) [20]. The forced ventilation damages the rind by causing the formation of cracks [137].

The ripening period depends on the type of cheese intended. Butter Serra Cheese ranges from 30 to 45 days [127, 132], old Serra Cheese needs a minimal of 120 days.

## Packing

The material used to pack the cheese must be innocuous and inert. This operation must occur under good hygienic practices (GHP) and good manufacturing practices (GMP). The packing has to ensure the conservation and the products characteristics during the normal period of storage and selling. Packing Serra Cheese can only occur in the delimited geographic zone [8].

## Preservation

The final product is stored to avoid contamination. The producer determines the conservation temperature, witch can not go over 22 °C. Prolonged conservation of Serra Cheese can occur at temperatures below -1 ºC, but producers establish how

long (it depends on the temperature used and the ripening phase). Afterwards, when cheese is taken from the prolonged conservation chamber it is submitted to a progressive increase of the conservation temperature.

This operation can only occur in a delimited geographic area and in producers with a Estrelacoop authorization [8].

**Cheese Yield**

The cheese yield, the amount of cheese obtained from amount of milk, in Serra Cheese is higher than others cheeses made with ewe or cow's milk.

For production of Serra Cheese with 1kg is necessary between 5.0 to 6.0 L of Serra ewe's milk, with final yield of 17-20%. In general, for 1 kg of ovine cheese was necessary between 6 to 7 liters of other ewe's milk (others different the Serra ewe) [14].

However, 7 to 8% of total curd in whey during the curd working is lost due to small size [128]. Seasons also influence the yield, being higher in January and lower in June due to weather conditions [127].

# Appendix B – Sum up of effect of HPP in literature

**Table 14. Effect of HPP treatments in pathogenic spoilage and beneficial microorganisms in some ewe milk cheese by several authors.**

| Microorganism evaluated | Cheese variety | Moment of applications (d) | Treatment conditions P (MPa) | t (min) | T (°C) | Log reductions | Obs. | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Effect in pathogenic and spoilage microorganisms** | | | | | | | | |
| *S. aureus* CECT 976 | Raw milk cheese | 50 | 500 | 2 | 10 | CI[2] (>5.3) | 60 d | [74] |
| *Staphylococci* | La Serena (raw milk cheese) | 2 | 300/400 | 10 | 10 | 0.49 / 1.45 | 3 d | [83] |
| *Staphylococci* | Raw ovine milk cheese | 1 | 400/500 | 10 | 10 | 0. 7/ 3.2 | 63 d | [85] |
| Listeria monocytogenes Scott A | Raw cow's milk cheese | 2 or 50 | 500 | 5 | 10 | 5.02 / CI (>6.34) | | [86] |
| Listeria monocytogenes ATTC 19115 | Turkish white raw ewe cheese | | 600 | 10 | 25 | 4.9 | | [87] |
| *E. coli* K-12 | Cheddar (raw milk cheese) | 1 | 350 | 3 | 50 | CI (>6.5) | | [88] |
| *E. coli* O157:H7 | Raw milk cheese | 2 or 50 | 500 | 3 | 10 | CI (>5) | 60 d | [82] |
| *Enterococci* | La Serena (raw milk cheese) | 2 | 300/400 | 10 | 10 | 2.05 / 2.68 | 3 d | [83] |
| *Enterococci* | La Serena (raw milk cheese) | 50 | 300/400 | 10 | 10 | 1.37 / 1.98 | 60 d | [83] |
| Coliforms | La Serena (raw milk cheese) | 2 | 300/400 | 10 | 10 | 4.13 / 5.50 | 3 d | [83] |
| Coliforms | La Serena (raw milk cheese) | 50 | 300/400 | 10 | 10 | 4.85 / >5 | 60 d | [83] |
| Spoilage yeasts | Fresh lactic curd cheese | 3 | 300 - 600 | 5 | 22 | > 4 | 6 wk | [84] |
| Yeasts and moulds | Baby Swiss cheese | 1 | 345 - 550 | 10 or 30 | 25 | CI (>3) | | [139] |
| **Effect in beneficial microorganisms** | | | | | | | | |
| Thermophilic LAB | Raw ovine milk | 1 | 400/500 | 10 | 10 | 1.9 / 2.7 | 62 d | [85] |
| Thermophilic LAB | Ewe raw milk cheese | 1 | 300 | 10 | 10 | 0.46 | 60 d | [90] |
| Mesophilic LAB | Raw ovine milk | 1 | 400/500 | 10 | 10 | 2.1 / 4.2 | 61 d | [85] |
| Mesophilic LAB | Ewe raw milk cheese | 1 | 300 | 10 | 10 | 0.68 | 60 d | [90] |

[2] Complete inactivation

**Table 15. Sum up of HPP effects on proteolysis and lipolysis during the storage in different types of cheese by several authors.**

| | Treatment conditions | | | | | |
|---|---|---|---|---|---|---|
| **Cheese variety** | **Moment of application** | **P (MPa)** | **t** | **T (°C)** | **Effects** | **Ref.** |
| **Proteolysis** | | | | | | |
| La Serena (raw ewe milk cheese) | 50 d | 300-400 | 10 min | 10 | Similar proteolysis level than control cheese on 60-days-old. | [71] |
| Irish blue-veined (raw ewe milk cheese) | 42 d | 400-600 | 20 min | 20 | Breakdown of β- and $\alpha_{s2}$-casein was accelerated; PTA/TN index increased on 28 days of storage. | [95] |
| Hispánico (raw cow and ewe milk cheese) | 15 d | 300-500 | 5 min | 10 | Casein hydrolysis accelerated and total FAA content increased on 45 days of ripening. | [94] |
| Ewe's milk (pasteurized ewe milk cheese) | 15 d | 500 | 10 min | 18 | Lower FAA contents after 75 days of ripening. | [91-93] |
| Cheedar (pasteurized cow milk cheese) | 1 or 4 mo[2] | 200-800 | 5 h | 25 | Deceleration of ripening at treatments ≥400 MPa; low levels of FAA at 500-800 MPa after 4 months of ripening. | [98] |
| Camember (pasteurized cow milk cheese) | 5 or 10 d | 0.1-500 | 4 h | 5 | Most intense proteolysis at 50 MPa on 10 d. | [99] |
| Gouda (pasteurized cow milk cheese) | 5 or 10 d | 50-100 | 20-100 min | 14 | No improving of the proteolysis rate. | [100] |
| Edam (pasteurized cow or goat milk cheese) | 4,6 or 8 wk[3] | 400 | 30 min | 25 | No changes in proteolysis indexes. | [101] |
| Garrotxa (pasteurized goat milk cheese) | 1 d | 400/50 | 5 min/72 h | 14 | High FAA levels at 28 days of ripening. | [102] |
| **Lipolysis** | | | | | | |
| Ewe's milk (pasteurized ewe milk cheese) | 15 d | 400-500 | 10 min | 12 | Low concentration of FFA at 400-500MPa after 45 days of ripening. | [93, 103] |
| Hispánico (raw cow and ewe milk cheese) | 15 | 300 - 400 | 10 min | 10 | At 300 lower FFA concentration than control cheese, but at 400 MPa was quantified high FFA levels after 45 days of storage. | [85, 90, 94, 122] |
| Garrotxa (pasteurized goat milk cheese) | 1d | 400 | 5 min | 14 | Lipolysis decelerated; lower content of FFA. | [140] |
| Full-fat Cheddar (pasteurized cow milk cheese) | 1 d | 400 | 10 min | 25 | Lipolysis was higher at 42 days but low at 180 days in treated cheese. | [104] |

**Table 16. Effect of HPP treatments on physical proprieties, on rheological properties and on sensorial properties in different kinds of cheese by several authors.**

| Cheese variety | Treatment conditions: Moment of Application (d) | P (MPa) | t (min) | T (°C) | Effects | Ref. |
|---|---|---|---|---|---|---|
| **Effect on physicochemical proprieties** | | | | | | |
| Ewe's milk (pasteurized ewe milk cheese) | 1 or 15 | 200-500 | 10 | 12 | Cheese treated showed low moisture content on d 1, but identical to control cheese on d 15, being increased the water retention capacity. | [107] |
| Ewe's milk (pasteurized ewe milk cheese) | 1 or 15 | 200-500 | 10 | 12 | High level of salt content in medium and interior cheese matrix. | [92] |
| Ewe's milk (pasteurized ewe milk cheese) | 1 | 300 | 10 | 18 | Lower L-values (visual lightness) and higher b-values (yellowness to blueness) at 15 days. | [92] |
| **Effect on rheological properties** | | | | | | |
| La Serena (raw ewe milk cheese) | 2 or 50 | 300-400 | 10 | 10 | High fracturability, hardness, and elasticity in treated on d 2 but analyzed on d 60. | [71] |
| Ewe's milk (pasteurized ewe milk cheese) | 1 or 15 | 200-500 | 10 | 12 | 200-300MPa on d 1 firmness improved; 500 MPa revealed high deformability and low fracturability and rigidity; texture was less crumbly and more elastic on d 30. | [103] |
| Hispánico (raw cow and ewe milk cheese) | 1 | 300-500 | 10 | 10 | Low fracturability, hardness and elasticity. | [85, 90] |
| **Effect on sensorial properties** | | | | | | |
| La Serena (raw ewe milk cheese) | 50 | 300-400 | 10 | 10 | No influence in volatile compounds profile and sensorial characteristics. | [111] |
| Ewe's milk (pasteurized ewe milk cheese) | 1 or 15 | 200-300 | 10 | 12 | No effects at 15 d. and 1 d at 200 MPa<br>Cheese treated at 300 MPa on 1d showed low taste, aroma and odour quality scores on d 30 and 60. | [92, 107] |
| Hispánico (raw cow and ewe milk cheese) | 15 | 400-500 | 10 | 10 | Not shown significantly differences in flavour intensity and quality at 45 days of ripening. | [94] |

# Appendix C – Standard curves

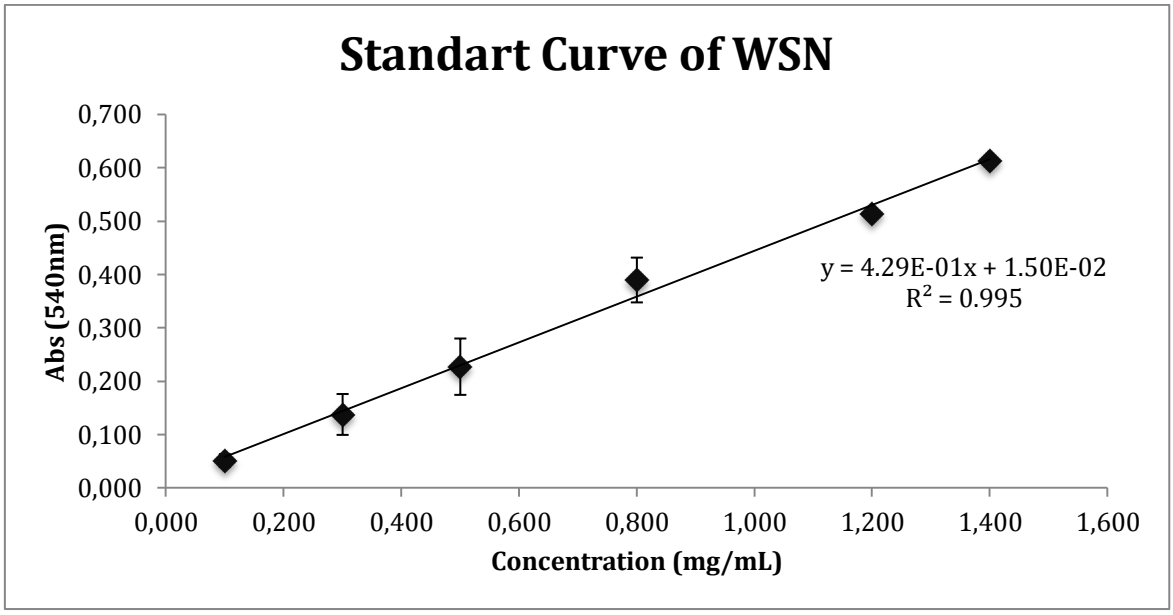


**Figure 22. Standard curve of water soluble nitrogen content by Bradford method with error bars.**

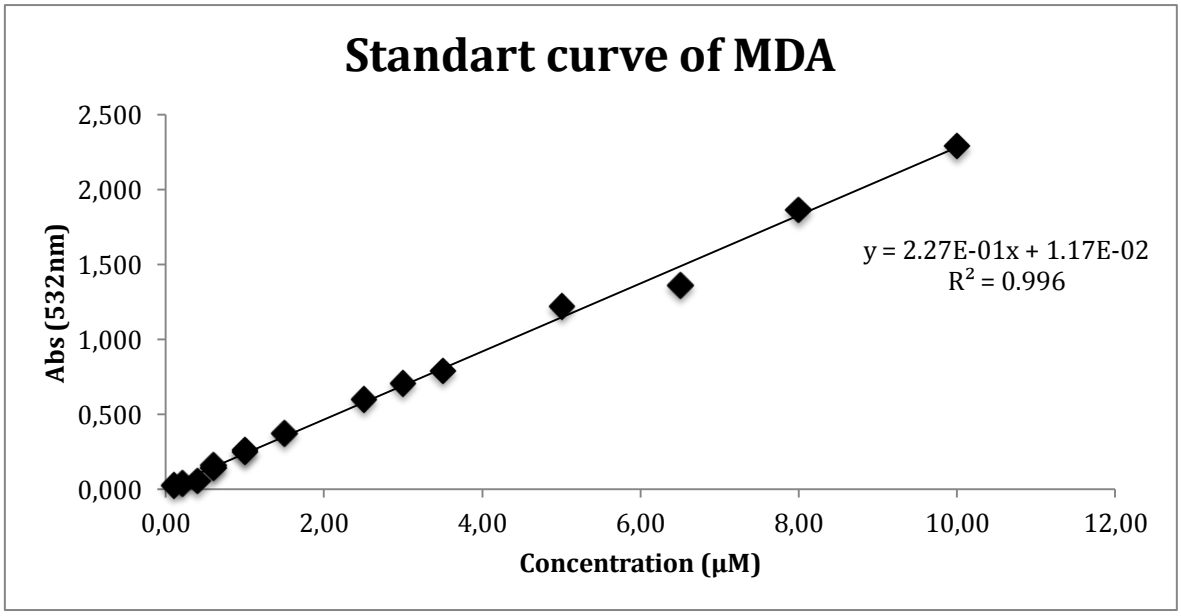


**Figure 23. Standard curve of malondialdehyde content by TBARS method with error bars.**

# Appendix D – Sum of results with statistic analyse

## Microbiology

**Table 17. Effect of HPP treatments on counts of total microflora, mesophilic LAB, *Enterobacteriaceae*, yeasts and moulds and *L. innocua* during storage with statistical analysis. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

| | NP | P400/10 | P500/5 | P600/3 |
|---|---|---|---|---|
| **Total Microflora** | | | | |
| Day 0 | 9.04 ± 0.071 $^{b,A}$ | 8.57 ± 0.033 $^{b,c,B}$ | 7.98 ± 0.000 $^{c,C}$ | 7.85 ± 0.000 $^{c,C}$ |
| Day 14 | 9.04 ± 0.122 $^{b,A}$ | 8.59 ± 0.138 $^{b,c,B}$ | 8.65 ± 0.072 $^{b,A,B}$ | 8.42 ± 0.009 $^{b,B}$ |
| Day 42 | 9.03 ± 0.065 $^{b,A}$ | 8.94 ± 0.090 $^{b,A,B}$ | 8.50 ± 0.275 $^{b,B,C}$ | 8.15 ± 0.035 $^{b,C}$ |
| Day 70 | 10.01 ± 0.000 $^{a,A}$ | 9.41 ± 0.038 $^{a,B}$ | 9.22 ± 0.043 $^{a,B,C}$ | 9.28 ± 0.109 $^{a,B,C}$ |
| Day 100 | 8.74 ± 0.067 $^{b,A}$ | 8.26 ± 0.059 $^{c,B}$ | 7.74 ± 0.272 $^{c,C}$ | 7.76 ± 0.081 $^{c,C}$ |
| **Mesophilic LAB** | | | | |
| Day 0 | 8.84 ± 0.291 $^{a,b,A}$ | 8.43 ± 0.000 $^{a,b,A,B}$ | 8.58 ± 0.000 $^{a,A,B}$ | 8.02 ± 0.000 $^{a,B}$ |
| Day 14 | 9.02 ± 0.165 $^{a,A}$ | 8.31 ± 0.001 $^{b,B}$ | 8.21 ± 0.092 $^{a,b,B}$ | 7.80 ± 0.029 $^{a,B}$ |
| Day 42 | 8.66 ± 0.007 $^{a,b,A}$ | 8.26 ± 0.071 $^{b,A,B}$ | 7.85 ± 0.022 $^{b,c,B,C}$ | 7.44 ± 0.335 $^{a,b,C}$ |
| Day 70 | 8.44 ± 0.019 $^{a,b,A}$ | 7.50 ± 0.025 $^{c,B}$ | 7.43 ± 0.031 $^{c,B}$ | 7.14 ± 0.016 $^{b,B}$ |
| Day 100 | 8.36 ± 0.077 $^{b,A}$ | 7.84 ± 0.055 $^{b,A}$ | 6.77 ± 0.444 $^{d,B}$ | 6.23 ± 0.132 $^{c,B}$ |

| | | | |
|---|---|---|---|
| ***Enterobacteriaceae*** | | | |
| Day 0 | 5.46 ± 0.054 [a] | < 2.0 | < 2.0 | < 2.0 |
| Day 14 | 4.56 ± 0.000 [b] | < 2.0 | < 2.0 | < 2.0 |
| Day 42 | 2.51 ± 0.023 [c] | < 2.0 | < 2.0 | < 2.0 |
| Day 70 | < 2.0 | < 2.0 | < 2.0 | < 2.0 |
| Day 100 | < 2.0 | < 2.0 | < 2.0 | < 2.0 |
| **Yeasts and moulds** | | | | |
| Day 0 | 5.58 ± 0.371 [b] | < 2.0 | < 2.0 | < 2.0 |
| Day 14 | 6.06 ± 0.117 [a,b] | < 2.0 | < 2.0 | < 2.0 |
| Day 42 | 6.16 ± 0.045 [a,b] | < 2.0 | < 2.0 | < 2.0 |
| Day 70 | 6.59 ± 0.006 [a] | < 2.0 | < 2.0 | < 2.0 |
| Day 100 | 6.52 ± 0.028 [a] | < 2.0 | < 2.0 | < 2.0 |
| ***L. Innocua*** | | | | |
| Day 0 | 8.58 ± 0.056 [a,A] | 3.80 ± 0.015 [B] | 3.73 ± 0.083 [B] | 3.74 ± 0.039 [B] |
| Day 14 | 7.03 ± 0.023 [b] | < 2.0 | < 2.0 | < 2.0 |
| Day 42 | 5.88 ± 0.009 [c] | < 2.0 | < 2.0 | < 2.0 |
| Day 70 | 5.39 ± 0.087 [d] | < 2.0 | < 2.0 | < 2.0 |
| Day 100 | 3.62 ± 0.153 [e] | < 2.0 | < 2.0 | < 2.0 |

## Physicochemical Parameters

**Table 18. Effect of HPP treatments on counts water content, water activity, pH-values, titratable acidity, water soluble nitrogen and lipid oxidation during storage with statistical analyze. Different letters denote significant differences ($p < 0.05$) between samples at the same conditions (non-capital letters) or between samples at the same time of storage (capital letters).**

| | NP | | P400/10 | | P500/5 | | P600/3 | |
|---|---|---|---|---|---|---|---|---|
| **Water Content** | **% (w/w)** | **STD** | **% (w/w)** | **STD** | **% (w/w)** | **STD** | **% (w/w)** | **STD** |
| **0** | 56.02 | ± 1.21E+00 a,A | 57.68 | ± 9.69E-01 a,A | 58.45 | ± 2.81E+00 a,A | 54.71 | ± 1.38E+00 a,A |
| **14** | 41.23 | ± 2.93E+00 b,A | 42.81 | ± 2.22E-01 b,A | 45.06 | ± 2.04E-01 b,A | 44.95 | ± 5.75E-01 b,A |
| **42** | 44.10 | ± 8.78E-01 b,A | 43.21 | ± 5.19E-01 b,A | 42.13 | ± 4.84E-01 b,A | 40.95 | ± 8.27E-01 c,A |
| **70** | 44.90 | ± 5.54E-01 b,A | 45.80 | ± 2.29E+00 b,A | 43.63 | ± 1.26E+00 b,A,B | 40.83 | ± 1.73E+00 c,B |
| **100** | 42.58 | ± 5.41E-01 b,B | 46.25 | ± 1.41E+00 b,A | 43.91 | ± 6.82E-01 b,A,B | 42.29 | ± 4.67E-01 b,c,B |
| **Water Activity** | **$a_w$** | **STD** | **$a_w$** | **STD** | **$a_w$** | **STD** | **$a_w$** | **STD** |
| **0** | 0.950 | ± 7.07E-04 a,A | 0.953 | ± 7.07E-04 a,A | 0.949 | ± 7.07E-04 a,A | 0.949 | ± 7.07E-04 a,A |
| **14** | 0.942 | ± 2.12E-03 b,B | 0.944 | ± 0.00E+00 b,B | 0.941 | ± 1.41E-03 a,A | 0.947 | ± 7.07E-04 a,A,B |
| **42** | 0.948 | ± 2.12E-03 a,A | 0.945 | ± 0.00E+00 b,A | 0.948 | ± 7.07E-04 a,b,A | 0.946 | ± 1.41E-03 a,A |
| **70** | 0.943 | ± 2.83E-03 a,b,A | 0.941 | ± 7.07E-04 b,A | 0.944 | ± 7.07E-04 b,A | 0.944 | ± 0.00E+00 a,A |
| **100** | 0.945 | ± 2.12E-03 a,b,A | 0.940 | ± 2.12E-03 b,A | 0.942 | ± 1.41E-03 b,A | 0.945 | ± 5.66E-03 a,A |
| **pH values** | **pH** | **STD** | **pH** | **STD** | **pH** | **STD** | **pH** | **STD** |
| **0** | 5.24 | ± 0.06 c,A | 5.31 | ± 0.05 b,c,A | 5.27 | ± 0.07 d,A | 5.32 | ± 0.01 c,A |
| **14** | 5.31 | ± 0.01 b,c,A | 5.40 | ± 0.10 b,A | 5.37 | ± 0.10 b,cA | 5.34 | ± 0.10 b,A |
| **42** | 5.38 | ± 0.06 a,b,B | 5.49 | ± 0.08 a,A | 5.47 | ± 0.09 a,A | 5.44 | ± 0.05 b,A,B |
| **70** | 5.41 | ± 0.03 a,B | 5.51 | ± 0.02 a,A | 5.45 | ± 0.03 a,b,A,B | 5.40 | ± 0.02 a,b,B |
| **100** | 5.33 | ± 0.06 a,b,A | 5.30 | ± 0.05 c,A | 5.33 | ± 0.07 c,d,A | 5.30 | ± 0.05 c,A |

| 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** | 0.133 | ± 7.98E-03 | c,A | 0.119 | ± 3.90E-03 | c,A | 0.128 | ± 4.39E-03 | b,A | 0.131 | ± 5.63E-03 | a,b,A |
| **14** | 0.146 | ± 1.87E-02 | b,c,A | 0.135 | ± 7.07E-03 | b,c,A,B | 0.134 | ± 1.29E-02 | a,b,A,B | 0.128 | ± 9.57E-03 | b.B |
| **42** | 0.145 | ± 7.37E-03 | b,c,A | 0.140 | ± 7.72E-04 | a,b,A | 0.140 | ± 5.55E-03 | a,A | 0.149 | ± 8.77E-03 | a,A |
| **70** | 0.150 | ± 6.24E-03 | b,A | 0.143 | ± 3.17E-03 | a,b,A,B | 0.140 | ± 6.13E-03 | a,A,B | 0.129 | ± 3.34E-03 | b,B |
| **100** | 0.184 | ± 8.09E-03 | a,A | 0.154 | ± 1.02E-02 | a,B | 0.145 | ± 1.07E-02 | a,B | 0.142 | ± 1.02E-02 | a,b,B |
| | | | | | | | | | | | | |
| **WSN** | **N (g/100g)** | **STD** | | **N (g/100g)** | **STD** | | **N (g/100g)** | **STD** | | **N (g/100g)** | **STD** | |
| **0** | 0.207 | ± 2.09E-02 | c,A | 0.212 | ± 1.25E-02 | b,A | 0.221 | ± 1.01E-02 | c,A | 0.221 | ± 2.25E-02 | c,A |
| **14** | 0.228 | ± 3.21E-02 | c,A | 0.231 | ± 2.91E-02 | b,A | 0.223 | ± 9.31E-03 | c,A | 0.219 | ± 1.18E-02 | c,A |
| **42** | 0.338 | ± 2.34E-02 | b,A | 0.351 | ± 2.04E-02 | a,A | 0.322 | ± 1.49E-02 | b,A | 0.323 | ± 1.24E-02 | b,A |
| **70** | 0.393 | ± 4.42E-03 | a,A | 0.376 | ± 8.87E-03 | a,A | 0.368 | ± 1.92E-02 | a,A | 0.378 | ± 2.28E-02 | a,A |
| **100** | 0.385 | ± 2.56E-03 | a,A | 0.381 | ± 3.33E-03 | a,A | 0.384 | ± 1.86E-02 | a,A | 0.382 | ± 5.94E-03 | a,A |
| | | | | | | | | | | | | |
| **Lipid oxidation** | **MDA (µg/g)** | **STD** | | **MDA (µg/g)** | **STD** | | **MDA (µg/g)** | **STD** | | **MDA (µg/g)** | **STD** | |
| **0** | 0.017 | ± 1.39E-03 | d,A | 0.018 | ± 4.13E-03 | b,A | 0.019 | ± 3.13E-03 | c,A | 0.021 | ± 7.97E-04 | d,A |
| **14** | 0.034 | ± 3.32E-04 | c,A | 0.020 | ± 9.38E-04 | b,B | 0.020 | ± 6.67E-04 | c,B | 0.023 | ± 2.39E-03 | c,d,B |
| **42** | 0.049 | ± 5.58E-03 | b,A | 0.035 | ± 4.67E-03 | a,B | 0.028 | ± 1.44E-03 | b,B | 0.035 | ± 1.41E-03 | b,B |
| **70** | 0.059 | ± 6.77E-03 | a,A | 0.038 | ± 9.00E-03 | a,B | 0.042 | ± 2.48E-03 | a,B | 0.043 | ± 5.04E-03 | a,B |
| **100** | 0.047 | ± 2.54E-03 | b,A | 0.034 | ± 2.08E-03 | a,B | 0.024 | ± 9.96E-04 | b,C | 0.030 | ± 5.83E-04 | b,c,B,C |
| | **NP** | | | **P400/10** | | | **P500/5** | | | **P600/3** | | |