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Effect of high pressure processing (HPP) on starter-free fresh cheese shelf-life

Doctoral Thesis

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HACEN CONSTAR: Que la Licenciada en Ciencias Veterinarias y Pecuarias Katherine Evert Arriagada ha realizado, bajo su dirección, el trabajo titulado "Effect of high pressure processing (HPP) on starter-free fresh cheese shelf-life" que presenta para optar al grado de Doctor en Ciencia de los Alimentos.

Y para que así conste firmamos el presente documentos en Bellaterra (Cerdanyola del Vallès), 25 de Febrero de 2013.

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"Was mich nicht umbringt, macht mich stärker"

Friederich Nietzsche

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Que tu formación te haga más sensible y no más arrogante, que los títulos te ayuden a cambiar el lente con el que miras la realidad. Que tu experiencia te ayude a acercarte a la gente y no a verla como menos…

SUMMARY

High pressure processing (HPP) is a non-thermal technology able to extend the shelf-life of a number of food products while maintaining their sensory and nutritional quality. Fresh cheese is unripened rennet or acid-coagulated type and is usually consumed after manufacturing. Traditional fresh cheese is obtained by rennet coagulation and it may serve as an ideal medium for bacterial proliferation, since no starter culture is added, and considering its high water activity and its high protein and fat contents.

The main objective of this thesis was to evaluate the effect of HPP technology for extending the shelf-life of a starter-free fresh cheese, and in order to obtain a high quality product. For this purpose, a preliminary study at pilot plant scale using small commercial cheeses (80 g) was carried out with the aim to determine the HPP capacity to inhibiting the outgrowing of spoilage microorganisms. In this sense, cheeses were treated at 300 and 400 MPa (5 min at 6ºC) and their microbiological, compositional and physico-chemical evolution during cold storage was evaluated. Results indicated that HPP did not affect cheese composition and provoked only little changes in colour (more yellow) and textural (firmer) properties of fresh cheese when compared with untreated cheese. Besides, 300 and 400 MPa were able to extend fresh cheese shelf-life to 14 and 21 days, respectively.

The next step was to determine the shelf-life of commercial fresh cheeses (250 g) treated at 500 MPa (5 min at 18ºC) at industrial scale, by following their microbiological, compositional, physico-chemical and sensorial evolution during cold storage of 21 days. This time, pressurised cheeses achieved a shelf-life of about 19-21 days compared to 7-8 days of the untreated cheese. However, 500 MPa significantly affected colour, microstructural, textural and sensory characteristics, but the preference for the treated cheese was not affected. It is also worth noting that HPP was able to maintain the aroma characteristics of cheese throughout its shelf-life, leading to a delay in the formation of volatile compounds responsible for off-flavours.

Additionally, an inoculation study with *Listeria* spp. was carried out in order to evaluate the effectiveness of the HPP (300-600 MPa for 5 min at 6ºC) to assure food safety. Three bacterial strains (*L. monocytogenes* Scott A and CECT 4031, and its surrogate *L. innocua* CECT 910) were selected to study the initial inactivation achieved,

the sublethal damage generated and microbial growth during 15 days of storage at 4°C. Treatment at 300 MPa did not inactivate any of the strains inoculated. Conversely, 500 and 600 MPa were effective to achieve significant reductions of pathogen counts in fresh cheese. On the other hand, treatment of fresh cheese within the range of 300-600 MPa did not produce significant sublethal injury. Regarding behaviour differences among *Listeria* strains, it was observed that *L. monocytogenes* CECT 4031 was the most sensitive and *L. monocytogenes* Scott A the most resistant to HPP. Finally, growth during cold storage was not prevented since viable cells remain after the treatment. Counts of cheeses treated at 300 MPa were not different from untreated cheese counts, for all strains and inocula level used. On the other hand, differences in rate of growth among strains were observed. Scott A strain showed the lowest rate for all conditions studied. These results confirm that the fresh cheese, due to their characteristics of pH and a_w, is able to support growth of *Listeria* spp.

RESUMEN

La Alta Presión Hidrostática (APH) es una tecnología no térmica capaz de extender la vida útil de los alimentos al mismo tiempo que mantiene sus características nutricionales y organolépticas. El queso fresco es una variedad no madurada, de coagulación enzimática o ácida, y que generalmente es consumido poco después de su elaboración. El queso fresco tradicional es obtenido por coagulación enzimática y es un medio ideal para el crecimiento bacteriano, ya que en su fabricación no se utilizan fermentos lácticos, posee una elevada actividad de agua y un alto contenido en proteína y grasa.

El principal objetivo de esta tesis fue evaluar el efecto de la APH para extender la vida útil de un queso fresco tradicional obteniendo un producto de alta calidad. Para este propósito, se realizó un estudio preliminar con quesos comerciales de pequeño formato (80 g) a nivel de planta piloto con el fin de determinar la capacidad de la APH para inhibir el crecimiento de microorganismos causantes de deterioro. Los quesos fueron tratados a 300 y 400 MPa (5 min a 6°C) y se evaluaron diferentes parámetros microbiológicos, de de composición y físico-químicos durante su almacenamiento en frío. Los resultados mostraron que la APH no modificó la composición del queso fresco y produjo sólo pequeños cambios en el color (más amarillo) y la textura (más firme), cuando se comparó con un queso no tratado. Además, la aplicación de 300 y 400 MPa aumentó la vida útil de los quesos a 14 y 21 días, respectivamente.

La siguiente fase fue determinar la vida útil de un queso comercial (250 g) tratado a 500 MPa (5 min a 18°C) bajo condiciones industriales siguiendo también su evolución microbiológica, de composición, físico-química y sensorial durante 21 días de almacenamiento en frío. En este caso, los quesos presurizados alcanzaron una vida útil de entre 19 y 21 días al compararlos con los quesos no tratados que fue de entre 7 y 8 días. Sin embargo, el tratamiento de 500 MPa afectó significativamente a las características de color, textura, microestructura y sensoriales del queso, pero no así su preferencia por parte de un panel de catadores. Es importante destacar que la APH fue capaz de mantener las características de aroma del queso a lo largo de su vida útil, retrasando la formación de compuestos volátiles responsables de sabores extraños.

Adicionalmente, se realizó un ensayo de inoculación con *Listeria* spp. con el fin de evaluar la efectividad de la APH (300-600 MPa durante 5 min a 6°C) para asegurar la

inocuidad alimentaria. Se seleccionaron tres cepas bacterianas (*L. monocytogenes* Scott A y CECT 4031, y su marcador, *L. innocua* CECT 910) para estudiar la inactivación y la generación de daño sub-letal después del tratamiento y su evolución durante 15 días de almacenamiento a 4°C. Con el tratamiento de 300 MPa no se consiguió inactivar ninguna de las cepas inoculadas. Sin embargo, tanto los tratamientos de 500 MPa y 600 MPa se lograron importantes reducciones de *Listeria* spp. Por otra parte, la presurización del queso fresco entre 300-600 MPa no produjo significativamente daño sub-letal, apreciándose diferencias en el comportamiento entre las cepas estudiadas. Así, se observó que *L. monocytogenes* CECT 4031 fue la más sensible a la APH y Scott A la más resistente. En los quesos tratados el crecimiento de esta bacteria durante el almacenamiento en frío no fue inhibido. Los recuentos en los quesos tratados a 300 MPa no fueron distintos a los del queso control para todas las cepas y nivel de inoculo utilizados. Por otra parte, se observaron diferencias en las tasas de crecimiento para las cepas utilizadas, la cepa Scott A fue la que presentó las menores tasas bajo todas las condiciones estudiadas. Estos resultados confirman que el queso fresco, debido a sus características de pH y a_w, es capaz de tolerar el crecimiento de *Listeria* spp.

PUBLICATIONS AND PRESENTATIONS RELATED TO THE THESIS

- **Publications**

Paper I

Evert-Arriagada, K., Hernández-Herrero, M.M., Juan, B., Guamis, B., Trujillo, A.J. (2012). Effect of high pressure on fresh cheese shelf life. *Journal of Food Engineering*, 110,248–253.

Paper II

Evert-Arriagada, K., Hernández-Herrero, M.M., Chacón-Gallardo, J. J., Juan, B., Trujillo, A.J. (2013). Effect of high pressure processing on volatile compounds profile of a free-starter fresh cheese. Submitted to: *Innovative Food Science & Emerging Technology*.

Paper III

Evert-Arriagada, K., Hernández-Herrero, M.M., Guamis, B., Trujillo, A.J. (2013). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. Submitted to: *Food Research International*.

Paper IV

Evert-Arriagada, K., Trujillo, A.J., Gervilla, R., Guamis, B., Hernández-Herrero, M.M. (2013). Fate of *Listeria* spp. inoculated in a commercial starter-free fresh cheese by means of HPP. To be submitted to *Food Microbiology*.

- **Conference presentations**

Evert-Arriagada, K., Hernández-Herrero, M.M., Guamis, B., Trujillo, A.J. (2012). *Effect of High pressure processing on starter-free fresh cheese shelf-life*. Encuentros Paris 2012. Knowledge for Economic and Social development. Paris, France, 4-6 July. Poster presentation

Evert-Arriagada, K., Hernández-Herrero, M.M., Juan, B., Guamis, B., Trujillo, A.J. (2011). T*exture, Sensory and Colour Characteristics of a starter-free fresh cheese treated by High hydrostatic pressure*. 49th EHPRG Conference. Budapest, Hungary. 28 August - 2 September. Oral communication.

Evert-Arriagada, K., Hernández-Herrero, M.M., Guamis, B., Trujillo, A.J. (2010). *Inactivation of Listeria monocytogenes and Listeria innocua inoculated into fresh cheese by means of High hydrostatic pressure*. EFFoST ANNUAL MEETING. Food and Health. Dublin, Ireland, 10-12 November. Poster presentation.

Evert-Arriagada, K., Hernández-Herrero, M.M., Juan, B., Guamis, B., Trujillo, A.J. (2010). *Effect of High hydrostatic pressure on fresh cheese shelf-life*. FoodInnova-2010. International Conference on Food Innovation., Valencia, Spain, 25-29 October. Oral communication.

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LIST OF ABBREVIATIONS

ALOA	Agar according to Ottaviani & Agosti
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BP-RPF	Bair Parker agar supplemented with rabbit plasma fibrinogen
CAGR	Compound Annual Growth Rate
CECT	Colección Española de Cultivos Tipo
CLA	conjugated linoleic acid
CLSM	Confocal Laser Scanning Microscopy
EC	European commission
EFSA	European Food Safety Authority
EU	European Union
FID	Flame ionization detector
FAA	Free amino acids
FFA	Free fatty acids
GC	Gas Chromatography
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
HP	High Pressure
HPP	High Pressure Processing
HT	Heat Treatment
HTST	High Temperature Short Time
HUS	Haemolytic Uremic Syndrome
IDF	International Dairy Federation
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
LAB	Lactic Acid Bacteria
LG	lactoglobuline
LOD	Limit of detection
MAP	*Mycobacterium avium* subsp. paratuberculosis
MAP	Modified Atmosphere Packaging
MRS	Man Rogose Sharpe agar

MS	Mass Spectra
n.d.	Not determined
N.D.	Not Detected
PCA	Plate Count Agar
PCA	Principal Component Analysis
PEF	Pulsed electric field
PTA	phosphotungstic acid
RD	Real Decreto
RTE	Ready-to-eat
RCT	Rennet Clotting Time
SD	Standard deviation
SEM	Scanning Electron microscopy
SFP	Staphylococcal Food Poisoning
SN	Soluble nitrogen
SPME	Solid phase microextraction
TAL	Thin Agar Layer
TN	Total nitrogen
TPA	Texture Profile Analysis
TSA	Tryptone Soy Agar
TSAYE	TSA enriched with 0.6% yeast extract
TS	Total solid
TSB	Tryptone Soy Broth
UAB	Universitat Autònoma de Barcelona
VRBG	Violet Red Bile glucose Agar
ΔE	Colour difference
μ max	maximum growth rate

Chapter 1

Introduction, objectives & working plan

1.1. Introduction

Since its development, food science research has been focused in accomplish different consumers demands. The most important concern of the consumers is the product security. In this sense, most of the efforts carried out by food scientist are in the aim of guaranteeing food safety, by improvement in processing and monitoring techniques. Nevertheless, during the last years, there is a change in the consumer demands for safe products. This change is being related to the preference for products in which the nutritional value and flavour characteristics remain without variations after treatment, perceived as a *fresh like products* or *natural products*.

To preserve foodstuff and inactivate pathogen microorganisms, the most common technique is thermal processing. However, the application of high temperatures during processing, leads important changes in the sensory, nutritional and functional characteristics (Hogan et al., 2005; Rico, et al., 2007; Norton & Sun, 2008). Consequently, most of the research realised in the food science area is related with the application of non-thermal technologies, in which, the same safety than in thermal processing can be achieved, but reducing the disadvantages of heat treatment.

Among the non-thermal technologies, the high pressure processing (HPP) has been one of the most applied in food products, which is able to ensure product microbial safety while preserving sensory and nutritional characteristics. HPP technology achieves microbial inactivation by inactivation of key enzymes involved in biochemical reactions and genetic mechanisms, such as DNA replication and transcription (Patterson et al., 1999). Moreover, the use of this technology in products with short

shelf-life presents an alternative to heat treatment, developing more convenient products for consumers.

The origin of cheese is very remote. In a simple definition, cheese is the fresh or ripened product obtained after coagulation and whey separation of milk, cream or partly skimmed milk, buttermilk or a mixture of these products. Cheese composition depends greatly on milk composition and the manufacture process. Moreover, it is well known that some other factors such as climate, season, animal feed, age and breed, stage of lactation and health state of the animal, could influenced the composition of milk (Farkye, 2004).

Regarding cheese production and consumption over the world, the last report of the International Dairy Federation (IDF, 2011) presented a firm increased during the last decade in both parameters. The increase in cheese production only in the European Union (EU) was from 7.2 million tonnes in 2000 to 8.5 million tonnes in 2010. Besides, this increasing tendency is also in agreement with cow's cheese world production, which during the last decade improved from 14.1 million tonnes to 17.6 million tonnes in 2000 and 2010, respectively.

Milk and dairy products are some of the principal elements on Spanish diet, being cheese the second most consumed dairy product (22.8%) in this country, with an annual consumption per capita of about 8 kg, being fresh cheese the most consumed (2.5 kg per capita) cheese variety, representing 31.7% of the cheese consumption (Ministerio de Agricultura, Pesca y Alimentación, 2012).

Fresh cheese is unripened rennet-coagulated type and is usually consumed after manufacturing. It may serve as an ideal medium for bacterial proliferation because of the absence of competing starter culture, its high water activity and its high, protein and fat content. Fresh cheese is prone to rapid bacterial deterioration, particularly if it is handled under abusive storage temperatures or poor hygienic conditions. Previous works presented the possibility to applied different non-thermal technologies, alone or in combination with other treatment, in order to prevent the growth of pathogenic and spoilage microorganisms in this cheese variety. The effects of different technologies such as non and starter lactic acid starter bacteria inoculation in milk cheese, modified atmosphere packaging, antibacterial effect of lactoperoxidase system, surface pasteurisation, active coating, and the use of bacteriocins such as nisin have been

studied (Nuñez et al., 1986; Santos et al., 1995; Rosenthal et al., 1996; Capellas et al., 2000; Papaioannou et al., 2007; Dermiki et al., 2008; Conte et al. , 2009; Settanni et al., 2011). Additionally, the reports published regarding the use of HPP on this kind of cheese and its effects on the food matrix are limited (Capellas et al., 2001; Daryaei et al., 2008; Okpala et al., 2010), creating a *window of opportunity* for the research in this area.

1.2 Objectives

General objective

The main objective of this thesis was to evaluate the effect of HPP technology for extending the shelf-life of a starter-free fresh cheese, in order to obtain a high quality product.

Specific objectives

- To evaluate the suitability of High Pressure (HP) treatment of fresh cheese for extending its shelf-life by applying this technology at a pilot-scale and following their microbiological, physicochemical, textural, and colour evolution during cold storage.
- To determine the shelf-life of commercial fresh cheeses treated under industrial-scale, by following their microbiological, compositional and physicochemical evolution during cold storage.
- To elucidate the influence of HP treatment on the texture, microstructure, colour, and sensorial characteristics with respect to unpressurised fresh cheeses.
- To study the volatile compound profile of untreated and treated fresh cheeses and its evolution during cold storage.
- To evaluate the effectiveness of the HPP against different strains of *Listeria* spp. inoculated into fresh cheeses by studying their inactivation and the presence of sublethal injuries.
- To study the influence of the HP treatment on the capability of *Listeria* spp. to survive, repair and grow during the shelf-life of fresh cheeses.

1.3 Working Plan

According to the objectives, Figures 1-3 schematically represent the experimental design of all the assays performed in the framework of this thesis.

A preliminary study was carried out in order to evaluate the effect of (HP) technology on the shelf-life of small commercial starter-free fresh cheeses (~80 g) by using a HP pilot plant scale equipment ACB GEC Alsthom (Nantes, France) with a 2 L capacity pressure chamber at the Universitat Autònoma de Barcelona (UAB) pilot plant. In this section, different HP conditions were applied in order to obtain the conditions with minor sensory and physicochemical changes and with the highest shelf-life (Figure 1).

The next step was to apply HP technology on the shelf-life of commercial starter-free fresh cheeses (~250 g) (Figure 2). In this section, real industrial conditions were applied and cheeses were treated in HP industrial scale equipment (Hiperbaric S.A. Wave 6000/135) with a 135 L capacity pressure chamber.

Additionally, a third step included the evaluation of the effectiveness of the HP treatment against pathogenic microorganisms that can be present in the fresh cheese (Figure 3). In this test, due to the high resistance of the Gram + bacteria to HPP and from a public health perspective the microorganism chosen was *Listeria* spp. (*L. monocytogenes* and its surrogate *L. innocua*), evaluating after HP treatment the initial inactivation achieved, the sublethal damage generated and the microorganisms evolution during storage at 4°C. Cheeses (~250 g) were treated in a 5 L HP system (Iso-Lab FPG11500, Stansted Fluid Power LTD, Stansted, Essex, UK) using the infrastructure at the UAB pilot plant.

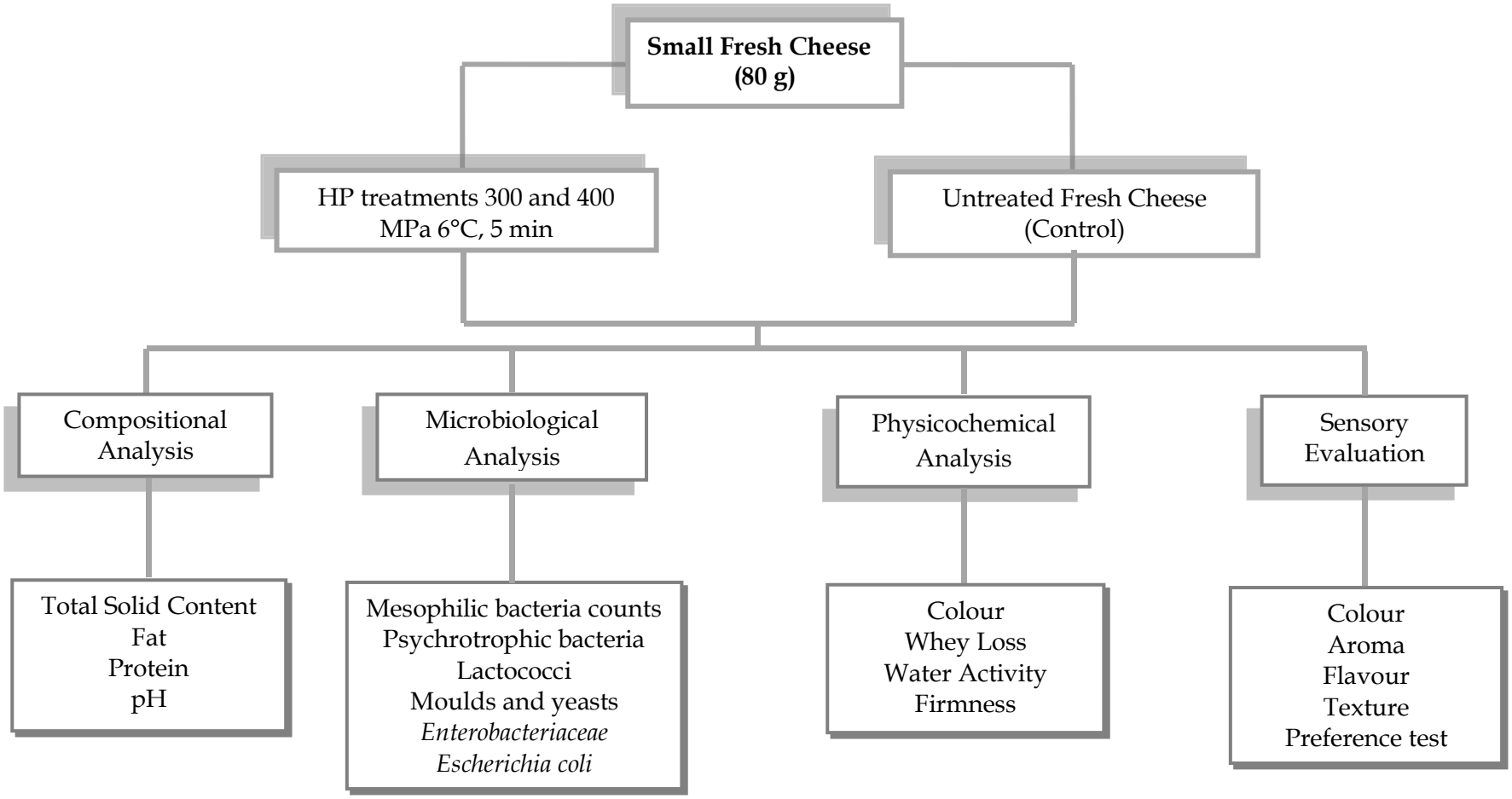

Figure 1. Working plan corresponding to the studies on Pilot Plant experiments.

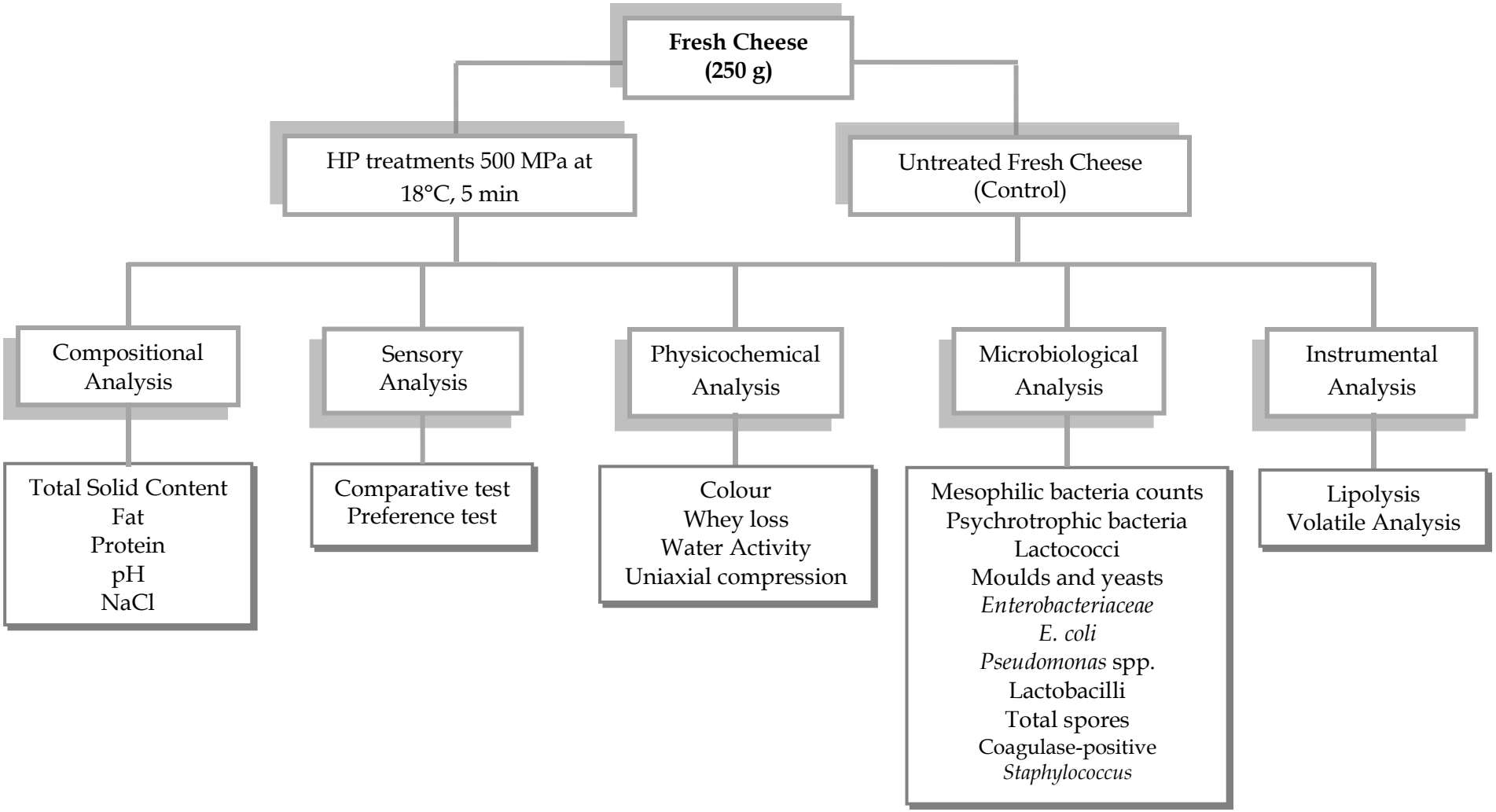

Figure 2. Working plan corresponding to the studies on Industrial Scale experiments.

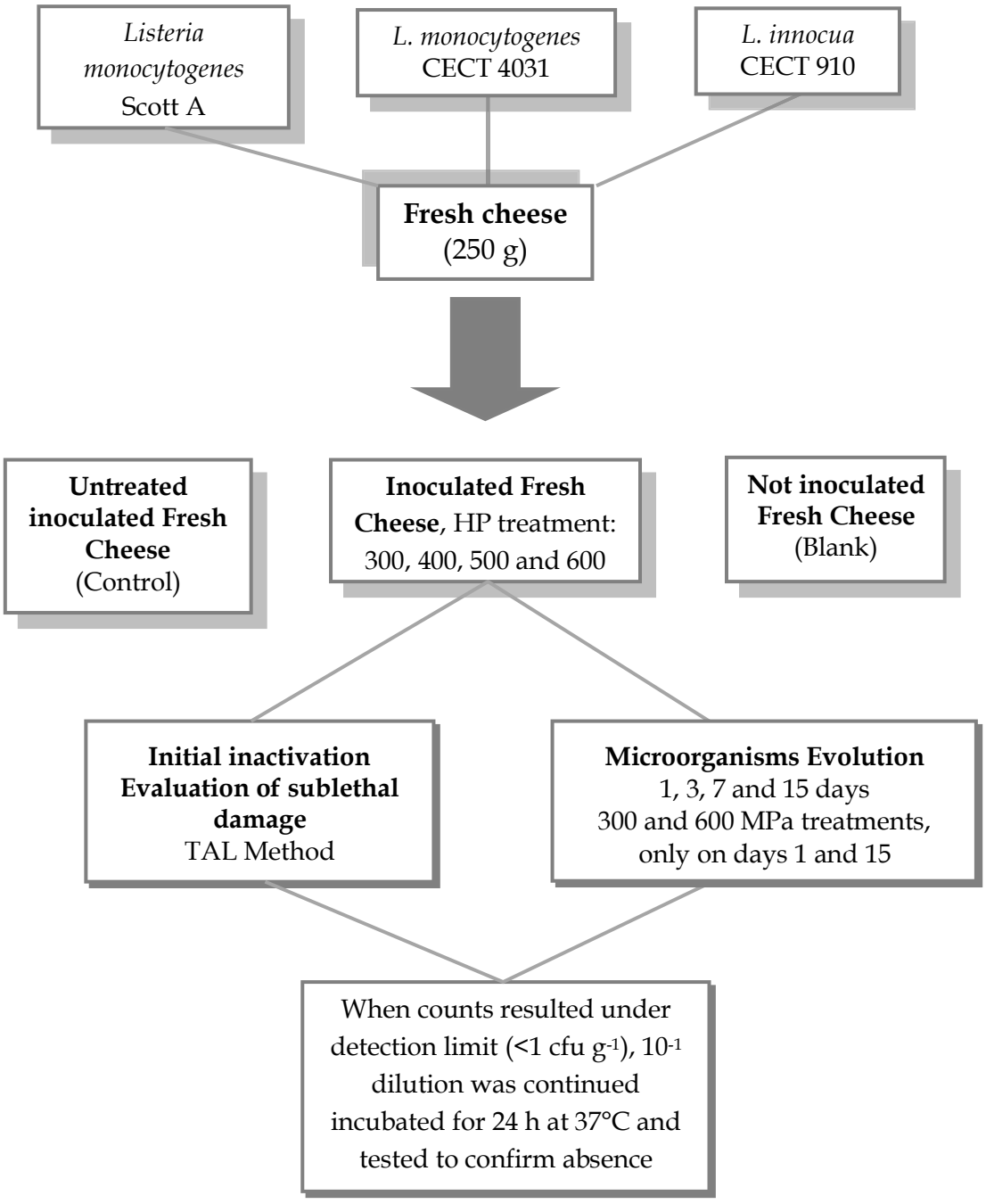

Figure 3. Working plan corresponding to the studies on Effect of HPP on *Listeria* spp. inoculated into fresh cheese.

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Chapter 2

Literature Review

2.1 Cheese production and consumption

Cheese is the generic name for a group of fermented milk-based food products, made in a wide range of flavours and forms throughout the world. Traditionally, it was elaborated as a way of preserving milk. According to the Codex Alimentarius, cheese is the ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk. Fresh cheese is the cheese which is ready for consumption shortly after manufacture. Cheese can be obtained by coagulating and draining wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents (Anonymous, 2007a). Cheese is considered as one way of preserving milk and increasing its economic and nutritional values. It is estimated that there are in excess of 1000 distinct cheese varieties currently produced worldwide (Fox & McSweeney, 2004). Cheese is an excellent source of protein, fat and minerals such as calcium, iron and phosphorus, vitamins and essential amino acids and consequently is an important food in the human diet. However, its composition greatly depends on milk composition and the manufacture process. It is well known that some other factors such as climate, season, animal feed, age and breed, stage of lactation and health state of the animal, could influenced the composition of milk (Farkye, 2004).

With regard to cheese production and consumption over the world, the last report of the IDF (IDF, 2011) presented a firm increased during the last decade in both parameters. The

increase in cheese production only in the EU was from 7.2 million tonnes in 2000 to 8.5 million tonnes in 2010. Besides, this increasing tendency is also in agreement with cow's cheese world production, which during the last decade increased from 14.1 million tonnes in 2000 to 17.6 million tonnes in 2010. Additionally, cheese consumption in the world presented also an increase during the last decade. The world average increase in cheese consumption was up to 4.5 million tonnes based in Compound Annual Growth Rate (CAGR). In 2011, cheese was the second most consumed dairy product (22.8%) in Spain, with an annual consumption per capita of about 8 kg. Fresh cheese was the most consumed (2.5 kg per capita) cheese variety, representing 31.7% of the cheese consumption (Ministerio de Agricultura, Pesca y Alimentación, 2012).

2.1.1 Starter-free fresh cheese

Traditional fresh cheese is a soft cheese that is manufactured with little or no starter culture and subsequently does not go through a fermentation or aging period. Due to the absence ripening, the cheese is normally consumed within 3-7 days of manufacture, although the labelled expiring date is usually of 14 days. This variety is typically white, have relatively high moisture (46-57%) and high pH (>6.1), contains about 18-29% fat, 17-2% protein and 1-3% salt (Hwang & Gunasekaran, 2001), it has a crumbly texture resulting from fine milling before salting (Van Hekken & Farkye, 2003). There is a substantial market for fresh cheese due to the demands for new cheese flavours, varieties and products, the nutritional benefits such as its high mineral and protein contents, as well as, the economic advantages due to its relatively short or no ripening time and high yield. In the U.S., for example, Hispanic cheeses such as fresh cheese are gaining in popularity due to the increasing ethnic diversity of the population (Young, 2008).

2.1.2 Fresh cheese manufacturing

Milk for fresh cheese-making is generally standardised for fat content and pasteurised at 70-80°C for 15-40 s. Once pasteurised, milk is adjust to 30-37°C and calcium chloride, rennet and salt are added. Sodium chloride is usually added directly to milk before coagulation for better homogeneity of salt content within the final product. Curd salting contributes to decrease water activity (a_w), control enzymatic activity and microorganism growth, affect biochemical and physico-chemical changes and enhance the flavour of cheese (Guinee & Fox, 2004). Coagulation time ranges from 15 to 45 min depending on the characteristics of the rennet used. The rennet-set curd is cut and cooked to desired firmness, and the whey is drained. When curd is drained, fat and casein in milk are concentrated between 6-12 fold times (Fox & McSweeney, 2004). The curd is then finely milled and packed into moulds of different shapes and sizes. Whey drainage is achieved without pressing through gravitation at 7-10 ^{o}C. For large cheeses (500 g), drained curds are turned upside down to facilitate the drainage of the upper part of the cheese.

2.1.3 Technological challenges

Traditional fresh cheeses varieties usually have low refrigerated shelf-life of approximately less than 14 days, mostly due to the growth of spoilage microorganisms. The potential export market is hindered by its relatively short shelf-life. Many producers are interested in extending the shelf-life of their products without having to use preservatives, and maintaining the desirable sensory attributes of the product to take advantage of export opportunities. Since the use of preservatives is restricted by law and its labelling is compulsory under European Directive 2000/13/EC (Anonymous, 2000), Spanish Real Decreto 1334/1999, Spanish Real Decreto 142/2002, and their modifications (Anonymous, 1999; 2007a; 2004a; 2004b; 2011a), and considering that sometimes producers omit the inclusion of some additives, such as sorbic acid in the labelling (Anonymous, 2007b); the implementation of alternative methods to extend the shelf-life for these products is necessary. Many works using different technologies have been carried out to achieve this goal. Some of them includes: inoculation of lactic starter in cheese (Nuñez et

al., 1986), modified atmosphere packaging (Dermiki et al., 2008), antibacterial effect of lactoperoxidase system (Santos et al., 1995), surface pasteurisation (Rosenthal et al., 1996), the use of bacteriocins such as nisin (Capellas et al., 2000), ultra-high-pressure homogenisation treatment of cheese milk (Zamora, et al., 2012), and high-pressure processing of fresh cheese (Capellas et al., 2000; Daryaei et al., 2008, Okpala et al., 2010, Koca et al., 2011).

2.2 Cheese safety

The origin of cheese is very remote. Even though, in the beginning, milk was contaminated with lactic acid bacteria, and later the acidification of milk created for these bacteria gave conditions for other microorganisms. Pathogens survival and growth in milk and milk products depend on several factors, including environment, udders, farm management practices and milking equipment (Holsinger et al., 1997; Oliver et al., 2005). In the case of cheese, time and temperature during ripening, presence of competing microorganisms, variations in pH and a_w, as well as biochemical changes during ripening may affect the safety during cheese-making process.

Milk is a nutritious medium, rich in carbohydrates, proteins and fats, which presents a favourable environment (near neutral pH) for the multiplication of microorganisms and supports a wide range of spoilage and pathogenic bacteria. There is an influence of the initial microbiological quality on the cheese, based on the quality of the raw material employed. According to U.S. standards, Grade A raw milk may not exceed 300,000 cfu mL^{-1} (Anonymous, 2011b) and in the European community the bacterial count should be lower than 100,000 cfu mL^{-1} (Anonymous, 2004c). However, pasteurised milk in the U.S. must contain less than 20,000 cfu mL^{-1} (Anonymous, 2011b). Although pasteurisation is regarded as an effective method for eliminating pathogens, the increasing number of outbreaks reports in 'pasteurised' milk and ready-to-eat (RTE) dairy products clearly indicates that pasteurisation may not destroy all food-borne pathogens presented in milk, which can survive and thrive in post-pasteurisation processing environments, leading to

recontamination of dairy products. Therefore, pasteurisation alone is not the final solution for the control of milk borne pathogens (Donnelly, 1990; Oliver et al., 2005).

Nowadays, special attention is focused on the safety of the food supply, in particular in milk and dairy products. Many cheese makers used raw milk or add raw milk to the cheese milk considering this essential for assuring the good flavour of the product, primarily due to the greater proteolysis and lipolysis activity of the raw milk microorganisms (Little et al., 2008). This presents a high risk in cheese process due to the presence of pathogen bacteria during elaboration that cannot be eliminated. On the other hand, consumers are more aware of the potential for food-borne pathogens and are concerned about the quality of their food. Public perception of food quality is critical in the marketing of any product. Therefore, dairy industry and public health regulators must remain vigilant to ensure that all measures are taken to prevent the entry and multiplication of pathogenic microorganisms during the handling and processing of milk and milk products to prevent any pathogen associated illness.

2.2.1 Pathogenic bacteria

Pathogenic microorganisms such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolotica*, and *Escherichia coli* are the main bacterial etiologic agents involved on food-borne outbreaks associated with milk or milk products consumption (De Buyser et al., 2001; Gillespie et al., 2003; Oliver et al., 2009). According to the data available in the Community Summary Report on zoonoses, zoonotic agents and food-borne outbreaks, from the total of 5,262 food-borne outbreaks reported (698 strong evidence outbreaks) cheese was involved in 2.3% of the strong evidence outbreaks (Anonymous, 2012a).

Many milk-borne infections exhibit a number of clinical presentations, but gastrointestinal disorders (diarrhoea, vomiting, nausea, fever, abdominal cramps) are the most common clinical manifestation. However, a certain percentage of the population can develop more severe clinical symptoms such as Guillain-Barré syndrome (*Campylobacter* spp.) and haemolytic uremic syndrome (HUS; *E. coli* O157:H7), or even death. Assuming a score of

severity on a scale from 1 to 4 (being 4 the most serious effect) for the pathogens frequently encountered in raw milk related outbreaks, a score of 3, 3, 4 and 4 can be assigned to *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes* and human pathogenic *E. coli,* respectively (Claeys, et al., 2013).

The European Community regulation on the hygiene of foodstuffs established that food industry operators shall ensure that all stages of the production, processing and distribution of food, under their control, satisfy the general hygiene requirements and shall put in place, implement and maintain a permanent procedure based on the Hazard Analysis Critical Control Point (HACCP) principles (Anonymous, 2009b). Nowadays the HACCP concept has international recognition and is one of the main guidance devices that set up an effective preventive system which leads to safe food production. In addition, HACCP system requires to be built upon a solid foundation of prerequisite programs which take into account the necessary conditions that each food industry segment must provide to protect food while it is under their control. This has traditionally been accomplished through the application of good manufacturing practices (GMP), due to the fact that microbiological quality of cheese is influenced by the equipment and environmental hygiene during manufacture, packaging and handling (Anonymous, 2009b). These conditions and practices are now considered to be prerequisite for the development and implementation of effective HACCP plans.

Contamination of fresh cheese can occur during its processing and shelf-life. Improvements in the safety and quality of fresh cheese need to be effective in inhibiting growth of pathogenic and spoilage organisms, while maintaining the properties and characteristics of this cheese variety.

There are several bacteria with human pathogenic potential that can be found in dairy products, some of them are described as follows:

• ***Mycobacterium paratuberculosis.*** Recently, considerable interest has generated the possible association between John's disease in ruminant animals caused by *Mycobacterium avium* subsp. paratuberculosis (*MAP*) and Crohn's disease in humans (Sanderson, 1992;

Greenstein, 2003). The heat resistance of this organism has been subject to extensive study during the past decade using various laboratory techniques and pilot scale High Temperature Short Time pasteurisation (HTST, 72ºC for 15 s) equipment (Grant, et al., 2002, Lund, et al., 2002). The use of pulse electric field (PEF) and HPP has been also studied with the aim to reduce counts of *MAP* in milk by means of these non-thermal technologies (Rowan et al., 2001; López-Pedemonte et al., 2006; Donaghy et al., 2007).

• ***Bacillus cereus.*** *B. cereus* is associated with two types of gastrointestinal disorders: the *emetic syndrome*, characterised mainly by vomiting and caused by ingestion of a preformed toxin in the food, and the *diarrhoeal syndrome*, caused by a different toxin that can be formed in the food but also in the small intestine (Granum & Lund, 1997; Ehling-Schulz et al., 2004). According to European Food Safety Authority report (EFSA; Anonymous, 2010), in 2008 this pathogen was responsible for 1132 cases of outbreaks. *B. cereus* not only can cause food-borne illness, but can affect the quality of dairy products, such as "bitty cream" (aggregation of the cream layer by the action of lecithinase) and "sweet-curdling" (coagulation without pH reduction) (Andersson, et al., 1995). This pathogen is generally classified as a mesophile, though psychrotolerant strains i.e. *B. weihenstephanensis,* has been proposed for this sub-group of *B. cereus* (Lechner, et al., 1998). These strains are not uncommon, particularly in raw and pasteurised milk (Jenson & Moir, 2003). Refrigerated foods, such as fresh cheeses, are a new niche for such bacteria and they have indeed become a problem for the food industry. Spores of *B. cereus* have shown extreme resistance to several preserving technologies such as heat, PEF, ultrasounds, HPP and bacteriocins (Pol et al., 2001; Ross et al., 2003; Black et al., 2007). Combinations of two or more non-thermal processes have been also evaluated in an effort to find a potential effective synergy (Ross et al., 2003). The combination of HPP and nisin or lysozyme was used to increase the inactivation of *B. cereus* spores in model cheese (López-Pedemonte, et al., 2003). Cheese inoculated with approximately 6 log cfu g^{-1} of *B. cereus* spores were subjected to a germination cycle of 60 MPa at 30°C for 210 min, followed by an inactivation cycle at 300 or 400 MPa at 30°C for 15 min. Only the combination with nisin

(1.56 mg L^{-1} of milk) resulted in an increase in sensitivity of the spores to HPP, obtaining a reduction of 2.4 log cfu g^{-1} of cheese.

• ***Staphylococcus aureus.*** Staphylococcal food poisoning (SFP) is caused not by ingestion of the organism itself, but by an enterotoxin produced by *S. aureus* growing in the food under suitable conditions prior to consumption (Stewart, 2003). Thus, absence or low numbers of *S. aureus* in a heat treated food product does not guarantee its safety; absence of the enterotoxin must also be demonstrated. SFP is one of the most prevalent causes of gastroenteritis worldwide (Hennekinne et al., 2012). Symptoms include nausea, vomiting, abdominal pain and diarrhoea, which usually occur approximately 2–6 h after the consumption of food containing enterotoxins (Tranter, 1990). In 2008, *S. aureus* was responsible for 595 reported cases of outbreaks caused by bacterial toxins (Anonymous, 2010). *S. aureus* is referred to as one of the most resistant non-sporulated Gram-positive bacteria to HPP (Patterson et al., 1995; Gervilla et al., 1999a, 2000; Alpas et al., 2003). Gervilla et al. (1999a) studied the effect of HPP on *S. aureus* CECT 534 in inoculated ewe's milk showed that this pathogen was highly resistant to HP treatments between 200-500 MPa at 4, 25 and 50°C; only pressurisation at 500 MPa (50°C, 15) min achieved reductions higher than 7.3 log units.

• ***Salmonella* spp.** Salmonellae are rod-shaped, motile, gram-negative bacteria that infect many animals including cattle, poultry, and swine. Pasteurisation of milk is very effective in killing *Salmonella* spp. However, pasteurised milk may become contaminated as a result of faulty processing procedures. Utensils used for cheese-making may also be contaminated with *Salmonella* spp. (Poppe, 2011). *Salmonella* infections, even though they have been mostly associated with poultry (Bryan & Doyle, 1995), have been also linked to outbreaks associated with the consumption of various types of cheese (De Buyser et al., 2001; Kousta et al., 2010). One of the largest outbreak of Salmonellosis in U.S. history occurred in 1985 with 'pasteurised' milk containing 2% milk fat. Investigation into the cause of this outbreak indicated no irregularity in processing but *Salmonella* was isolated

from various points within the processing plant, especially from valves linking the raw and pasteurised milk tanks (Flowers et al., 1992).

The inactivation of *Salmonella* by HPP has been investigated in different food substrates such as milk, cheese and poultry (Chen et al., 2006; De Lamo-Castellví et al., 2007, Guan et al., 2005; Morales et al., 2009; Erkmen, 2009; Tananuwong et al., 2012). In model cheese, produced with and without starter culture, De Lamo-Castellví et al. (2007) investigated the impact of the pressurisation at 300 and 400 MPa for 10 min on viability of 2 strains of *Salmonella enterica*. However, when starter culture was not present, the maximum lethality was only observed in cheese inoculated with *S. E*nteritidis and treated at 400 MPa. These authors concluded that HP treatments can be useful to accelerate the reduction of initial levels of *Salmonella* in cheese, but it is necessary to combine this technology with the low pH and the presence of the starter culture to inhibit recovery and growth and to increase the death rate of the injured population.

• ***Campylobacter* spp.** *Campylobacter* infections have been founded to be more common than *Salmonella* infections, with an infective dose as low as 2 to 3 cells mL^{-1} (Flowers, et al., 1992). Campylobacteriosis has been associated with abdominal pain, reactive arthritis, Reiter's syndrome and the Guillain-Barré syndrome (Smith, 1996). Humans get infected through ingestion of contaminated non-pasteurised milk, not properly pasteurised milk, untreated water, and raw or improperly cooked poultry (Oliver et al., 2009). In the U.S., *C. jejuni* accounts for 99% of the reported *Campylobacter* spp. from human disease, with *C. coli* accounting for the majority of the remaining 1% (Wallace & Hocking, 2003). According to Holsinger et al. (1997), this microorganism does not survive pasteurisation and proper refrigerated storage prevents growth in contaminated milk. Therefore *C. jejuni* is not considered a problem in properly processed dairy products. However, there would be a possible post-pasteurisation contamination as a result of faulty processing procedures.

The effect of HPP (50-400 MPa, 10 min at 25ºC) on the inactivation of two strains of *C. jejuni* in buffer, broth and food substrates, such as whole and skim ultra-high temperature milk, was studied by Solomon & Hoover (2004). They found that HP treatment at 300-

325 MPa for 10 min was sufficient to reduce viable numbers of *C. jejuni* by 8 log units or more in broth or phosphate buffer, whereas in milk the same treatment gave only a 2-3 log decrease, suggesting that food products offered a protective effect and additional 50-75 MPa were required to achieve similar levels of inactivation when compared with broth and buffer.

• ***Escherichia coli.*** *E. coli* is a Gram-negative, non-spore forming, rod-shaped organism. Different groups have been recognised, namely, enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative and enterohaemorrhagic (Eley, 1996). Enterohaemorrhagic *E. coli* O157:H7 is recognised as an important food-borne pathogen that has caused food-borne outbreaks related to consumption of raw and dairy products (Anonymous, 2010). The infectious dose is very low (10–100 organisms), causing a wide range of clinical symptoms, including bloody diarrhoea (haemorrhagic colitis), HUS, and death (Harris et al., 2003). The application of non-thermal technology for the control of this pathogen has been studied in fresh goat's milk cheese. Capellas et al. (1996), working with inoculated Mató cheese achieved reductions of 6.7 to 8.7 log units in the population of *E. coli* in cheeses treated at 400 and 500 MPa for 5 to 15 min at temperatures of 2 to 25°C.

2.2.2 *Listeria monocytogenes*

Listeria spp. is a small, motile Gram-positive rod, catalase positive and psychotropic bacteria. It can grow in a wide pH and temperature range (Lado & Yousef, 2007, Rocourt & Buchrieser, 2007). The genus *Listeria* contains six species: *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, *L. seeligeri* and *L. monocytogenes*. Among all *Listeria* species, only *L. monocytogenes* has been described as a food-borne pathogen (Rocourt & Buchrieser, 2007). *L. monocytogenes* is widely distributed throughout the natural environment. Its ubiquity and psychrotrophic character, appear to be the main causes of its high prevalence in various food products including refrigerated RTE food (Lianou & Sofos, 2007), which present a potential hazard for pathogen transmission through improper pasteurisation, or contamination occurring in the cheese processing step, from the environment and/or

equipment (Kozak, 1996; Todd, 2011). This pathogen is often isolated from the environment (e.g. floors, drains) of cheese companies even when good sanitation and hygiene protocols are in place (Kornacki & Gulter, 2007).

L. monocytogenes has become a great concern to the dairy industry, because of the extent of the outbreaks and the high overall mortality rate compared with those of other food-borne bacteria (Donnelly, 2004). The incidence of listeriosis varies between 0.1 and 11.3 per million people in different countries (Anonymous, 2004e). However, listeriosis epidemics constitute the most severe infections among those traced to milk and milk products; inappropriate handling of pasteurised milk could result in bacterial growth and substantially increase the potential risk to consumers of pasteurised milk and its products (De Buyser et al., 2001; Ryser, 2007). Table 1 shows the prevalence of several listeriosis outbreaks involving different type of cheese. In 1985, consumption of a Hispanic soft cheese was linked to 300 estimated cases of listeriosis in U.S. with a death rate of 34% (Linnan et al., 1988); unpasteurised milk was likely illegally added to pasteurised milk used in cheese-making. Recalls of dairy products, contaminated by this pathogen are frequent nowadays, with subsequent economic losses (Arqués et al., 2005; Todd, 2007; Anonymous, 2012b). Therefore, controlling contamination and growth of *L. monocytogenes* during cheese manufacture, ripening, and storage are an important safety concern and consumer demand.

Listeriosis, usually presents as septicaemia, meningitis or abortion, and cases occur in well-defined risk groups, including immune-compromised individuals, elderly, pregnant women, and newborns, even though the level of contamination play also a major role for the pathogen to cause infection (Ryser & Marth, 2007).

Harmonisation of *Listeria* regulations within the European Community, through EU regulation EC 2073/2005 (Anonymous, 2005) as amended by regulation 1441/2007 (Anonymous, 2007d), have been complicated by the different standards implemented within the member states i.e. Germany have tolerance limits for *Listeria* where others, such as Spain, implemented a zero-tolerance (absence of the organism in 25 g) policy (Todd, 2007).

Table 1. Prevalence of *L. monocytogenes* in different types of cheese. Adapted from Kousta et al. (2010).

Cheese type	*Type of milk used*	*Prevalence % (total samples)*	*Country of origin*	*Publication year*
Soft cheese	Pasteurised	0.0 (36)	France	1987
Soft cheese	Unknown	5.2 (19)	France	1987
Soft cheese	Raw milk	65.0 (14)	France	1987
Soft cheese	Raw milk	46.0 (63)	Portugal	2005
Soft cheese	Unspecified	40.0 (10)	Greece	2008
Soft cheese	Unspecified	6.2 (192)	Italy, France Austria, Germany	2001
Soft ripened cheese	Unspecified	8.2 (769)	England and Wales	1991
Soft unripened cheese	Unspecified	1.1 (366)	England and Wales	1991
Semi-soft	Pasteurised	2.1 (1489)	Italy	2005
Soft and semi-soft cheese	Raw	41.9 (31)	France, Germany, Italy	1995
Soft and semi-soft cheese	Pasteurised	2.3 (302)	France, Germany, Italy	1995
Fresh cheese	Unspecified	1.3 (78)	Spain	2008
Semi-soft cheese	Unspecified	7.6 (92)	Italy, Germany, Austria, France	2001
Fresh cheese	Unspecified	4.0 (50)	Portugal	2004

This in effect provided the option of either moving towards a European wide zero-tolerance policy or adopting the risk-based approach. Under the regulation, high risk foods, where growth of this microorganism can occur, should test negative for *Listeria* in a 25 g sample at the point of release from the processor. An additional option proposed by the authority is therefore to tolerate 100 cfu g^{-1} throughout the shelf-life provided that the manufacturer is able to demonstrate that the product will not exceed this limit during the

product shelf-life. For RTE foods that support growth of *L. monocytogenes*, it is impossible to predict with high degree of certainty that the level will or will not exceed 100 cfu g^{-1} during the shelf-life of these products. Thus, applying this option may result in accepting a probability that foods with more than 100 cfu g^{-1} will be consumed. The impact on public health would depend whether the levels markedly above 100 cfu g^{-1} are reached.

2.3 High pressure processing

Nowadays, one of the most applied non-thermal technologies in food treatment is the HPP. It was first reported by Hite (1899) who used this technology to increase the shelf-life of milk. HPP has been able to scale from laboratory to pilot plant and industrial operation during the last 20 years. Since then several reviews of the application of this technology on different food products have been published (Farr, 1990; Cheftel, 1995; O'Reilly et al., 2001; Trujillo et al., 2000a, 2002; San-Martín et al., 2002; Torres & Velázquez, 2005; Rastogi et al., 2007; Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Martínez-Rodríguez et al. 2012). Moreover, HPP is the emerging technology with more products sale in the market such as fruit juices, york ham, fruits and vegetables, sauces, desserts, milk products, seafood, eggs and guacamole, with high consumer acceptance (Bermúdez-Aguirre & Barbosa-Cánovas, 2011). Some of the current uses of HPP technology are shown in Table 2.

HPP technology is based in two main principles, the Pascal principle: "The pressure at any point of a fluid is the same in every direction, exerting equal force on equal surfaces". In other words, the HP acts immediately and in every direction in the pressure fluid and independently the size and shape of the product treated (Knorr, 1993; Cheftel, 1995; Smelt et al., 1998). The other principle is the Le Chatelier principle: "If a system at equilibrium is disturbed, this system shifts to counteract the change, reaching a new position of equilibrium".

Table 2. Uses of HPP in food industry. From Bermúdez-Aguirre & Barbosa-Cánovas (2011).

Specific use	*Pressure range*	*Food product related*
Microbial inactivation in raw products	200–800 MPa	Fruits and vegetables, meat, milk, dairy products, seafood
Microbial reduction in processed and packaged products	400 MPa	Deli meats and ready-to-eat foods
Enzyme inactivation	200–600 MPa	Mainly fruit- and vegetable-based products
Modification in texture	200 MPa	Meat
Sterilisation	Above 600 MPa plus heat (60°C and higher)	Low-acid foods
Reduction of curdling time in cheese-making and ripening	200–670 MPa	Cheddar, mozzarella
Enhancement of green colour	Depends on product	Green beans, broccoli, basil, spinach
Reduction of fat globule size	200 MPa	Milk, cheese, yogurt

During HP treatment, any reaction related with a reduction in volume (such as change in molecular configuration, transition phase or chemical reactions) will be enhanced and the opposite (Cheftel, 1995). Besides, many changes take place as a result of a volume change caused by pressure. However, these changes depend on several parameters such as treatment intensity, temperature, process length and pressure build-up and decompression system among others (Patterson et al., 1995; Smelt, 1998). Also, HPP has some effects on the material pressurised by acting on non-covalent interactive forces which stabilise the structure of biopolymers such proteins and polysaccharides. As a consequence, the process may induce denaturation, aggregation and gel formation of food major components (Heremans, 2001). In the case of enzymes, the HPP could result in an

activation or inactivation, depending on the kind of enzyme and the substrate nature (Trujillo et al., 1997; Patterson et al., 1999; Rastogi et al., 2007). Additionally, HPP affects microorganisms as it induces changes to their morphology, biochemical reactions, genetic mechanisms and cell membranes and walls (Hoover et al., 1989).

2.3.1 High hydrostatic pressure equipments

HP technology is traditionally a batch process that can be used for liquids and solids products. The industrial pressure vessels have capacities from 35 to 350 L and reached up to 600-800 MPa (Knorr et al., 2011), and for research purposes, 1400 MPa equipments with pressure vessel models of 0.5 L have been developed (Stansted Fluid Power, Inc). Industrial HP treatment of foods is currently conducted in batch or semi-continuous systems of vertical or horizontal design. Depending on the product, treatments are carried out in discontinuous (solid or liquid packed products) or semi-continuous (unpacked liquid products) equipments. In the first case, the product is put in the pressurisation container and this into the vessel. The pumps are used to inject the transmission fluid (usually water) into the vessel. Once the desired pressure is reached, the pump is stopped, the valves are closed and the pressure is maintained without any energy input (Figure 4).
Pressure is transmitted rapidly and uniformly throughout the pressure fluid to the food and as it is equal from all sides, the pressure does not significantly affect the product shape (Smelt, 1998; Rastogi et al., 2007). In the case of liquids, the vessel can be filled with the product, which becomes itself the pressure transmission fluid. After the treatment, the liquid products can be transferred to an aseptic filling machine (Patterson, 2005). The packing material used in the process should be flexible, with a seal and barrier system that guarantees that there is not interaction between the product and the environment. The presence or air pockets reduce the treatment efficiency, for this reason, the product has to occupy the entire container (Mertens, 1993).

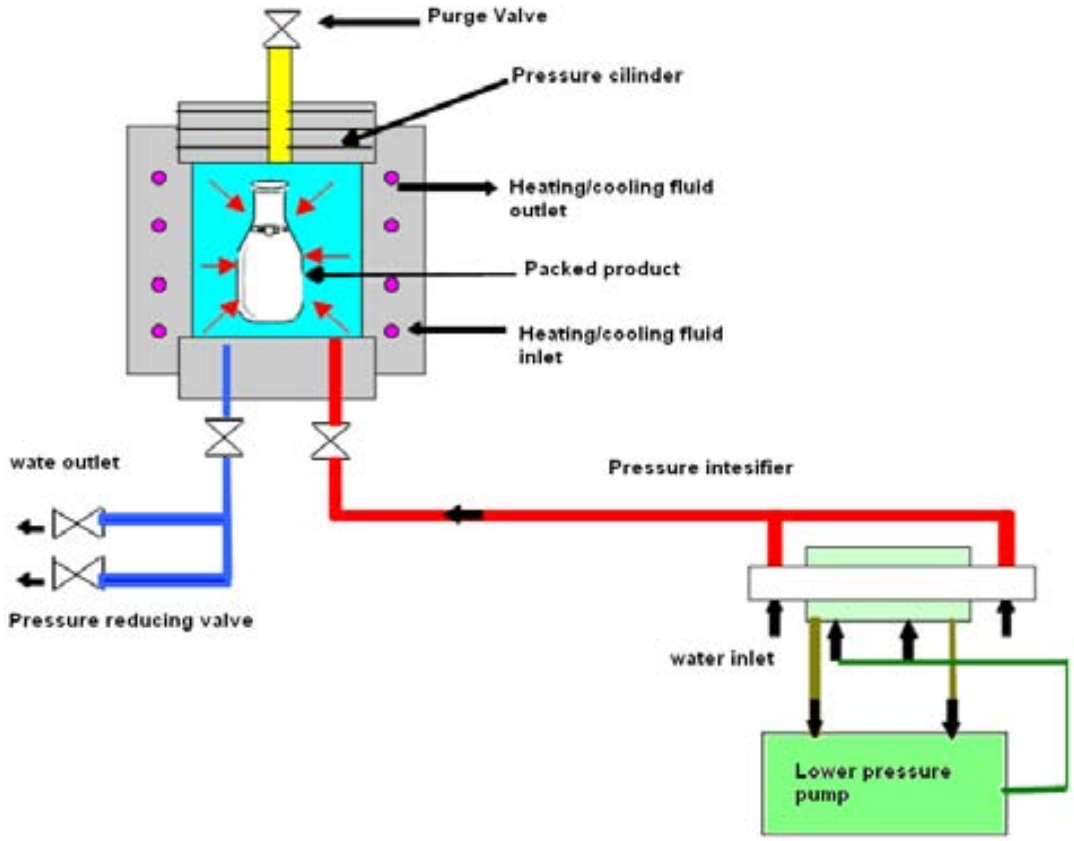

Figure 4. High pressure equipment diagram from Centre Especial de Recerca Planta de Tecnologia dels Aliments, Universitat Autònoma de Barcelona (courtesy of ACB-GEC Alsthom, Nantes, France).

The key of the HP equipments are the pressure vessels, the generating pumps or pressure intensifiers and the pressure transmission fluid (Figure 5). The pressure intensifiers operate based on the principle of ratio of areas. The pistons of two cylinders with different bore diameters are connected through a rod. The low pressure fluid (i.e. oil) contained in the first cylinder and acting on the larger piston exerts a force to the smaller piston. This force is transferred mechanically by the rod and then a higher pressure is generated by the smaller piston to the fluid in the second cylinder. Oil at low pressure (i.e. 20 MPa) is fed on the high oil pressure side of the main pump piston which has a high area ratio (i.e. 20:1 or

30:1) with respect to the HP fluid piston displacing into the HP vessel the pressure transmitting fluid.

When the main piston reaches the end of its displacement, the system is reversed and high pressure oil is then fed to the other side of the main piston and the HP fluid exits on the other pump side.

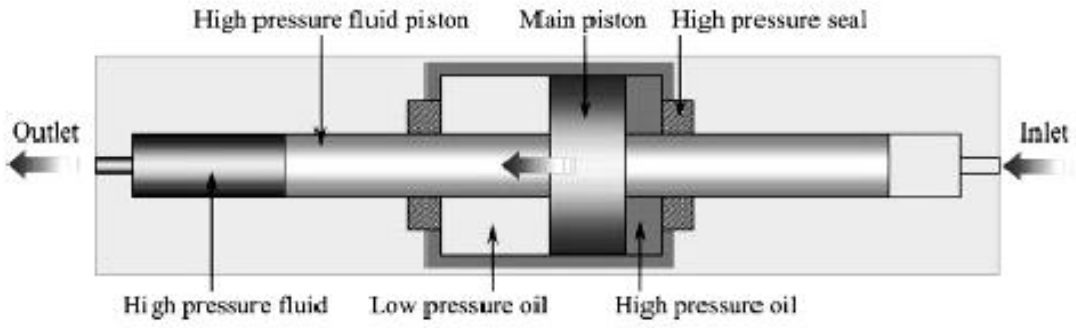

Figure 5. High hydrostatic pressure pump or intensifier (Adapted from Torres & Velázquez, 2005)

During the last 20 years there has been a relevant increase in the number of industrial facilities for HPP around the world. The number increased from one company in Japan in 1990 to about 160 companies around the world in 2010. Also, there is an increase in the variety of products treated, from meat products in the early 90's to vegetables and seafood at the end of the last decade, giving more options to consumers (Figure 6) (Mújica-Paz et al. 2011; Bermúdez-Aguirre & Barbosa-Cánovas, 2011).

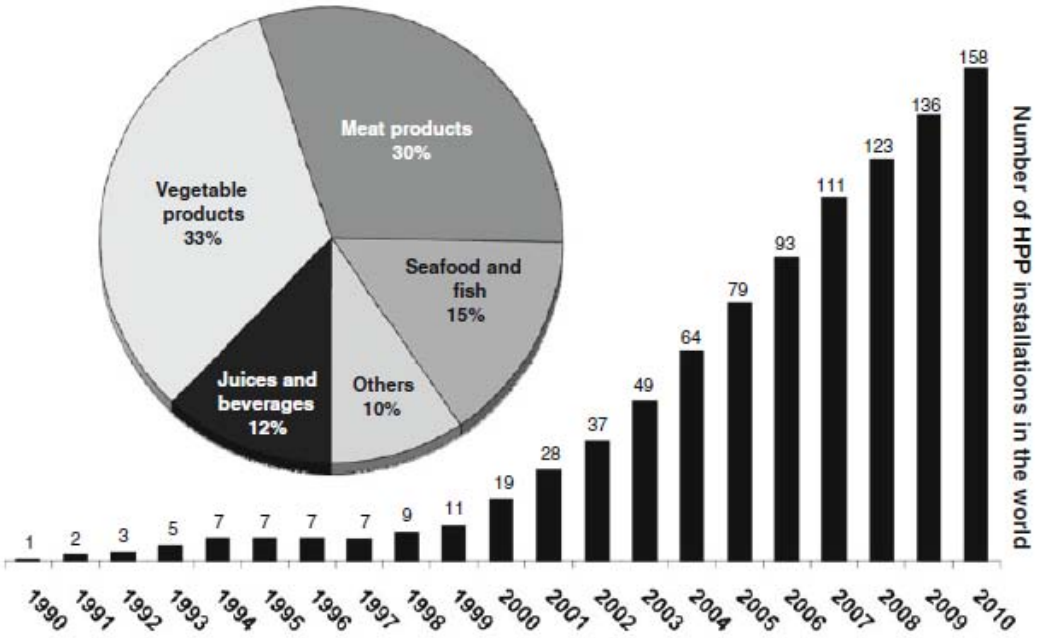

Figure 6. World growth of the food industry use of HP technology. Source: Hiperbaric S.A., Burgos, Spain (http://www.hiperbaric.com/) and Mújica-Paz et al. (2011).

Nowadays, the current HPP equipment manufacturers include: *Hiperbaric*® S.A. (Spain), company which offers high-pressure equipment for food processes, laboratory and industrial applications; *Stansted Fluid Power*® Ltd (United Kingdom), that manufactures and supplies a wide range of industrial and research HP applications and components for food, pharmaceuticals or cosmetics; *Elmhurst Research, Inc.* which designs and manufactures ultra high pressure vessels for food processing; *Engineering Pressure Systems International EPSI*® (Belgium), company which supplies laboratory scale and industrial HPP equipment; *Kobe Steel*® (Japan) that offers a whole range of laboratory and industrial high-pressure processors; *Resato International*® (The Netherlands), company specialised in the design and manufacture of high-pressure components, HP test equipment, and complete systems; *Avure* ® (U.S. and Sweden), which manufactures both batch presses and semi-continuous systems, with a wide variety of commercial applications developed; *UHDE*® (Germany), that offers high-pressure processing equipment for the treatment of

food, pharmaceuticals or cosmetics, and *ACB Pressure System-Alstom Hyperbar®* (France) which design and prototyping of a HP intensifier incorporating shear seal technology. Most of these companies work in collaboration with researchers and the food industry on the specific requirements a food product needed to ensure its microbial quality when undergoing minimal processing (Bermúdez-Aguirre & Barbosa-Cánovas, 2011).

2.3.1.1 Cost of HPP

In the past, the production and commercialisation of pressurised products was focused on high value-added foods because of two reasons, low availability of HPP equipments in many countries and the scarcity of HP processed products. However, during the last few years, there were a fast development of HPP technology due to its high potential acceptance (Butz et al., 2003; Deliza et al., 2005; Olsen et al., 2010); in consequence, more pressurised products are now available in local supermarkets, reducing their cost considerably. Besides, the reduction of cost in HP products is related to the design of larger units with an increase in vessels capacity, which increased the production (Bermúdez-Aguirre & Barbosa-Cánovas, 2011).

For cost analysis of the whole process, some aspects need to be considered during the installation of the HP plant, i.e., the cost of equipment, automation level, installation, plan preparation, labour, utilities, product design, and packaging. The main cost involved is the equipment and its installation, but because of current availability of HP equipment and companies, the cost of products processed by this technology has dropped significantly in recent years, making these products accessible to consumers. According to Purroy et al. (2011), the initial investment on commercial HPP machines can range from 500,000 € up to 2,000,000 € depending upon equipment capacity (55 to 425 L); therefore, amortisation of the equipment is responsible for about 60% of the processing cost. Parts replacement represents 36% of the cost. The energy cost is less than 4% and water consumption is negligible. HPP costs at fixed processing times are approximately 0.070 € kg^{-1} when treated at 300 MPa, 0.082 € kg^{-1} at 400 MPa, 0.094 € kg^{-1} at 500 MPa, and 0.106 € kg^{-1} at 600 MPa. The cost of wear parts are 0.011 € kg^{-1}, 0.019 € kg^{-1}, 0.025 € kg^{-1}, and 0.036 € kg^{-1} for the

stated pressure conditions, respectively which makes HPP technology suitable for products of premium quality (Mújica-Paz, et al., 2011). Besides, an advantage of HPP technology is the possibility of reducing or eliminating additives and preservatives from food products. The coming years will be very important for the development of new pressurised products which can help to reduce the production cost even more.

2.3.1.2 Temperature changes during HPP process

Uniformity in temperature during HPP may not be suitable because of compression-heating differences between the product and the pressure media. Also, there is a process of heat loss and heat gain between the sample, media and pressure vessel. In theory, the adiabatic temperature change of water is predictable being typically 3ºC/100 MPa, near room temperature from 400 to 1000 MPa, and approximately 9ºC/100 MPa for olive and soy oils (Balasubramanian & Balasubramaniam, 2003; Hogan et al., 2005; Balasubramaniam & Farkas, 2008; Norton & Sun, 2008). If the pressure transmitting media exhibits high compression heating, the inactivation data could include unintended thermal effects. In this sense, pressure-media fluid upon compression could heat the sample (i.e. 50% mixtures of castor-oil and water, glycol and water). This error can be important when a small sample size is used with a large quantity of fluid media. With the exception of products with very high oil content, the food and water medium can be treated as the homogeneous content of the pressure vessel. Despite the fact that the liquid and the pressure vessel could started at the same temperature, upon pressurisation, the compressed liquid is hotter than the metal chamber surrounding it. The difference between the chamber wall temperature and the content temperature results in the transfer of heat from the content into the chamber wall and a lowering of temperature during processing. For this, upon decompression, content temperature falls below the initial starting temperature of the test. This is essentially similar to a refrigeration cycle at work in which the refrigerant is the content of the pressure chamber (Ting et al., 2002; Rasanayagam et al., 2003).

Temperature increasing reported during HP process vary depending on the equipment materials in the rig, its geometry, location and type of the temperature probes and fluid used (Ting et al., 2002). Based on this, a common strategy is to determine this temperature gain, place the samples below target temperature and then using this gain to reach it. Afterwards, refrigerating/heating devices keep the pressure chamber and its content at constant temperature.

2.3.2 Effects of HPP on microorganisms

As one of the main objectives of the emerging technologies, many studies on HP effects on microorganisms had been carried out (Farkas & Hoover, 2000; O'Reilly et al., 2001; Trujillo et al., 2002; Lado & Yousef, 2002; Rastogi et al., 2007; Considine 2011; Martínez-Rodríguez et al., 2012). From these studies, some of the microbial mechanisms of inactivation are: changes in the morphology, wall and cell membranes; inhibition of ATPase enzyme activity; modification of biochemical reactions and genetic mechanisms; crystallisation of membrane phospholipids (which causes irreversible changes in cell permeability) and cellular transcription interference (Cheftel, 1995; Patterson et al. 1995; Smelt, 1998; Abee & Wouters 1999). Figure 7 presented the main effects of HPP on microorganisms depending on the pressure applied. In this figure, important inactivation effects take place at pressures above 50 MPa and close to 200 MPa there is a region called *threshold of lethality*, meaning that after this pressure, the inactivation rate obtained presented a shoulder (Lado & Yousef, 2002).

Besides, microbial inactivation by HPP is not only dependant on the pressure level, holding time of pressurisation (holding or exposure time), and process temperature applied, but it is also affected by the type, form, genus, species and strain of microorganism, and the composition, pH, a_w and other characteristics of the dispersion medium (San Martín et al., 2002). In addition, the efficiency of inactivation of microorganisms by HPP can be strongly influenced by the growth phase, the temperature and the presence of different solvents which may have either a protective or synergy effect against pressure inactivation (Cheftel, 1995; Rastogi et al., 2007).

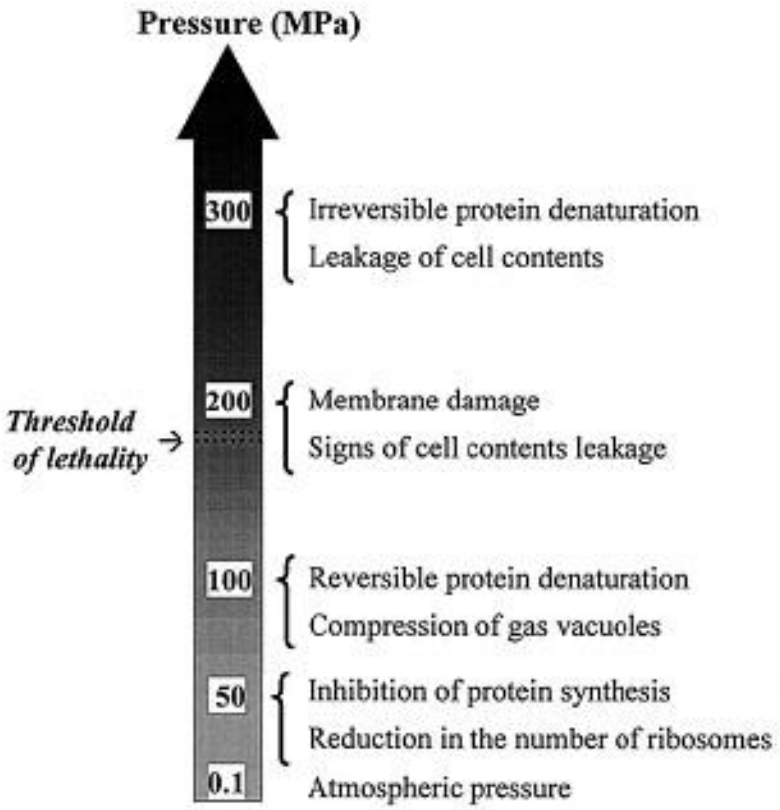

Figure 7. Structural and functional changes in microorganisms at different pressures (From Lado and Yousef, 2002)

Moreover, pulsed-HP treatment, like the application of consecutive, short pressure treatments interrupted by brief decompressions, has shown the possibility to substantially enhance the inactivation of pathogens such as *L. monocytogenes*, *E. coli* and *Salmonella* E*nteritidis* (Garcia-Graells et al., 1999; Vachon et al., 2002; Buzrul, 2009; Pilavtepe-Çelik et al., 2011) by increasing the sensitivity of cells to pressure, offering a promising alternative for the cold pasteurisation of milk and possibly other low-acid liquid foods (Vachon et al., 2002).

Some studies have tried to compare the inactivation of some pathogenic bacteria by HPP with heat treatment (HT), in order to establish the equivalence of treatments. Table 3 presented a comparison between heat and HPP treatments, in the inactivation of some selected microorganisms (Smelt, 1998).

Table 3. Approximate heat and pressure resistance for some pathogenic bacteria

Microorganism	***D value at 60°C (min)***	***Inactivation (log units) after 15 min pressure treatment (MPa)***			
		300	***400***	***500***	***600***
Aeromonas hydrophila	0.1-0.2	>6			
Pseudomonas aeruginosa	0.1-0.2	>6			
Campylobacter spp.	0.1-0.2	>6			
Salmonella spp.	0.1-2.5	1-4.5			
Yersinia enterocolitica	2-3	>6			
Escherichia coli	4-6	1-2			
E. coli 0157:H7	2.5	1-2		2.5	
Salmonella Senftenberg	6-10	3			
Staphyloccus aureus	1-10	3	0.1	1.9	2.1
Listeria monocytogenes	3-8	3	1-3	>6	

2.3.2.1 Factors affecting HP microbial inactivation

Temperature: Higher inactivation rates are generally observed at temperatures above or below 20ºC; although, most of the studies suggest operating at temperatures higher than 50ºC in order to increase inactivation achieved and eliminate the problem of pressure resistant strains. Temperatures higher than 70ºC have been proposed in order to achieve pressure sterilisation or, at least pressures of 400 MPa at 50ºC during 30 min are necessary to obtain a certain degree of commercial sterility (Moerman et al., 2001; Moerman, 2005). On the other hand, refrigerating temperatures can also enhance pressure inactivation in

the case of non-psychrotrophic cells (Cheftel, 1995; Patterson et al., 1995; Gervilla et al., 1999b; Patterson, 2005). Lower temperatures, down to −20ºC have proved to produce extremely effective bacterial reductions (Hayakawa et al., 1998; Yuste et al., 2002; Picart et al., 2004; Picart et al., 2005). The effect of the sub-zero temperatures on the inactivation obtained is related to the ice crystal formation and phase transition of the water in the product (Dumay et al., 2006; Moussa et al., 2007; Shen et al., 2009).

Strains and phase of growth: Several authors reported significant variation in pressure sensitivity among different strains of the same specie (Cheftel, 1995; Patterson et al. 1995; Simpson & Gilmour 1997; Benito et al., 1999; Alpas et al., 1999; Alpas et al., 2000; San Martín et al., 2002; Alpas et al., 2003; Tay et al., 2003; De Lamo, 2005 et al.; López-Pedemonte et al., 2007; Rastogi et al., 2007; Martínez-Rodríguez et al., 2012). Concerning this matter, Benito et al. (1999) suggested that differences in membrane permeability could explain this phenomenon. Regarding phase of growth, cells in stationary phase have been found to be more resistant to HP than those in exponential phase as they have a more robust cytoplasmic membrane that can better tolerate pressure treatment (McClements et al., 2001; Mañas & Mackey, 2004; Mañas & Pagán, 2005). Patterson et al. (2006) associated this behaviour to the synthesis of new proteins when bacteria enter the stationary phase, protecting cells against adverse conditions. Moreover, the resistance to HP treatment can be affected by the morphology of the cells. The most sensitive bacteria are rod-shaped and the most resistant ones are spherical (Ludwig & Schreck, 1997; San Martín et al., 2002; Yuste et al., 2004; Patterson, 2005), possible due to rod-shape cell wall is more susceptible to stress than spherical bacteria (Koch, 1995). This was confirmed by Juan et al. (2007a) and (Espinosa-Pesqueira, 2010) who observed that lactobacilli bacteria were more sensitive to HPP than lactococci; however, lactococci inoculated into 10% (w/v) reconstituted skim milk showed more sensitivity to HP treatment (100-350 MPa) than lactobacilli (Casal & Gomez, 1999).

pH: The pH plays a particular role in microbial inactivation. Based on this, the pH of a food, being not optimal for a particular species, cannot only enhance inactivation during treatment but also inhibit outgrowth of sublethally injured cells (Norton & Sun, 2008). Bacterial spores are generally the most resistant to the direct effects of pressure treatment at neutral pH (Hogan et al., 2005). Besides, the pH of acidic solutions and food matrices decreases as pressure increases (pH drops 0.2 per 100 MPa), and when pressure is released pH reverts to the original value. However, it is not known whether this change in pH affects microbial survival in a synergic effect to the effect on pressure (Hoover et al., 1989; Roberts & Hoover, 1996).

It is common that at low pH values, sublethally injured cells may not recover, enhancing the inactivation rate obtained. For instance, the pressure resistance of *E. coli* O157:H7 in orange juice is dependent on the matrix pH, the degree of inactivation increasing as pH decreases. Further, survival of *E. coli* O157:H7 in orange juice during storage is also dependent on pH (Hogan et al., 2005). In a recent study, Daryaei et al. (2010) found that acidic conditions (pH 4.30, 5.20 and 6.50) during refrigerated storage prevented or delayed the recovery of *Lactococcus lactis* subsp. *lactis* and *Candida* spp. cells in fermented milk test system treated at 300 and 600 MPa for 5 min, and stored at 4^{o}C for up to 8 weeks. These authors concluded that HPP could be used to control further acidification in fermented dairy products including fresh lactic curd cheeses.

Water activity (a_w): Liquid water is essential for the existence of all kind of living organisms. The amount of water available for microbial growth is generally expressed in terms of the a_w of the system. Decreasing the a_w of a food can significantly influence the growth of food spoilage or pathogen microorganisms that may be present in the raw materials or introduced during processing; this is the principle of the very old method of food preservation by drying (Hogan et al., 2005). In HPP, reducing the a_w seems to protect microbes against inactivation, and it is visibly observed in foods with values below 0.9 (Rendueles et al., 2011); however, the recovery of sublethally injured cells can be inhibited

at low a_w (Hogan et al., 2005). Consequently, the effect of a_w on microbial inactivation by HP treatment may be difficult to predict.

Cell membranes and wall: The cell membrane is generally recognised to be a primary site of pressure damage in microorganisms. The membrane is composed by a bilayer of phospholipids with embedded functional proteins that, among others, play an important role in transporting ions and other substances across the membrane (San Martín et al., 2002). HPP altered membranes due to the rupture of ionic bonds, some hydrogen bonds and hydrophobic and electrostatic interactions (Rendueles et al., 2011). The evidence of physical damage to the cell membrane has been demonstrated as leakage of ATP or UV-absorbing material from bacterial cells subjected to pressure (Smelt et al., 1994) or increased uptake of fluorescent dyes such as propidium iodide that do not normally penetrate membranes of healthy cells (Benito et al., 1999). Exponential-phase cells are normally less pressure resistant than stationary-phase cells. Mañas & Mackey (2004) have proposed that exponential-phase cells are inactivated under HP by irreversible damage to the cell membrane. On the other hand, stationary-phase cells have a more robust cytoplasmic membrane that can better withstand pressure treatment. This conclusion was based on the fact that exponential-phase cells showed changes in their cell envelopes that were not seen in stationary-phase cells. These changes included physical perturbations of the cell envelope structure, a loss of osmotic responsiveness and a loss of protein and RNA to the extracellular medium. Also, loss of membrane functionality resulting from pressure treatment has also been described by Wouters et al. (1998), who reported that in *Lactobacillus plantarum*, pressure treatments at 250 MPa were able to reduced F_0F_1-ATPase activity. The ability to maintain a ΔpH was also reduced and the acid reflux was impaired.
Comparing the cell wall and membrane damage by HPP, it can be observed that the cell wall is less affected by HP than the membrane and generally no morphological changes can be observed in prokaryotes and lower eukaryotes by observation under a light microscope, although intracellular damage can be observed using electron microscopy. Ritz et al. (2001), using scanning electron microscopy (SEM), reported that small fractures

appeared on the cell surface of *L. monocytogenes* after a 10 min pressure treatment at 400 MPa in citrate buffer. Won Park et al. (2001) studied the effect of pressure on the ultrastucture of *Lactobacillus viridescens*. Nodes on cell walls of organisms treated at 400 MPa (5 min at 25°C) were observed using SEM. Transmission electron micrographs indicated empty cavities between the cytoplasmic membrane and the cell wall after treatment.

Genetic mechanism and injured population: Nucleic acids are relatively resistant to HP. After treatment, there is a disruption in the enzyme-mediated steps involved in DNA replication and transcription, but the DNA helix, largely resulting of hydrogen bond formation is stable under pressure. Also, it has been reported that HP causes a condensation of nuclear material and at elevated pressures the DNA comes into contact with endonucleases that cleave the DNA. The microorganism become inactivate by disenabling the enzyme responsible for renaturation, making the cell will no longer be able to multiply (Mackey et al., 1994; Chilton et al., 1996; Smelt, 1998; Wouters et al., 1998; Patterson, 2005). Besides, ribosomes have been suggested that play an important role in pressure sensitivity (Earnshaw et al., 1995; Patterson et al., 1995). In this sense, Bozoglu et al. (2004) applied differential scanning calorimetry to detect *in vivo* damages of ribosomes. Since ribosomes have been implicated as temperature sensors, cold and hot shock proteins may also been important in stress response under inactivation conditions such as HP. Additionally, Welch et al. (1993) have shown that exposure of *E. coli* to HP induces a unique stress response, which results in higher levels of cold-shock proteins as well as other proteins that appear only in response to HP. Special attention has been paid in the inactivation kinetics with the intention of observe mutants microorganisms that can survive HP (Lechowich, 1993; Hauben et al., 1997). However, this resistance in mutants proved to be unrelated to barotolerant growth (Hauben et al., 1997). Moreover, if barotolerant strains can survive commercial pressurisation treatments, they can become a serious threat to the safety and stability of pressure-processed foods.

HP treatment is known to cause sublethal injury to microbes, which is a particularly important consideration for any preservation method (Norton & Sun, 2008). After HP treatment, not all the microbial population is inactivated, but instead, the treatment can produce injuries to a portion of the population. Recovery of the damaged cells will depend on conditions in the environment after treatment such as pH, a_w, storage temperature of the product, as well as type of organism and food matrix; when the accumulated damage exceeds the cell's ability to repair, death occurs (Rendueles et al., 2011). In addition, microorganisms are resistant to selective chemical inhibitors due to their ability to exclude such agents from the cell, mainly by the action of the cell membrane; however, if the membrane becomes damaged, this tolerance is lost (Norton & Sun, 2008). Therefore, a common mistake is the use of selective agar, because they can give an inaccurate estimation of the number of survivors (Patterson, 2005). However, when working with food matrix, like cheese, where the presence of background microorganisms cannot be avoid, the use of selective media is required. Attending to this fact, Kang & Fung (1999) developed the thin agar layer (TAL) method, which consists on a selective medium overlaid with non-selective medium; injured cells will grow on the non-selective layer while the selective medium agents spread to the top layer. Thus, the microorganisms are able to perform most reactions that normally do on selective medium. Several studies have been carried out using TAL method with different pathogens strains in different food matrixes exposed to different stressful conditions, such as heat, acid and pressure injury (Wu & Fung, 2001; Yuste et al., 2004; López-Pedemonte et al., 2007; Miller et al., 2010) and significant recovery of injured cells were observed. López-Pedemonte et al. (2007) reported that when applying 300-400 MPa (20°C for 10 min) in model cheese inoculated with two strains of *L. monocytogenes* was possible to recover 0.7-1.1 log cfu g^{-1} injured cells, depending on the bacterial strain and pressure level applied.

2.3.3 Effects of HPP on cheese

At the present moment, there are some cheeses and cheese-related products processed with HP technology available in the European market (Martínez-Rodríguez et al.,

2012).This fact represents the next step from the initial stages on the application of HPP, where more effort was paid in safety areas such as making cheese from pressurised milk or applying pressure (alone or in combination with bacteriocins) directly to the cheese in order to inactivate pathogen and spoilage microorganisms, and accelerate or arrest cheese ripening (O'Reilly et al. 2000a, 2000b, 2001; Trujillo et al., 2002; Patterson, 2005; Martínez-Rodríguez et al., 2012).

In cheese technology, most of the studies have been focused on the application of HPP in the inactivation of microorganisms (Capellas et al., 1996, 2000; Trujillo et al., 2000, 2002; Daryaei et al., 2006, 2008; López-Pedemonte et al., 2003, 2006, 2007; De Lamo-Castellví et al., 2007; Hnosko et al., 2012) as well as on the modifications of cheese maturation (deceleration or acceleration) (Yokoyama et al., 1994; O'Reilly et al., 2000a, 2000b; Saldo et al., 2001; Wick et al., 2004; Garde et al., 2007; Juan et al, 2007a, 2000b, 2008; Rynne et al., 2008; Voigt et al. 2010, 2012). Other applications of HPP on cheese includes accelerating brine absorption (Messens et al., 1998, 1999a; Pavia et al., 2000), increasing cheese yield (Drake et al., 1997; Trujillo et al., 1999; Huppertz et al., 2004), and improving low-fat cheese texture (Sheehan et al., 2005). Also, some studies about the effect of HP on enzymes, volatile composition, rheological changes, lipolysis and proteolysis in traditionally made cheeses were conducted by Buffa et al. (2001a, 2001b), Saldo et al. (2003), Garde et al. (2007), Juan et al. (2004, 2007a, 2008), Rynne et al. (2008) and Voigt et al. (2010).

Some of the most recent research in microbiology, physico-chemical properties and other characteristics of cheese treated by HPP are described in the next sections.

2.3.3.1. Effect of HPP on cheese microbiology

Most of the studies applying HP on cheese are concerning an improvement in safety and shelf-life, by inactivating pathogenic or spoilage microorganisms (native or inoculated) using two main methods: (a) raw milk treated by HPP and then used to elaborate cheese and (b) cheese elaborated with pasteurised or raw milk and then treated by HPP (Trujillo et al., 2002; Patterson, 2005; Chawla et al., 2011; Martínez-Rodríguez et al., 2012).

The use of HPP to control microbial growth and extend the shelf-life of pasteurised fresh cheese elaborated with goat milk was studied by Capellas (1998). In that study, cheese milk was inoculated with *E. coli* CECT 405 to obtain 8 log cfu g^{-1} of cheese, with the resultant cheese being treated using combinations of pressure (400-500 MPa), temperature (2, 10 or 25°C) and time (5-15 min) and subsequently stored at 2-4°C. The results presented no survival of *E. coli* after treatment, except when 400 MPa at 25°C for 5 and 10 min were applied. For these conditions, a reduction of 6.7 and 7.7 log units were observed, respectively. Afterward, no surviving cells were detected in cheese at days 15, 30 or 60 days of storage, in all the pressure conditions tested. However, that study did not examine the possibility of sublethally injured cells. Similarly, Szczawinski et al. (1997) and Gallot-Lavallée (1998) reported a significant inactivation of *L. monocytogenes* inoculated in cheese. The conditions applied by these authors were 500 MPa for 15 min and 450 MPa for 10 min or 500 MPa for 5 min, respectively. A reduction of 6 log units and 5.6 log units respectively was observed. Besides, a significant decrease of cheese microbiota was achieved, without significant changes in the sensory characteristics of the treated cheese.

In order to increase the inactivation rate of high baroresistant microorganisms and bacterial spores, Capellas et al. (2000), working with fresh goat milk cheese, adopted different strategies like the increase in treatment temperature (10-50°C), application of multiple HPP levels (400-500 MPa) at different times (5-210 min), or addition of nisin (3.4 ppm) for the inactivation of *Staphylococcus carnosus* and *Bacillus subtilis* spores. The increase in treatment temperature (500 MPa at 50°C for 5 min) was able to enhance the inactivation acquired of *S. carnosus,* from 2 log units up to 7 log units. Multiple HP-cycles of 500 MPa at 10°C for 5 min also improved the inactivation rate, but the combination of nisin showed the best results. On the other hand, a reduction of 4.9 log of *B. subtilis* spores was observed when germination treatments of 60 MPa (40ºC for 210 min) were followed by vegetative cells inactivation treatments of 500 MPa (40ºC for 15 min).

Moreover, HP is a useful technology for the inactivation of pathogen and spoilage microorganisms in cheese (O'Reilly et al., 2001; Trujillo et al., 2002; Rastogi et al., 2007; Chawla et al., 2011; Martínez-Rodríguez et al., 2012). In this sense, Casal & Gomez (1999)

studied the effect of HP (100-400 MPa for 20 min at 20ºC) on the viability, acidifying and peptidolytic activities of lactococci and lactobacilli isolated from goat's milk cheese. The results showed that lactoccoci bacteria were more sensitive than lactobacilli to pressures of 100 to 350 MPa. These results are contrary to those presented by Juan et al. (2007a) and (Espinosa-Pesqueira, 2010) in which HPP treatments of 200-500 MPa for 10 min at 12°C in raw ewes' cheese and 400 MPa for 10 min at 2°C in caprine and ovine raw milk cheeses, respectively. In these trials, lactoccoci bacteria were more resistance to lactobacilli bacteria. Some works on the application of HPP in fresh cheese (also known as *Queso Fresco*) had been published during the last years. Capellas et al. (2000) applied HP treatments of 500 MPa at 25ºC during 5 to 30 min increasing the shelf-life of refrigerated (4ºC) vacuum-packed fresh cheese up to 2 and 3 months, respectively, and also, up to 4 months when nisin was added. In addition, Sandra et al. (2004) applied 400 MPa (20°C for 20 min) to Queso Fresco. These authors reported that HP treatment of cheese was not able to inactivate completely coliforms and moulds and yeasts. Further, Daryaei et al. (2008) applied HP treatments at 200-600 MPa for 5 min at 25°C in fresh lactic curd cheeses. After treatment, cheeses were evaluated for 8 weeks in *Lactococcus* starter and spoilage yeasts. The inactivation acquired in *Lactococcus* starter was about 7 log units in selected conditions (600 MPa incubated at 4°C in aerobic conditions) and the spoilage yeasts remained controlled during the 8 week of storage in treatments above 300 MPa. Moreover, with the intention to observe the inactivation by HPP of a specific pathogenic microorganism inoculated in Queso Fresco, Hnosko et al. (2012) employed HP treatments of 400, 500 and 600 MPa at 21°C from 1 to 20 min, inoculating three strains of *L. innocua*. The results showed a reduction of 5 log units at set point pressures of 500, 550, or 600 MPa when held for at least 15, 3, or 1 min, respectively. However, there is some controversy in the author conclusions, because as no complete inactivation was achieved, the use of HPP for Queso Fresco was not recommended.

2.3.3.2 ***Effect of HPP on physico-chemical properties of cheese***

Several studies had proved that HPP do not change significantly the composition (total solid, ash, fat, protein, moisture content) and nutrient contents of cheese (Capellas et al., 2001; Sandra et al., 2004; Rynne et al., 2008; Koca et al., 2011); however, it has an effect on pH, in an extent that depends on treatment conditions and cheese age. Different studies has showed an increase in pH in HP-treated cheeses compared to control cheeses, in varieties such as Cheddar (Rynne et al., 2008), ewes' milk cheese (Juan et al., 2007a, b, 2008), Garrotxa (Saldo et al., 2000, 2002a); fresh cheese (Sandra et al., 2004; Okpala et al., 2010), Edam (Iwánczak & Wísniewska 2005), Paillardin (Messens et al., 2001), Gouda (Messens et al., 1998, 2000), Manchego (Pavia et al., 2000), Mozzarella (Johnston & Darcy, 2000), La Serena (Arqués et al., 2007, Garde et al., 2007) and Camembert (Kolakowski et al., 1998) cheese. Also, there is an increase in the pH values when higher pressure levels and longer exposure times are applied. This raise is due to the release of colloidal calcium phosphate into the aqueous phase of cheese, lactic acid bacteria (LAB) inactivation, or reduced ability of LAB to produce acid even when there is no apparent loss of cell viability as a result of damage in the glycolysis system. Nevertheless, pH differences between treated and control samples become less significant during the ripening process (Martínez-Rodríguez et al., 2012).

With regard to moisture content in cheese, HPP had shown to alter the water distribution and water loss in treated cheeses by denaturation of whey proteins and micelle fragmentation (Buffa et al., 2001b; Trujillo et al., 1999). Torres Mora et al. (1996) reported a reduction in variability of moisture content in reduced fat Cheddar cheese (HPP conditions not specified). By the contrary, Trujillo et al. (1999) and)Saldo et al. (2001), applying HPP in raw milk and in cheese respectively, described higher moisture content in semi-hard goat milk cheese applying HPP treatments of 500 MPa, 15 min, 20°C and 400 MPa for 5 min followed by 50 MPa for 72 h at 14°C, respectively. Moreover, Messens et al. (1999) and Saldo et al. (2001) observed a reduction in water loss during brining of Gouda and Garrotxa cheeses at pressures from 300 to 500 MPa and 400 MPa for 5 min followed by 50 MPa for 72 h at 14°C, respectively. In the same sense, (Saldo, et al., 2002c) applying HP

treatments at 400 MPa for 5 min in goat's cheese, evaluated the bond and free water in HP treated and pasteurised cheeses. The results presented that HP-treated cheese had 12.7% free water and 27.6% bound water, while control cheese had 18.9% free water and 21.4% bound water. Also, it was observed that solute diffusion improved by pressure treatments by enhancing salt distribution in treated cheese.

Colour is another parameter significantly affected in cheese by HPP, being treatment temperature, pressure intensity, and holding time, the factors that influence this attribute the most. In all cases, the most affected parameter of colour is the b^* followed by L^*. Johnston & Darcy (2000) treated immature Mozzarella cheese at 200 MPa for 60 min and compared it with samples matured by holding at 4°C. The results showed changes in the parameters L^* and b^*, obtaining a more yellow cheese, compared to control cheese. Capellas et al. (2001) evaluated the colour of Mató cheese treated at 500 MPa for 5, 15, and 30 min at 10ºC and 25ºC; the results showed an important change in total colour difference, being the b^* value that changed the most in all treatments, increasing as pressure holding time increased, followed by a decrease in luminosity (L^*). The authors related changes in lightness and yellowness of the cheese surface to microstructural changes. Similar results were reported by Sheehan et al. (2005), by employing HPP at 500 MPa for 5, 15 and 30 min at 10ºC or 25ºC, L^* value decreased with yellowness while greenness (a^*) increased as tempering at 21ºC for 16 h. Further, Okpala et al. (2010) treated fresh cheese with HPP. In that study, an important increase in b^* parameter was reported when the pressure was elevated from 50 to 291 MPa and from 5 to 25 min at 25ºC. More recently, Koca et al. (2011) applying HPP in white-brined cheese (50, 100, 200, and 400 MPa at 22ºC for 5 and 15 min) found higher values for b^* parameters, what can be interpreted as a more yellow cheese, with no significant changes in the L^* parameter.

2.3.3.3 Effect of HPP on sensory characteristics of cheese

Texture is very important for consumers' perception of food quality; it is considered as one of the four quality factors of food products, the other three being flavour, appearance and nutritional value (Rosenberg et al., 1995). It has been defined as "the attribute of a

substance resulting from a combination of physical properties and conformation of constituent structural elements, and perceived by the senses of touch (kinaesthesia and mouthfeel), sight and hearing" (Jowitt, 1974). In the particular case of cheese texture, a more recent definition was set by O'Callaghan & Guinee (2004) who described it as the effort required to masticate the cheese or alternatively the level of mastication achieved for a given level of chewing.

Sensory characteristic of cheese is also one of the most important properties for the consumers. Moreover, sensory characteristics of cheeses, are human responses to perceptions of stimuli that are experienced with the cheeses, and can generally been described using terms defined within the categories of appearance, flavour and texture.

Cheese is viscoelastic in nature and exhibits both solid (elastic) and fluid (viscous) behaviour (Konstance & Holsinger, 1992). It consists of a continuous protein matrix with a loose and open structure with dispersed fat globules. The mechanical properties of cheese are related to cheese composition (moisture, protein, fat, salt and pH), matrix structure, and interactions among molecules within the cheese network and their changes during ripening (Visser, 1991; Lucey et al., 2003).

The evaluation of texture and sensory categories determines the eating quality of the cheeses (Delahunty & Drake, 2004). For these reasons, HP studies have focused on the changes presented in cheese after treatment and during storage (Martínez-Rodríguez et al., 2012).

Uniaxial compression test is a standard and simple test to evaluate texture in cheese and it is based on large destructive deformations which are especially important in determining fracture properties. The parameters that could be extracted from the force displacement data obtained from the compression test gives information about the fracture and mechanical cheese properties large scale deformation, which is relevant for comparison with data from sensory texture where cheese is degraded during mastication (Zoon, 1991). O'Callaghan & Guinee (2004) suggested that fracture stress describes strength of cheese matrix, fracture strain describes longness of cheese, and elastic modulus describes elasticity.

Most of the textural properties evaluated in cheese are part of the texture profile analysis (TPA), in which the cheese is double-compressed with a specific amount of force. From this method, hardness, springiness, cohesiveness, gumminess and chewiness of the cheese are evaluated. In some cases, a compression method is applied, in order to evaluate only the hardness of the cheese (also known as firmness) (Szczesniak, 2002; O'Callaghan & Guinee; 2004).

Some of the cheeses studied regarding changes in their textural and sensory properties by means of HPP are Cheddar, Mató, fresh cheese, ewes' cheese, La Serena, goats' cheese, Hispánico cheese and Mozzarella, among others (Capellas et al., 2001; Buffa et al., 2001b; Ávila et al., 2006; Delgado et al., 2012; Garde et al., 2007; San Martín et al., 2007; Juan et al., 2007a, 2007b; Okpala et al., 2010; Koca et al., 2011). One of the first studies on textural properties of cheese treated with HP was carried out by Messens et al. (1999) in Gouda cheese. These authors manually observed that texture of the cheese varied according to the treatment (50-400 MPa for 20-100 min), and although the water content and proteolysis indices were unaffected, the viscoelastic properties of the cheese treated at 225 and 400 MPa for 1 h differed significantly to those untreated, cheeses were less rigid and solid-like, more viscoelastic, and had less resistance to flow at longer times. Later, Molina et al. (2000) studied the effect of HT (65ºC, 30 min), HP technology (400 MPa, 22ºC for 15 min) and combined treatments (HT-HP) on cheese milk for the elaboration of fat-reduced cheese. The results showed an important difference in the cheese hardness; at day 1, the HT-HP cheese was the cheese with lowest hardness followed by the HP cheese, and with the highest hardness, the heat-treated cheese. Also, the hardness of the cheese after 60 days of ripening was lower comparing to the initial value, in all samples. In the same sense, Delgado et al. (2012) evaluated the effect of HPP on textural properties of raw milk goats' cheese. Ibores cheeses (days 1, 30 and 50 of ripening) were HP-treated at 400 and 600 MPa for 7 min at 10ºC. These authors observed that the hardness and the cohesiveness of the cheeses were reduced in reference to raw milk cheese. Moreover, textural properties on Hispanic cheese elaborated with a mix of ewe and cow milk (20-80%, respectively) were evaluated by Ávila et al. (2006). In that study, the application of HP (400 MPa for 5

min at 10°C) joined to bacteriocin-producing strains or bacteriocin non-producing, showed a reduction in hardness among the pressurised and control cheeses. Also, during cheese ripening, a reduction in hardness, fracturability and elasticity was observed. On the other hand, Garde et al. (2007) reported higher values of hardness and elasticity in La Serena cheeses treated by HP (300 and 400 MPa for 10 min at 10°C) than control cheeses, elaborated with ewe's raw milk. In Mozzarella cheese, Sheehan et al. (2005) applied HP treatments of 400 MPa for 5 min at 21°C, studying the firmness of the cheese during its storage at 4°C. The results showed no significant difference between the control and HP samples.

In fresh cheeses, Capellas et al. (2001) applied HP treatments in Mató cheese, in treatments of 500 MPa at 10 or 25°C, for 5, 15 and 30 min, and 500 MPa 25°C 5, 15 and 30 min. The results presented an increase in the fracture stress, which is an indicator of firmness in cheese. Besides, Daryaei et al. (2006) reported an increment in firmness in fresh lactic curd cheese treated with HP (300 and 600 MPa at 22°C for 5 min) after 3 weeks of storage of, but this increment was not significant from 3 weeks to the end of the storage time (8 weeks). Similar results were reported by Okpala et al. (2010) in a long spectrum of pressure (9, 50, 150, 250 and 291 MPa) and several combinations of time (1, 5, 15 and 25 min). In this study an increase in hardness was linked to a reduction in the adhesiveness of the cheese samples.

2.3.4.4 Changes in volatiles compounds profile by HPP

The flavour compound development in cheese is made by different biochemical reactions in milk components. In this process, three main pathways are involved: glycolysis (lactose), lipolysis (fat) and proteolysis (caseins). Lipolysis and proteolysis are catalysed mainly by enzymes from milk coagulant or microorganisms, while glycolisis and related reactions are caused by microorganisms (Fox & McSweeney, 2004).

HPP has an important effect on cheese ripening and therefore, it has many effects in the development of volatile compounds. Nevertheless, the focus on these characteristics in HP cheese has been evaluated just a few years ago. One of the first studies published

evaluating the effect of HPP in volatile compound profile in cheese was carried out in Hispanic cheese by Ávila et al. (2006). In that study, the HPP was employed in 15-day ripening cheeses at 400 MPa, 5 min at 10°C. The results showed an increase in hexanal, 3-hydroxy-2-pentanone, 2-hydroxy-3-pentanone, and hexane and a reduction in the levels of ethanal, ethanol, 1-propanol, ethyl acetate, ethyl butanoate, ethyl hexanoate, 2-pentanone, and butanoic acid, when the HP cheese was compared to untreated samples.

Besides, there is a relationship between the ripening time and HPP, in which, when the ripening has been carried out for some days, the effect of HPP on the volatile compound development tend to decrease. Base on this, Juan et al. (2008) treated ewes' cheese by HPP (300 MPa, 10 min) in two different ripening stages (1 and 15 days) evaluating the volatile compound profile in both cheeses. The results showed the effect of HPP on day 1 of ripening by the presence of low amounts of aldehydes, ketones, short-chain free fatty acids (FFA) and terpenes, and high levels of ethanol and ethyl esters. By the contrary, cheeses pressurised on day 15 of ripening were more similar to untreated ones than those HP-treated on the first day. Cheeses treated at 300 MPa were characterised by a higher content of short-chain FFA and pyruvaldehyde, and a lower abundance of secondary alcohols than control cheeses.

In the same way, Arqués et al. (2007) treated La Serena cheese (elaborated with ewes' raw milk) with HPP at 300 and 400 MPa for 10 min on days 2 and 50 of ripening. The results showed that HP at day 2 of ripening enhanced the formation of branched-chain aldehydes and of 2-alcohols except 2-butanol, but retarded the presence of n-aldehydes, 2-methyl ketones, dihydroxy-ketones, n-alcohols, unsaturated alcohols, ethyl esters, propyl esters, and branched-chain esters. Moreover, the differences between HP-treated and control cheeses in the levels of some volatile compounds tended to disappear during ripening. Besides, the differences in volatile compound profile between control and HP cheese treated at day 50 of ripening were not significant after 60 days of ripening.

In addition, Voigt et al. (2010) evaluated the effect of HP treatment (400 and 600 MPa) on ripening of mature 42-day-old Irish blue-veined. Even when the results showed changes in the proteolysis in the HP-treated cheese, there was no significant change in the level of

flavour compounds. Similarly, Delgado et al. (2012) evaluated the volatile compound profile of goat cheese treated by HPP (400 or 600 MPa for 7 min) in order to evaluate if the HPP has an effect on the ripening (1, 30 or 50 days). The volatile compound profile of the cheeses presented a mix of about fifty compounds: 12 acids, 16 alcohols, 5 esters, 8 ketones and 9 other compounds. HP cheeses treated at day 1 presented a decrease in the abundance of most volatile compounds, but at the same time an increase in the formation of ketones and other compounds. On the other hand, the changes were less intense when treatment was applied at the end of maturation.

2.3.3.5 Technological application of HPP in cheese

2.3.3.5.1 Effect of HPP on cheese yield and rennet coagulation time

Cheese yield is another property that is affected by HP technology; it is of particular interest, for economic reasons. Changes in milk components as a result of HPP (such as whey protein denaturation, micelle fragmentation, and mineral equilibrium alteration), modify the technological capacity of milk to make cheese, improving the rennet coagulation and yield properties (López-Fandiño et al., 1996, 2006; Trujillo et al., 1999; Buffa et al., 2001b; O'Reilly et al., 2001). In this sense, Drake et al. (1997) in pressurised milk (3 cycles of 1 min at 586 MPa) for produce Cheddar cheese, observed an improvement in cheese yield without diminishing the flavour. Some studies in cow milk reported also an increase in cheese yield in HP treatments above 300 MPa (López-Fandiño et al., 1996; O'Reilly et al., 2001; Huppertz et al. 2002, 2006). Lately, Huppertz et al. (2005) presented a very interesting study about the effect of pressure in yield. In that study, one group of milk was heat treated (90°C for 10 min), and other group was treated with HP (0-30 min at 100-600 MPa at 20°C) after heat treatment (HT-HP). The results showed an increase in yield (about 15%) in HT-HP cheeses (250-600 MPa for 0-30 min) compared with control raw milk cheese. This increase in yield was attributed to the incorporation of denatured whey proteins into the cheese curd as a result of heat treatment.

The coagulation of milk is the result of two processes, the enzymatic hydrolysis of the κ-CN which destabilises the casein micelles, and aggregation of micelles leading to the formation of a gel. These processes are governed by stability of casein and mineral balances in milk, especially calcium and pH. HPP of milk has been shown to cause reduction in colloidal calcium phosphate concentration and reduction of enzymatic coagulation time, due to an increase in Ca^{2+} activity (Schrader et al., 1997). Furthermore, pressure treatment of milk affects milk proteins, including reduction in the size of casein micelles and denaturation of β-LG, probably followed by interaction with micellar κ-CN (O'Reilly et al., 2001). Moreover, changes in the rennet clotting time (RCT) can be induced by HPP depending on the pressure level, treatment, duration and temperature, and milk pH (López-Fandiño et al., 1996, 2006; O'Reilly et al., 2001; Huppertz et al. 2002, 2004, 2006). López-Fandiño et al. (1996) observed that RCT of raw bovine milk was reduced by HP treatment up to 200 MPa, but increased as the pressure was raised from 200 to 400 MPa to reach values comparable to those of untreated milk. However, in the particular case of milk treated at 300 MPa for 30 min the RCT decreased by 19% and increased the curd firming rate and the curd firmness by 39 and 58%, respectively. In addition, a significant reduction in the RCT of raw skimmed milk after treatment at 100 or 250 MPa (20°C, 30 min) was described by Zobrist et al. (2005), but treatment at 400 or 600 MPa resulted in an RCT similar to, or ~15% greater than that of untreated milk, respectively.

2.3.3.5.2 Effect of HPP on cheese ripening acceleration

Cheese ripening is a slow and expensive process depending on storage time and temperature; cheese composition, especially moisture and salt levels; and the types and activities of rennet, indigenous milk enzymes and microorganisms present (Farkye & Fox, 1990). Therefore, an efficient way to reduce aging time without significantly affecting other quality attributes would provide significant savings to cheese manufacturers (Martínez-Rodríguez et al., 2012).

Proteolysis is considered as the most complex and the most important biochemical event in ripening, especially in characteristic cheeses such as Cheddar cheese, although

glycolysis and lipolysis also play important roles (Fox, 1989; McSweeney, 2004). The primary proteolysis of caseins in cheese is carried out by the residual coagulant and plasmin activities, and this process lead to generation of polypeptides and peptides that can be degraded by enzymes produced by the LAB (O'Reilly et al., 2001). The general reaction steps are: initial hydrolysis of caseins by residual coagulant and plasmin to large peptides; breakdown of large peptides by starter proteinases and peptidases into medium and small peptides; and further hydrolysis of medium and small peptides by starter peptidases into dipeptides, tripeptides and free amino acids (Farkye, 2004).

Proteolysis characterisation is most commonly used as a maturity index. There are several methods to quantify proteolysis in cheese. Most of them are based on (1) separating, quantifying and characterising nitrogenous compounds from cheese during cheese ripening i.e., separation into solvent-soluble and solvent-insoluble fractions followed by nitrogen determination and electrophoretic and chromatographic analyses, and (2) analysis of amino groups and free amino acids throughout reactions with a chromophore compound (Polychroniadou, 1988; Farkye & Fox, 1990; McSweeney & Fox, 1999).

The evaluation of ripening in cheese treated by HPP has been an important issue for numerous research groups. This is based on the idea of reducing the ripening time without significantly affecting other quality attributes that would provide significant savings to cheese manufacturers (Trujillo et al., 2002). The HP treatments that have been studied can be classified into: low to moderate pressure level applied for long periods of time (50 to 200 MPa for up to 82 h), high pressure level employed for short times (300 to 600 MPa for 5 to 20 min), and finally, a combination of both treatments. Table 4 presents the most important characteristics of cheese ripening treated by HPP (Martínez-Rodríguez et al., 2012).

Yokoyama et al. (1992) described in a patent the potential use of HPP for accelerating the ripening of Cheddar cheese. Cheese samples were exposed to pressure from 0.1 to 300 MPa at 25ºC for 3 days, and found that it was possible to decrease the ripening time of Cheddar cheese from six month to three days by 50 MPa at 25ºC. O'Reilly et al. (2000b) tested the same conditions on conventional Cheddar cheese, and showed an increase in

the proteolysis rates, but not as significant as suggested by the Japanese group. It should be noted that the manufacture of cheese reported by Yokoyama et al. (1992) was different from conventional procedure and the level of starter bacteria added to the cheese milk was at least 10-fold higher than conventional inoculation rates.

Based on the fact that most of the ripening reactions are carried out by enzymes, the HPP has an important effect in two specific conditions for enzyme reactions such as pH, and water distribution. HPP has shown that can increase the pH and modify the water distribution of certain cheese varieties (*see 2.3.3.2 Effect of HPP on physico-chemical properties of cheese*), leading to enhanced conditions for enzymatic activity (Saldo et al., 2002a, 2002c). The main mechanisms involved in the acceleration of cheese ripening by HPP are: alterations in enzymes activities, some conformational changes in the casein matrix making it more susceptible to the action of proteases, and/or bacterial lysis enhancing the release of microbial enzymes that promote the biochemical reactions taking place in the ripening (Messens et al., 1998; O'Reilly et al., 2000a, 2000b; Saldo et al., 2000, 2002a; Garde et al., 2007; Voigt et al., 2010).

The potential application of HPP for cheese ripening is evident from the results described above; however it depends on the stage of ripening and the variety of cheese. It seems that the application of HPP during the early stages of ripening tends to have a greater effect on ripening process than when HP is applied at a later stage of ripening. Furthermore, the application of higher pressures (≥ 500 MPa) drastically reduces microbial counts and inactivates enzymes, so it may be useful to arrest the ripening of cheeses and maintain the optimum characteristics in a given time (Juan et al., 2004).

Table 4. Effect of HP treatments on the ripening process of different cheese varieties.

Cheese variety	*Moment of application*	*Treatment Applied* *P (MPa)/t (min, h[d])/T (◦C)*	*Effects*
Proteolysis			
Cheddar	After salting	50/72 h/25	Similar taste and FAA[e] content of a 6 mo-old commercial cheese obtained in 3 d (Cheddar: 26.5 mg g^{-1}, Parmesan: 76.7 mg g^{-1})
Cheddar	2, 7, 14, or 21 d[a]	50/72 h/25	Faster α_{s1}-casein hydrolysis and accumulation of α_{s1}-I-casein. Increased pH 4.6 SN[f]/TN[g] and FAA levels
Cheddar	1 d	70–400/3.5–81.5 h/25	Maximum accumulation of a_{s1}-I-casein at 100 MPa and greatest increase in levels of pH 4.6 SN/TN below150 MPa. Total FAA decreased as pressure increased
Cheddar	1 or 4 mo[b]	200–800/5/25	Ripening deceleration at pressure treatments ≥ 400 MPa
Camembert	5 or 10 d	0.1–500/4 h/5	Most intense proteolysis at 50 MPa on d 11
Blue-veined	42 d	400–600/10/20	Accelerated breakdown of β- and α_{s2}-casein and increased levels of PTA[h] SN/TN
Gouda	After brining, 5 or 10 d	50 or 500/20–100/14	No changes in pH 4.6 SN, PTA SN/TN, FAA content and SDS-PAGE profiles
Edam	After salting, 4, 6, and 8 wk[c]	200 or 400/30/25	No changes in different fractions of nitrogen compounds
Garrotxa	1 d	400/5/14 followed by 50/72 h/14	Ripening period reduced from 28 to 14 d
Ewes' milk cheese	1 or 15 d	200–500/10/12	Increased peptidolytic activity and highest amount of FAA at 300 MPa applied on d 1. Treatments of 500 MPa decelerated primary proteolysis
Hispanico	15 d	400/5/10	Accelerated casein hydrolysis and increased total FAA content

La Serena	2 or 50 d	300 or 400/10/10	Levels of proteolysis were higher when HP treatments were applied at 400 MPa on d 2 compared to other treatments
Lipolysis			
Garrotxa	1 d	400/5/15	Decelerated lipolysis due to lactic acid bacteria or lipolytic enzymes inactivation.
Ewes' milk cheese	1 or 15 d	200–500/10/13	Lowest concentration of total FFA[i] at pressure treatments of 400 to 500 MPa applied on d 15 after 60 d of ripening compared to other treatments. Highest levels of FFAs were obtained at 300MPa applied on day 1 compared to other treatments
Hispanico	15 d	400/5/11	Esterase activity was not modified. Negligible differences in individual FFA levels compared to control
Full-fat Cheddar	1 d	400/10/26	Lipolysis was not significantly different from control over 180 d
Blue-veined	42 d	400–600/10/21	Reduced lipolytic activity of *P. roqueforti*.
Glycolysis			
Full-fat Cheddar	1 d	400/10/26	Concentration of total lactate in HP-treated cheese was significantly lower compared to the control after 180 d of ripening

[a]d = day; [b]mo = month; [c]wk = weeks; [d]h = time in hours when specified; [e]FAA = free amino acids; [f]SN = soluble nitrogen; [g]TN = total nitrogen; [h]PTA = phosphotungstic acid; [i]FFA = free fatty acids.

2.3.3.5.3 Other HPP applications in cheese

Besides the reduction of microbial counts, the increase in cheese yield, the modification of cheese ripening and the development of products with new sensory characteristics, other applications of high pressure in cheese have been proposed.

It was assayed the possibility of accelerating cheese brining by HP treatment in Gouda (Messens et al. 1998) and Manchego (Pavia et al., 2000) cheeses, but the salt uptake and salt diffusion were not accelerated by the pressure conditions tested (100-500 MPa, 15-130 min in Gouda cheese and 50-200 MPa in Manchego cheese).

Other application proposed was the use of HPP to attenuate starter bacteria for using as adjuncts in cheese manufacture. Casal & Gómez (1999) suggested that *Lactococcus lactis* subsp. *lactis* treated at 300 MPa and *Lactobacillus casei* subsp. *casei* treated at 350 MPa may be added during cheese making to give an extra supply of enzymes with potential debittering properties, which may be use to cheese ripening acceleration. In agreement, Upadhyay et al. (2007) observed that pressurisation at 200 MPa for 20 min at 20ºC was successfully used to attenuate *Lactococcus lactis* subsp. *cremoris*, which may be used in combination with primary strains in Cheddar cheese-making, producing higher levels of free amino acids and acceleration of secondary proteolysis in cheese.

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Chapter 3

Material & methods

3.1 Pilot Plant Experiments

3.1.1 Cheeses and high pressure treatment

Fresh cheeses were supplied by a local cheese producer (Albal, Valencia, Spain) on the cheese making day. Briefly, fresh cheeses were made from cow milk pasteurised at 75°C for 40 s, cooled up to 37ºC and salt (1% w/v) and calcium chloride (0.01% v/v) were added. Milk coagulation was performed at 37ºC for 45 min after addition of microbial rennet at 0.03% (v/v). Curd was cut into grains (diameter ca. 1 cm), and were poured into polypropylene moulds. Filled moulds were allowed to drain at 8ºC for 60 min and packaged cheeses (~80 g) were kept at 4ºC.

Before HP treatment, cheeses were vacuum packaged in polyethylene bags under laminar flow cabinet. Untreated vacuum packaged cheeses were used as control samples.

Vacuum packaged samples were HP-treated (day 1) at 300 and 400 MPa for 5 min at 6°C in a ACB GEC Alsthom (Nantes, France) discontinuous HPP unit with a 2-L capacity pressure chamber (Figure 8). The time needed to achieve maximum pressure (400 MPa) was 1.5 min. The chamber and water (hydrostatic fluid medium) inside were cooled to treatment temperature with a constant flow of ethylene glycol-water (1:1) solution within the walls of the vessel. Time, temperature, and pressure parameters were selected on the basis of previous studies for increasing shelf-life and to avoid drastic changes in cheese texture. Cheeses were maintained into the polyethylene bags during cold storage at 4ºC,

and for microbiological examination at 4 and 8ºC. Sampling and analyses were carried out only during cheese shelf-life.

3.1.2 Cheese composition

Gross composition of cheeses was determined on day 1. Triplicate samples were assayed for total solid content (IDF, 1982), fat (ISO, 1975) and protein (IDF, 2002). The pH was measured with a pH meter (MicropH 2001, Crison, Alella, Spain) on a cheese: distilled water (1:1) slurry.

Figure 8. HPP unit from Alsthom (Nantes, France**)**

3.1.3 Whey loss and water activity

Cheese whey loss was estimated by centrifugation using the method described by Guo & Kindstedt (1995). A sample of 15 g of cheese homogenates was centrifuged (Sigma 4K15, Postfach, Germany) at 12,500 *g* for 75 min at 25°C. The supernatant was weighed and whey loss percentage was calculated. Water activity (a_w) was measured in a water activity analyser (Aqualab®, Model Series 3 TE, Decagon Devices, Inc., Pullman, WA). For each analysis, each treatment was analysed in quadruplicate, during respective cheeses shelf-life, on days 1, 7 and 14.

3.1.4 Microbiological analysis

Cheese analysis was performed by homogenizing 10 g of sample in 90 mL peptone water (Oxoid, Basingstoke, UK) for 1 min in an electromechanical homogenizer (Stomacher, Lab-blender 300, Seward Medical, London, UK). Decimal dilutions were prepared in peptone water and plated in duplicates on different media. Aerobic mesophilic counts and psychrotrophic bacteria were enumerated on Plate Count Agar (PCA, Oxoid) and incubated at 30°C for 48 h and 10°C for 5 days, respectively. Lactococci were enumerated on M17 agar (Oxoid) and incubated at 30°C for 48 h. *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBG, Oxoid) and total coliforms and *Escherichia coli* on Chromocult® (Merck, Darmstadt, Germany) were both incubated at 37°C for 18-24 h. Moulds and yeasts were enumerated on Rose Bengal Agar Base Chloramphenicol and incubated for 5 days at 25°C.

Cheeses were stored under two temperatures (4 and 8°C) and analysed on days 1, 7, 14 and 21 for cheeses stored at 4°C (optimal storage conditions), and days 4, 7 and 11 for cheeses stored at 8°C (simulating a conventional fridge). The results were expressed as log cfu per gram of cheese (log cfu g^{-1}), and it was considered the end of cheese shelf-life when total counts were >6 log cfu g^{-1}.

3.1.5 Colour and texture

The colour was measured with a portable Hunter Lab colorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, Virginia, USA) using illuminant D65, with a 10° observer. CIE L*-, a*- and b*–values were read at five different points of the inner surface of cheeses cut in halves. The L*-value, that ranges between 0 and 100, is a measure of lightness. Negative to positive values of a* and b* indicate the green–red and blue–yellow components, respectively. Total colour difference (ΔE) as $(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$ was calculate to compare control and pressurised cheeses. Fresh cheese samples were analysed, during respective cheeses shelf-life, on days 1, 7 and 14.

A penetration test was performed using a TA-XT2 Texture Analyser (Stable Microsystems, Surrey, UK). Cheeses were penetrated to 60% of their original height with a constant speed

penetration of 2.0 mm s^{-1}. The analysis was carried out 5 times for each batch on days 1, 7 and 14. The maximum penetration force was obtained using the software Texture Expert (version 1.16 for Windows, Stable Micro Systems Ltd., Surrey, UK) and was reported as firmness in Newtons (N).

3.1.6 Sensory analysis

Sensory evaluation of the cheese samples were conducted by a panel of 8-10 university faculty and staff members, who were familiar with fresh cheese. The analysis was performed on days 2 and 8 of storage, after microbiological analysis, in order to assure food safety. Comparative sensory analysis (see annex) was carried out asking the panel to identify and quantify differences in sensory attributes (colour, firmness, elasticity, grainy, pasty, watery, aroma, flavour and off-flavour) between cheeses treated by HP at 300 and 400 MPa, and freshly made cheeses (reference cheese). Differences of sensory attributes were scored on a 9-point negative to positive scale (0 = no differences respect to the reference; ±1 = minimal differences; ±2 = noticeable differences; ±3 = considerable differences; ±4: very considerable differences; algebraic sign, i.e., negative or positive, indicates lower or greater perception respect to the reference). Samples were equilibrated to room temperature (20^{o}C) and presented to the panellist on white plates coded with a three-digit random numbers. Panellists had free access to water and unsalted crackers to aid in palate cleansing between samples. The sensory scores for each sample of cheese were obtained by averaging the individual scores for the sub-samples. On day 2, the reference cheese used was the control cheese.

3.2 Industrial scale Experiments

3.2.1 Cheeses and high pressure treatment

As describes in section 3.1.1 fresh cheeses were supplied by a local cheese producer (Albal, Valencia, Spain) on the cheese making day. The cheeses used in this experiment (~250 g)

were treated in their original packaging at industrial scale using a Hiperbaric equipment, the Wave 6000/135 (Hiperbaric S.A., Burgos, Spain), with a 135 L volume vessel and a diameter of 300 mm (Figure 9). Untreated cheeses were used as control samples.

HP conditions (500 MPa, 5 min) were chosen based on previous studies using the same industrial equipment: 400, 500 and 600 MPa were tested, and changes in cheese shelf-life and texture were assessed. The application of 600 MPa produced cheeses with drastic changes in texture, which were firmer compared with those treated at 400 or 500 MPa. On the other hand, treatment performed at 500 MPa produced cheeses with similar or increased shelf-life than those treated at 600 or 400 MPa, respectively, avoiding the drastic changes in texture described for cheeses treated at 600 MPa (data not shown).

Cheeses were stored at 4ºC and for microbiological examination at 4 and 8ºC. Sampling and analyses were carried out only during cheese shelf-life, i.e. on days 1 and 7 for control cheeses, and days 1, 7, 14 and 21 for HP-treated cheeses, except for sensory analysis which was performed on days 4, 8, 15 and 22 of storage, and for microbiological analysis which was evaluated for both cheeses over 21 days of storage. For microstructure observation, micrographs of cheeses were acquired on day 4 of storage.

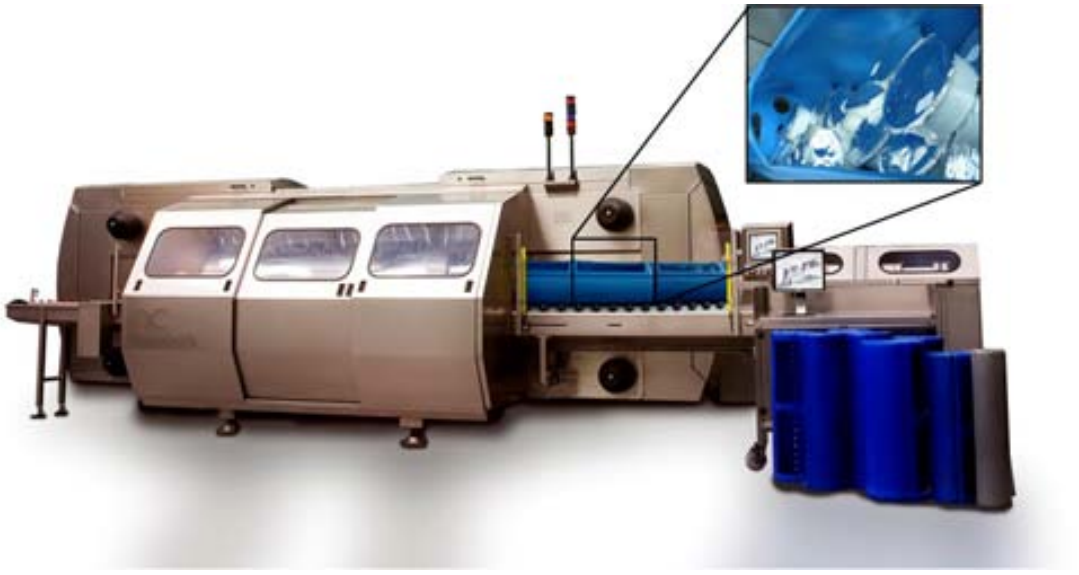

Figure 9. HPP unit Model Wave 6000/135 from Hiperbaric S.A., Burgos, Spain

3.2.2 Physicochemical analysis

Gross composition of cheeses was determined in triplicate and the results were expressed on a dry basis. Samples were assayed for total solid content, fat, protein and pH as described in section 3.1.2. The sodium chloride was determined in triplicate using chloride analysis (Corning 926 Chloride Analyzer, Sherwood Scientific Ltd. Cambridge, UK). Cheese whey expulsion during storage was calculated by removing cheeses from the packages and weighing both cheese and whey separately. The amount of expelled whey was expressed as percentage of the packaged cheese weight.

3.2.3 Microbiological analysis

Microbiological analysis and sampling dates were performed as describe in the pilot plant experiment section (see 3.1.4). Additionally, lactobacilli were enumerated on Man-Rogosa-Sharpe agar (MRS, Oxoid), and incubated at 30°C for 48 h. Counts of *Pseudomonas* spp. were made on Pseudomonas agar base supplemented with Pseudomonas CFC supplement (SR 103E, Oxoid), plates were incubated for 48 h at 20°C. Counts of coagulase-positive *Staphylococcus* were made by surface spreading 0.1 mL of decimal dilutions on Baird Parker agar supplemented with rabbit plasma fibrinogen (BP-RPF agar, Oxoid) and incubated at 37ºC for 24-48 h. To enumerate spores, 10 mL of the primary dilution were heated at 80°C for 10 min and immediately cooled in a water ice bath, followed by plating in appropriate dilutions on PCA. Plates were incubated at 30°C for 48h.

3.2.3.1 Estimation of cheeses shelf-life by predictive microbiology

Predictive microbiology is an efficient and effective method to determine food shelf-life (McMeekin et al., 1993); applying these models and the detected quantity of microorganisms, it is possible to estimate the shelf-life of a product. The Baranyi & Roberts (1994) model was fitted to the raw growth data, for mesophilic aerobic bacteria, using DMFit software (Institute of Food Research, Norwich, UK) obtained from http://modelling.combase.cc/DMFit.aspx to estimate maximum growth rates and lag time and their respective standard errors, and with them the shelf-life.

3.2.4 Texture

A uniaxial compression test was carried out with a TA-XT2 Texture Analyser (Stable Microsystems, Surrey, UK). Six cubes were cut from each cheese sample and held at 20ºC till the assay. Cheeses were compressed to 80% of their original height using a 245 N load cell and a compression cylinder of 36 mm of diameter, with a crosshead speed of 80 mm s^{-1}. True stress and true strain were calculated according to Calzada & Peleg (1978) by the following Equations:

$$\text{True stress: } \sigma_{(t)} = F_{(t)} \times A_{(t)}^{-1}$$

Where $\sigma_{(t)}$ (Nm^{-2}) is the true stress at time (t); $F_{(t)}$ (N) is the force at time (t); and $A_{(t)}$ (m^2) is the area at time (t).

$$\text{True strain: } \varepsilon = \ln [H_0 \times (H_0 - \Delta H)^{-1}]$$

Where ε is the true strain; H_0 (m) is the original height; and ΔH is the change in height.
The elastic modulus was calculated by fitting the following mathematical model:

$$\text{Modulus: } \sigma_{(t)}(\varepsilon) = E_C \times \varepsilon \times \exp(\varepsilon \times K)$$

Where ε and $\sigma_{(t)}$ are the true strain and true stress, respectively; E_C is the elastic modulus (i.e. the tangent to the stress strain curve at the origin); *K* is a constant and it was regarded as a fitting parameter.

3.2.5 Microstructure

Confocal laser scanning microscopy (CLSM) was used to study the distribution of fat and protein in cheeses. Cheese samples were sliced with a razor blade (ca. 1 mm thick) and then stained by submerging them for 15 min in a 0.02% (w/v) Nile Blue A solution (Sigma, Steinheim, Germany). Slices were washed twice and placed on microscope slides and

covered with non-fluorescent observation medium and a cover slip. Images were captured by a Leica TCS SP2 AOBS (Heidelberg, Germany) using a 63× magnification objective lens with a numerical aperture of 1.4. Confocal illumination was provided by an argon laser (488 nm laser excitation). Images were processed with overlays of the two channels using the Leica LAS AF Lite software (Leica Microsystems, Inc., Germany).

3.2.6 Colour

See section 3.1.5 for colour analysis.

3.2.7 Free fatty acids determination and quantification

Free fatty acids (FFA) were extracted by the modified method proposed by (De Jong & Badings, 1990). Ground cheese samples (1 g) were placed into a screw-capped tube, and mixed with 0.3 mL of H_2SO_4 (2.5 M), 3 g of anhydrous Na_2SO_4 and 20 µL of internal standard solution (heptanoic acid 16 mg mL^{-1} and decaheptanoic acid 17.8 mg mL^{-1}, Sigma Aldrich GmbH, Steinheim, Germany). Diethyl ether/heptane was added (3 mL, 1:1 v/v) and the mixture was shaken for 1 min using a vortex mixer. After centrifugation at room temperature (230 ×*g* for 2 min), the supernatant was transferred to a screw-capped tube containing 1 g anhydrous Na_2SO_4. This operation was repeated twice adding each time 5 mL of the diethyl ether: heptane mixture. In order to isolate the FFA, the lipid extract was applied to an aminopropyl column Spe-ed NH_2 500 mg mL^{-1} (Applied Separations, Allentown, PA, USA), previously conditioned with 10 mL of heptane. To eliminate glycerides a mix of Hexane/2-propanol (20 mL, 3:2 v/v) was used. FFA were eluted with 5 mL of diethyl ether containing 2% formic acid. This solution (1 µL) was directly used for gas chromatographic analysis. Two independent extractions were carried out for each sample, and one chromatographic injection was made for each extract.

FFA were analysed on a Hewlett Packard unit (HP 6890, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) in splitless mode at an inlet temperature of 320 °C. Free fatty acids were separated in a fused silica capillary column DB-FFAP (30 m × 0.32 mm × 0.25 mm), protected by a fused silica deactivated

ward column (5 m × 0.32 mm × 0.32 mm), both from Agilent (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was high purity helium at a flow rate of 40 mL min^{-1}. High purity hydrogen and compressed air were supplied to the FID. The oven temperature was raised from 100 to 240°C at 5°C min^{-1}, and then held at this temperature for 51 min. The output signal was integrated using HP ChemStation software (v. B.02.05). Identification of individual FFA was done by comparing the relative retention times from the sample peaks to those of the standard mix, supplied by Sigma (Sigma–Aldrich GmbH, Steinheim, Germany). Increasing concentrations of individual fatty acids and fixed concentrations of internal standards were used for the calculation of calibration curves. In order to quantify the FFA presented in cheese samples, heptanoic acid and decaheptanoic acid were used as internal standards for short-medium chain and long chain FFA, respectively.

3.2.8 Sensory analysis

Sensory evaluation was performed on days 4, 8, 15 and 22 of storage as described in section 3.1.6. Besides, a preference test was carried out: panellists were asked to say how much they "like" or "dislike" the cheese samples served using a 9-point hedonic scale, where 0 = "dislike extremely" and 9 = "like extremely" (Peryam & Pilgrim, 1957). Cheese samples were presented individually and different codes from comparative analysis were used.

3.2.9 Volatile compounds analysis

Control cheeses were analysed, according to their shelf-life, on days 1 and 7, and pressurised cheeses on days 1, 7, 14, and 21. After high pressure treatment, cheese samples were cut into sections, frozen, and stored at −20°C until volatile analysis was conducted.

3.2.9.1 SPME-GC-MS

Extraction of compounds was performed by solid-phase microextraction (SPME) and identification by gas chromatography coupled to quadrupolar mass selective spectrometry (GC-MS).

Volatile compounds were extracted using an 85 µm CAR/PDMS fibre (Supelco, Bellefonte, PA, USA) by SPME. The gas chromatograph system (HP 6890 Series II) was equipped with a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a HP 5973 Mass Selective Detector (Hewlett-Packard, Heilbronn, Germany). The fibre preconditioning at 300°C for 1 h was performed. Cheeses samples (ca. 1.50 g) were pre-equilibrated at 80°C for 5 min in a 10 mL amber vial fitted with a Teflon septum, followed by the volatile compounds extraction for 30 min. The injection port was used in splitless mode. Time of desorption fibre was 15 min at 280ºC.

Carrier gas was helium (1 mL min^{-1} flow-rate) and the column temperature was initially maintained at 40°C for 5 min, heated to 110°C at a rate of 5°C min^{-1}, then by 10°C min^{-1} to 240°C, where it was held for 15 min, giving a total run time of 52 min. The mass spectra were obtained by electron impact at 70 eV. The chromatograms were recorded by monitoring the total ion current in the 33-250 mass range.

Identification of the volatile compounds was based on comparison of the spectra with those of the NIST08 and Wiley 7n1 libraries. Main, molecular, and qualifier ions were selected for each compound indentified. Retention indices, relative to C_8-C_{26} *n*-alkanes were also determined by injecting 1 µl of each standard solution (Alkane standard solution C_8-C_{20} from Sigma-Aldrich, and Connecticut ETPH calibration mixture C_9-C_{36} were used as standards) in triplicate with a split ratio of 1:200. Signals were processed using Agilent MSD Productivity ChemStation Enhanced Data Analysis software (Agilent technology, Santa Clara, CA, U.S.)

Confirmation of the identity of some volatile compounds was achieved by comparing the retention times and mass spectra of individual components with those of authentic reference compounds injected under the same operating conditions. Semi-quantification was based on arbitrary units of the total main ion area counts divided by 10^4. Limit of

detection (LOD) was calculated for each compound by measuring a series of 7 blank samples, the mean blank value and the standard deviation (SD). The LOD is the mean blank values plus 3 SD.

3.3 Fate of *Listeria* spp. inoculated in a commercial fresh cheese by means of HPP

3.3.1 Bacterial strains and culture conditions

L. monocytogenes CECT 4031 (serotype 1/2a) and *L. innocua* CECT 910 were obtained from the Spanish Type Culture Collection (University of Valencia, Spain) and *L. monocytogenes* Scott A (serotype 4b) was kindly provided by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA; Madrid, Spain). Scott A strain was chosen due to its highly virulence. It has been reported that a majority of listeriosis outbreaks are caused by strains of serotype 4b (Swaminathan & Gerner-Smidt, 2007). Freeze-dried cultures were rehydrated in tryptone soy broth (TSB; Oxoid Ltd., Basingstoke, Hampshire, England) at 37ºC for 24 h and subsequently used to inoculate tryptone soy agar plates (TSA; Oxoid). Individual colonies were collected and agar slopes of TSA enriched with 0.6% yeast extract (TSAYE) were prepared and kept at 4ºC to provide stock cultures for the assays. For preparation of the inocula, each strain was subcultured twice before use in experiments to assure they were on stationary phase of growth. Briefly, the strains were revived by placing one bead in TSB and incubated at 37ºC for 18 h. This broth was used to streak the culture on TSAYE, and was incubated for 37ºC for 18 h. Once the purity of the culture was verified, cell suspensions were prepared in 10 mL of tryptone sodium chloride solution (1 g L^{-1} of tryptone pancreatic casein digestion, Oxoid) and 8.5 g L^{-1} of sodium chloride (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain a final concentration of approximately 8 to 9 log cfu mL^{-1}.

3.3.2 Preparation of samples and inoculation

Commercial fresh cheeses (ca. 250 g) were supplied by a local company (Albal, Valencia, Spain). Cheese inoculation was carried out in a Class II biosafety cabinet (Bio-II-A, Telstar, Terrasa, Spain) using a 50-200 µL multichannel pipette. Samples were deep inoculated 4 times with bacteria suspensions in order to obtain two target levels of *Listeria* counts of approximately 3 and 6 log cfu g^{-1}. Once inoculated, cheeses were cut in halves, placed into vacuum polyethylene bags, and vacuum packaged using a packaging machine EVT-7VT (Tecnotrip, Barcelona, Spain). Inoculated samples were stored at 6°C for 1 h to allow for cell attachment before high pressure treatment.

3.3.3 High pressure treatment

The equipment used (Figure 10) was a Stansted 5 L capacity unit model ISO-LAB FPJ 11500 (Stansted Fluid Power Ltd., Essex, UK). Cheeses were treated at 300, 400, 500, and 600 MPa for 5 min at 6ºC. Untreated inoculated cheeses vacuum packaged were used as control samples, and a pool of cheeses no inoculated and no treated was used as blank sample.

3.3.4 Microbiological analyses

Viable and injured bacterial counts were enumerated after HPP treatment by means of differential plating method using Agar Listeria according to Ottaviani and Agosti (ALOA; AES, Chemunex, Combourg, France) and Thin Agar Layer (TAL) Method onto ALOA covered with Tritone Soy Agar TSAYE (Kang & Fung, 1999). Cheeses were stored at 4°C and analysed on days 1, 3, 7, and 15 for cheeses treated at 400 and 500 MPa, and days 1 and 15 for cheeses treated at 300 and 600 MPa.

Figure 10. HPP unit Model ISO-LAB FPJ 11500 from Stansted Fluid Power Ltd., Essex, UK

At each sampling time, packages were opened aseptically and 10 g from each bag were transferred into sterile filtered bags, and 90 mL of buffered peptone water (Oxoid) were added to each bag. Samples were homogenised for 1 min in an electromechanical homogenizer (Stomacher, Lab-blender 300, Seward Medical, London, UK). Initial dilution

was incubated during ca. 1 h to revive injured microorganisms according to ISO 11290-2 procedure for detection and enumeration of *L. monocytogenes* (Anonymous, 2004). Ten-fold serial dilutions were prepared in buffered peptone water and surface-plated onto ALOA and ALOA+TSAYE for enumeration of uninjured and injured *Listeria*, respectively. Inoculated plates were incubated (37ºC, 18-24 h) and typical colonies of *L. innocua* and *L. monocytogenes* were enumerated after incubation according to the ISO 11290-2 stipulations, if after 18-24 h no suspect colonies were evident, plates were re-incubated for an additional period of 24 h. The counts were expressed as log cfu g^{-1}. Incubation of initial enrichment dilution was continued at 37ºC for a total of 24 h to allow multiplication of *Listeria* strains to levels that were enough for detection of the organism when cell levels decreased below the detection limit (1 log cfu g^{-1}). Consequently, the enrichment culture was streaked on ALOA in order to determine presence of *Listeria* spp. Lethality was calculated as the difference between the logarithms of colony counts of the untreated (N_0) and treated (N) samples (log N/N_0).

3.3.4.1 Maximum growth rate determination of Listeria strains

Additionally, the values for log cfu g^{-1} of *L. innocua* and *L. monocytogenes* were plotted against time. Maximum growth rate (μ max) was calculated using DMFit software from Institute of Food Research (Combase; Norwich, UK. http://modelling.combase.cc/DMFit.aspx).

3.4 Statistical analysis

One-way analysis of variance (ANOVA) was performed using IBM® SPSS® statistics 19 software (SPSS Inc., U.S.) to test the main effects of HPP and time on physicochemical, microbiological, sensorial, colour and uniaxial compression test, as well as in *Listeria* experiment. Significant differences were assessed by Student Newman Keuls test with significance level set for $p<0.05$.

For volatile profile analysis, data were analysed using statistical package SAS® 9.2 program (SAS Institute Inc., Cary NC, U.S.). Mean values for the individual constituents were calculated from triplicate analyses. One way and two way ANOVA were used to investigate the effect of HPP on the volatiles production, time and their interaction. Tukey test was used to compare sample data, which were considered to be statistically different when $p<0.05$. R-software (R Development Core Team (2011); R Foundation for Statistical Computing, Vienna, Austria) was used to perform the principal components analysis (*PCA*) in order to provide an easy visualisation of the relationship between the application of HPP and the development of volatile compounds during storage.

The *Listeria* spp. experiment involved three complete replications each with duplicate analysis using a different batch of fresh cheeses (n=6). Two-samples t test was performed using SPSS® to determine difference between ALOA and TAL method, differences with a p-value <0.05 were considered statistically significant.

3.5 References

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Chapter 4

Effect of HPP on starter-free fresh cheese shelf-life. Pilot plant scale experiment

This chapter consists of the paper I

4.1 Introduction

Nowadays, consumers are demanding minimally processed RTE products which provide increased convenience, while maintaining fresh-like characteristics of flavour, texture, colour, aroma and overall appearance.

In Spain, fresh cheese is the most consumed (2.5 kg per capita in 2011) cheese variety (Anonymous, 2013), and is characterised for being soft, white, with fresh flavour and traditionally made from pasteurised cow milk, without addition of any starter culture, which means that its pH is almost neutral; additionally, it has high moisture content and water activity. All these characteristics enhance the growth of possible pathogenic and spoilage microorganisms, reducing considerably its shelf-life (about one week). The concern of achieving microbiologically safe dairy products such as cheese, with an extended shelf-life, has increased the interest in non-thermal preservation technologies such as HPP, which is able to ensure product microbial safety while preserving sensory and nutritional characteristics (Cheftel, 1992).

Limited work has been conducted to date on the use of HPP for shelf-life extension of starter-free fresh cheese. Previous studies on different fresh cheese varieties such as fresh goat's milk cheese, Queso Fresco cheese, fresh lactic curd cheeses and rennet-coagulated

fresh Scottish cheese (Capellas et al., 1996, 2000, 2001; Sandra et al., 2004; Daryaei et al., 2006, 2008; Okpala et al., 2010) showed that the application of 300-600 MPa for 5-30 min increased their shelf-life by reducing microbial counts, without detrimental effects to the product quality. Therefore, the aim of this work was to study the application of HPP (300 and 400 MPa for 5 min at 6°C) in a starter-free fresh cheese (pH 6.72) to obtain long shelf-life while maintaining acceptable sensory characteristics during cold storage. The methodology applied for this purpose is described in Chapter 3 (see 3.1) and includes the study of the physicochemical, microbial, colour, texture and sensorial characteristics.

4.2 Results and discussion

4.2.1 Cheese composition

The composition of control and HP cheeses is shown in Table 5. Total solid content of HP-cheeses was higher than control cheeses ($p<0.05$) possibly due to compaction caused by high pressure, which was reflected on a higher expelling whey retained in packaging (data not shown).

Pressurization did not change fat and total protein contents, or pH values. Similar results were observed by Capellas et al. (2001) and Sandra et al. (2004), in Mató and Queso Fresco cheeses, respectively. However, opposite results were observed by Okpala et al. (2010) who reported that HP treatment influenced significantly the moisture, fat and protein contents of soft Scottish cheese. These authors reported that fat content increased as moisture decreased after HP treatment of above 100 MPa. Moreover, protein content of HP-treated fresh cheese remained lower than the control cheese.

Table 5. Composition of control and pressurised cheeses on day 1 stored at 4°C

	Treatment		
	Control	***300 MPa***	***400 MPa***
Total solids (TS, %)	32.17 ± 1.74[a]	36.19 ± 1.37[b]	35.04 ± 3.14[b]
Fat (% on TS)	47.50 ± 3.30	46.80 ± 6.50	43.29 ± 3.56
Total protein (% on TS)	38.26 ± 1.44	39.23 ± 1.56	37.30 ± 6.52
pH	6.72 ± 0.01	6.72 ± 0.01	6.72 ± 0.02

Values represented by mean ± standard deviation
[a, b] Different superscript in the same row indicates significant differences ($p<0.05$).

4.2.2 Whey loss and water activity

No differences in a_w between treatments were observed, and a clear effect of HP on cheese whey loss was not evidenced (Table 6). On day 1, cheeses treated at 400 MPa expelled more whey than control cheeses, but throughout cheeses shelf-life, HP-treated cheeses presented less whey loss ($p<0.05$) possibly because these cheeses expelled more whey at the beginning of shelf-life. Capellas et al. (2001) studied the effect of HP on physico-chemical characteristics of Mató cheese and they observed that treated cheeses (500 MPa for 5, 15 and 30 min at 10 or 25ºC) expelled significantly more whey than control cheese during cold storage at 4ºC.

Table 6. Whey loss and water activity of control and pressurised cheeses stored at 4°C

		Treatment		
	Day	***Control***	***300 MPa***	***400 MPa***
Whey loss (%)	1	43.13 ± 3.54[b]	43.64 ± 1.21[abA]	45.89 ± 2.90[aA]
	7	42.99 ± 4.13[a]	39.46 ± 3.31[bB]	41.75 ±1.26[abB]
	14	*N.d.*	37.55 ± 1.35[C]	38.29 ± 0.88[C]
Water activity (a_w)	0	0.997 ± 0.003	0.997 ± 0.004	0.995 ± 0.003
	7	0.996 ± 0.002	0.995 ± 0.002	0.994 ± 0.002
	14	*N.d.*	0.994 ± 0.003	0.996 ± 0.002

Values represented by mean ± standard deviation
[a, b] Different superscript in the same row indicates significant differences ($p<0.05$).
[A, B, C] Different superscript in the same column indicates significant differences ($p<0.05$).
N.d. = not determined.

4.2.3 Microbiological analysis

The effect of pressure on the microbial growth, of cheeses stored at 4°C, is shown in Table 7. The aerobic mesophilic and lactococci counts showed a similar trend during cheeses shelf-life, and a delay in bacterial growth in pressurised cheeses was observed. Thus, while the control cheeses reached counts near to 6 log cfu g^{-1} at 7 days; HP-treated cheeses at 300 and 400 MPa had a shelf-life of about 14 and 21 days, respectively.

HPP did not reduce the initial mesophilic counts and specifically lactococci counts, whose origin in cheese was probably associated with residual lactococci resistant to heat treatment and cross-contamination in dairy plant. An explanation of this phenomenon is the tailing effect. This mechanism is still poorly understood (Smelt, 1998), and it could be a result of heterogeneous microbial population, whose phenotypic variation, occurs in particular resistant to the stress applied (Patterson, 1999; Simpson and Gilmour, 1997). However, other authors (De Lamo et al., 2007; López-Pedemonte et al., 2007) applying pressures between 300 and 500 MPa for 10 min, observed reductions ca. 2-3 log cfu g^{-1} of lactococci in model cheeses containing starter (*Lactococcus lactis* subsp. lactis and *Lactococcus lactis* subsp. cremoris), when lactococci was present at high levels in cheese (~ 8 log cfu g^{-1}). Cheeses treated at 400 MPa showed the lowest rate of growth of psychrotrophic bacteria, reaching ca. 6 log cfu g^{-1} at 21 days. HP was also able to delay the development of moulds and yeasts as well as *Enterobacteriaceae*. Daryaei et al. (2008) observed yeasts growth after 6 weeks in cheeses (fresh lactic curd) treated under similar conditions. According to O'Reilly et al. (2000) at high pressures, the more severely damaged microorganisms bacteria take longer to recover. This fact possibly explains the later presence of moulds and yeasts counts in cheeses treated at 400 MPa.

Counts of microbial groups of cheeses stored at 8°C (Table 8) showed similar trends to those maintained at 4°C, although the shelf-life of the former cheeses decreased considerably. Control cheeses and cheeses treated at 300 and 400 MPa, stored at 8°C, exhibited a shelf-life of 3, 7 and 10 days less than their respective homologous cheeses stored under optimal conditions (4°C). These results show that breaks in the cold chain could compromise the potential of HP to ensure food safety and cheese quality.

Table 7. Microbiological counts (log cfu g^{-1}) of control and pressurised cheeses stored at 4°C.

		Treatment		
Microbial group	*Day*	*Control*	*300 MPa*	*400 MPa*
Aerobic mesophilic	1	3.57 ± 0.50^{A}	3.80 ± 0.35^{A}	3.42 ± 0.59^{A}
	7	6.29 ± 1.33^{aB}	4.13 ± 0.54^{bA}	2.54 ± 1.20^{cA}
	14	*N.d.*	6.50 ± 1.54^{aB}	3.91 ± 1.01^{bA}
	21	*N.d.*	*N.d.*	6.20 ± 1.49^{B}
Lactococci	1	3.20 ± 0.59^{A}	3.39 ± 0.72^{A}	3.03 ± 0.69^{A}
	7	6.40 ± 1.43^{aB}	4.04 ± 0.56^{bA}	1.81 ± 1.12^{cA}
	14	*N.d.*	6.38 ± 1.53^{aB}	3.27 ± 1.31^{bA}
	21	*N.d.*	*N.d.*	5.84 ± 1.60^{B}
Psychrotrophs	1	0.99 ± 1.54^{A}	N.D.	N.D.
	7	6.65 ± 1.69^{aB}	4.35 ± 0.65^{bA}	0.94 ± 1.45^{cAB}
	14	*N.d.*	6.33 ± 1.59^{aB}	3.06 ± 1.43^{bB}
	21	*N.d.*	*N.d.*	6.12 ± 1.45^{C}
Enterobacteriaceae	1	0.32 ± 0.78	N.D.	N.D.
	7	1.45 ± 2.25	N.D.	N.D.
	14	*N.d.*	0.35 ± 0.54	N.D.
	21	*N.d.*	*N.d.*	0.33 ± 0.66
Moulds and yeasts	1	N.D.	N.D.	N.D.
	7	5.22 ± 0.70^{a}	1.99 ± 3.09^{b}	N.D.
	14	*N.d.*	2.26 ± 2.56	2.37 ± 3.00^{A}
	21	*N.d.*	*N.d.*	4.83 ± 1.46^{B}

Values represented by mean ± standard deviation.
$^{a,\ b,\ c}$ Different superscript in the same row indicates significant differences ($p<0.05$).
$^{A.\ B.\ C}$ Different superscript in the same column indicates significant differences ($p<0.05$).
N.d. = Not determined.
N.D. = Not detected.

Table 8. Microbiological counts (log cfu g^{-1}) of control and pressurised cheeses stored at 8°C

		Treatment		
Microbial group	*Day**	*Control*	*300 MPa*	*400 MPa*
Aerobic mesophilic	4	6.32 ± 0.33^{aA}	3.67 ± 0.62^{bA}	1.97 ± 2.28^{bA}
	7	7.58 ± 0.35^{aB}	6.79 ± 0.53^{aB}	4.26 ± 0.69^{bAB}
	11	*N.d.*	*N.d.*	6.29 ± 1.00^{B}
Lactococci	4	6.13 ± 0.45^{aA}	3.43 ± 0.74^{bA}	1.20 ± 1.38^{cA}
	7	7.47 ± 0.34^{aB}	6.71 ± 0.48^{aB}	4.14 ± 0.75^{bB}
	11	*N.d.*	*N.d.*	6.20 ± 1.00^{C}
Psychrotrophic bacteria	4	5.88 ± 0.33^{aA}	3.52 ± 0.72^{bA}	0.33 ± 0.65^{cA}
	7	7.74 ± 0.64^{aB}	6.68 ± 0.52^{aB}	2.07 ± 2.45^{bA}
	11	*N.d.*	*N.d.*	6.61 ± 0.60^{B}
Enterobacteriaceae	4	3.33 ± 0.58^{A}	N.D.	N.D.
	7	5.74 ± 0.57^{B}	2.96 ± 3.42	N.D.
	11	*N.d.*	*N.d.*	1.12 ± 1.30
Moulds and yeasts	4	5.11 ± 0.74^{a}	1.43 ± 1.66^{bA}	N.D.
	7	5.91 ± 0.25^{a}	4.55 ± 0.97^{aB}	1.71 ± 1.97^{b}
	11	*N.d.*	*N.d.*	3.01 ± 3.48

Values represented by mean ± standard deviation.
$^{a, b, c}$ Different superscript in the same row indicates significant differences ($p<0.05$).
$^{A, B, C}$ Different superscript in the same column indicates significant differences ($p<0.05$).
N.d. = Not determined.
N.D. = Not detected.
* Counts of day 1 are presented in Table 3.

4.2.4 Colour and texture

HP did not cause significant changes in colour coordinates L* and a* or in total colour difference values. However, the cheeses pressurised at 400 MPa tended to be more yellow (higher b* values) than control cheeses (Table 9). Previous research on cheese colour reported an increase of b* values when cheeses were HP-treated (Capellas et al., 2001;

Okpala et al., 2010). According to Saldo (2002) changes in cheese colour should be related to microstructure changes after pressurization. Likewise, Juan et al. (2008) noted that changes produced by HP in matrix proteins could explain the colour differences obtained in ewe milk cheeses treated at 300 MPa for 10 min.

Table 9. Colour and firmness of control and pressurised cheeses stored at 4°C

		Treatment		
	Day	***Control***	***300 MPa***	***400 MPa***
L*	1	95.32 ± 1.01	95.42 ± 1.18	94.98 ± 1.35
	7	95.15 ± 1.06	94.48 ± 1.06	94.42 ± 1.17
	14	*N.d.*	94.69 ± 1.13	94.0 ± 1.14
a*	0	-0.50 ± 0.06^{A}	−0.45 ± 0.10	-0.47 ± 0.09^{B}
	7	-0.40 ± 0.13^{B}	−0.42 ± 0.19	-0.41 ± 0.19^{AB}
	14	*N.d.*	−0.52 ± 0.08	-0.52 ± 0.06^{A}
b*	0	9.45 ± 0.16^{b}	9.80 ± 0.14^{ab}	10.66 ± 0.50^{a}
	7	9.11 ± 0.80^{b}	10.2 ± 0.85^{ab}	10.36 ± 0.76^{a}
	14	*N.d.*	10.62 ± 1.14^{a}	11.11 ± 1.49^{a}
ΔE	1		0.72 ± 0.34	1.02 ± 0.44
	7		1.11 ± 0.82	1.17 ± 0.33
Firmness (N)	1	3.40 ± 0.70^{a}	4.48 ± 0.83^{b}	4.48 ± 0.39^{bA}
	7	3.26 ± 0.10^{a}	4.62 ± 0.64^{b}	4.69 ± 0.32^{bA}
	14	*N.d.*	4.76 ± 0.89^{b}	6.48 ± 0.81^{cB}

Values represented by mean ± standard deviation.
[a, b, c] Different superscript in the same row indicates significant differences ($p<0.05$).
[A, B] Different superscript in the same column indicates significant differences ($p<0.05$).
N.d. = Not determined.

In general, HP cheeses were significantly firmer than control cheeses (Table 9). Similar results were found in Mató cheese (Capellas et al., 2001), ewe milk cheese (Juan et al., 2007) and soft Scottish cheese (Okpala et al., 2010). Daryaei et al. (2006) also observed a gradual increase in firmness during storage of a fresh lactic curd cheese, but these differences were not significant.

The increase in firmness observed in HP-cheeses is probably due to lower water content with respect to control cheeses and as Juan et al. (2007) suggested, HP treatment causes changes in the cheese protein network, forming a new more compact cheese structure.

4.2.5 Sensory analysis

The objective of this analysis was to assess differences between a freshly made cheese and pressurised cheeses. The panellists identified the pressurised cheeses in relation with the reference cheese as more yellow, firmer, and less watery, but they did not appreciate off-flavours or great differences in flavour and aroma (Table 10). The results for colour and firmness observed by the panel were in agreement with the respective instrumental analysis. Sandra et al. (2004) also observed HP cheese as more yellow, but they did not find differences in firmness. Daryaei et al. (2006) showed that untrained panellists could not distinguish any significant differences between untreated and pressure-treated fresh lactic curd cheeses. In this study, the sensory panel plainly noted the appreciable influence of storage time in cheeses treated at 300 MPa. The cheeses became less watery and firmer than untreated cheeses. This makes sense since firmness and watery characteristics are negatively correlated; this effect was also observed in cheeses treated at 400 MPa.

Table 10. Sensory scores of control and pressurised cheeses stored at 4°C

		Treatment	
	Day	*300 MPa*	*400 MPa*
Colour	2	1.15 ± 0.78[a]	1.12 ± 0.82[a]
	8	1.15 ± 0.93[a]	1.15 ± 0.81[a]
Firmness	2	1.12 ± 1.24[aA]	1.77 ± 1.11[b]
	8	1.80 ± 0.95[bB]	2.05 ± 0.99[b]
Elasticity	2	–0.23 ± 1.5	–0.35 ± 1.67
	8	–0.20 ± 1.7	0.10 ± 1.65
Grainy	2	–0.15 ± 1.38	–0.04 ± 1.54
	8	0.70 ± 1.17	1.00 ± 1.21
Pasty	2	0.00 ± 1.39	0.04 ± 1.73
	8	0.15 ± 1.84	-0.05 ± 1.9
Watery	2	–0.77 ± 1.21[aA]	–1.65 ± 1.06[b]
	8	–1.75 ± 0.85[bB]	–1.75 ± 0.97[b]
Aroma	2	–0.38 ± 1.27	–0.23 ± 1.24
	8	0.00 ± 0.56	0.05 ± 0.76
Flavour	2	0.08 ± 0.93	0.27 ± 1.28
	8	0.30 ± 1.03	0.20 ± 1.15
Off-flavour	2	–0.04 ± 0.77	0.08 ± 0.93
	8	0.60 ± 0.75[a]	0.70 ± 0.92[a]

Values represented by mean ± standard deviation

[a, b] Different superscript in the same row indicates significant differences between treatments and respect to the reference cheese (p <0.05).

[A, B] Different superscript in the same column indicates significant differences throughout cheeses shelf-life (p <0.05).

Scoring scale: 0 = no differences with control; ±1 = minimal differences; ±2 = noticeable differences; ±3 = considerable differences; ±4: very considerable differences.

4.3 Conclusions

HP is a promising technology for increasing shelf-life of starter-free fresh cheese. Microbiological results indicate the possibility of improving microbiological quality of cheeses during storage, especially when 400 MPa are applied, compared to untreated cheese, obtaining a higher shelf-life of 14 to 21 days depending on the pressure level applied. On the other hand, HP treatment caused only little changes in some sensory characteristics of cheeses such as colour and firmness. There appears to be little published work on the application of this technology on the extension of shelf-life of fresh cheeses, and this study has demonstrated the effectiveness of such treatment. However, further investigation should be conducted in order to study if these results can be extrapolated to industrial processes to satisfy consumers, producing safe and high-quality fresh cheeses.

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Chapter 5

Effect of HPP on free-starter fresh cheese shelf-life. Industrial scale experiment

This chapter consists of the paper III

5.1 Introduction

In the last 20 years, the use of HPP has been widely studied to reduce microorganisms in different food matrices (Farkas & Hoover, 2000; Rastogi et al., 2007; Considine et al., 2008; Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Martínez-Rodríguez et al., 2012). HPP has already become a commercially implemented technology, spreading from its origins in Japan (1990), and slowly introduced into other countries such as USA, Spain, and France. Equipment for large-scale production of HPP products are commercially available nowadays, showing a fast increasing in the number of units installed during the last 10 years (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Mújica-Paz et al., 2011). Some of the products currently available on the market are guacamole, ham, oysters, meat, and fruit juices.

Fresh cheese is characterised by having a high pH and water activity, it is high in moisture content, has a mild milky flavour, and a short shelf-life (Hwang & Gunasekaran, 2001). Traditionally it is made from pasteurised milk without starter cultures addition. Milk pasteurisation kills and substantially reduces the number of spoilage microorganisms. However, it does not control bacterial cross-contamination, especially during and after curd production (Zottola & Smith, 1993). Storage of fresh cheeses under aerobic conditions

results in rapid spoilage, usually in less than 10 days, while cheese treated by HPP may extend storage life to 18-21 days (Evert-Arriagada et al., 2012).

As it was described in Chapter 4, HPP application to enhance microbiological quality of fresh cheese has been studied in different cheese varieties (Capellas et al., 2000, 2001; Daryaei et al., 2008; Sandra at al., 2004; Okpala et al., 2010). These studies were focused in finding the optimum conditions to reduce microbial counts on cheeses, affecting minimally physico-chemical and sensory characteristics. However, all these studies were carried out using model cheeses or small size cheeses made in a pilot plant and treated by using prototypes or lab high-pressure devices. In this study a more realistic scenario in the application of this novel technology was set. The aim of this study was to evaluate the effect of HPP under industrial conditions (500 MPa, 5 min, 18ºC) on physico-chemical (see 3.2.2), microbial (see 3.2.3), colour (see 3.1.5), texture (see 3.2.4), microstructure (see 3.2.5) and sensorial properties (see 3.2.9) during the shelf-life of a commercial starter-free fresh cheeses.

5.2 Results and discussion

5.2.1 Physicochemical analysis

Changes in physicochemical attributes (total solid, fat, protein, salt content, pH) and whey loss were studied during storage at 4°C of cheese samples (Table 11). With regard to changes in all attributes studied, high pressure-treated cheeses had no significant ($p \geq 0.05$) effect at day 1 compared to control cheeses. Similar results for total solids, fat and protein contents, as well as pH values, were observed in different types of fresh and white brined cheeses after the application of HPP (Capellas et al., 2001; Sandra et al., 2004; Koca et al., 2011; Evert-Arriagada et al., 2012). The total solids content increased ($p<0.05$) within the last 7 days of storage for pressurised samples. Although no differences were observed between pH values of control and pressurised cheeses, for cheese treated by HPP, the pH value at day 21 of storage was slightly but significantly ($p<0.05$) lower than in the previous days of sampling. This result could be related with the increase of lactococci and lactobacilli counts during cheese storage (Figure 11).

Table 11. Composition and whey loss of control and HP-treated cheeses stored at 4°C

		Treatment	
	Day	*Control*	*500 MPa*
Total solids (TS, %)	1	36.74 ± 0.54	36.58 ± 1.64^{B}
	7	37.11 ± 1.60	36.41 ± 1.24^{B}
	14		37.93 ± 1.99^{B}
	21		39.32 ± 1.21^{A}
Fat (% TS)	1	45.13 ± 1.53	46.23 ± 1.68
	7	44.00 ± 1.30^{b}	47.21 ± 2.74^{a}
	14		47.28 ± 1.07
	21		47.88 ± 1.11
Total protein (% TS)	1	38.62 ± 1.19	38.03 ± 1.62
	7	38.05 ± 0.81	37.17 ± 1.57
	14		37.33 ± 1.63
	21		37.61 ± 1.28
NaCl (%)	1	1.21 ± 0.12	1.22 ± 0.22
	7	1.24 ± 0.22	1.15 ± 0.18
	14		1.12 ± 0.17
	21		1.13 ± 0.13
pH	1	6.71 ± 0.05	6.70 ± 0.03^{A}
	7	6.66 ± 0.01	6.64 ± 0.02^{A}
	14		6.64 ± 0.02^{A}
	21		6.56 ± 0.10^{B}
Whey loss (%)	1	2.98 ± 0.88	3.34 ± 2.78^{B}
	7	3.92 ± 2.44	5.67 ± 2.32^{B}
	14		9.39 ± 4.61^{A}
	21		11.51 ± 3.46^{A}

Values represented by mean ± standard deviation

$^{a, b}$ Different superscript in the same row indicates significant differences ($p<0.05$).

$^{A, B}$ Different superscript in the same column indicates significant differences ($p<0.05$).

Regarding the amount of whey expelled from cheese matrix at day 1, all cheeses lost ~3% of their weight as free whey, and although higher amounts of whey were recovered from

the container in cheeses treated at 500 MPa, compared to control samples, these differences were not significant ($p \geq 0.05$) during the first 7 days of storage.

However, a significant increase ($p<0.05$) in whey loss 14 in the same cheese samples was observed from day and the highest measure of free whey at day 21 fits with the highest amount of total solids observed at the same sampling day (Table 11). A similar trend was observed by Van Hekken et al. (2012) in starter-free Queso Fresco stored at 4ºC during 8 weeks. According to these authors, the free whey that pools between curd particles is expelled as the fresh cheese undergoes the last stages of syneresis.

5.2.2 Microbiological analysis

Microbial counts of cheeses stored at 4 and 8°C are presented in Figures 11 and 12, respectively. In this study, *E. coli* and coagulase-positive *Staphylococcus* were not detected in any sample, probably due to the strict hygienic conditions implemented by the food company which supplied the cheeses. HPP significantly ($p<0.05$) reduced the counts of psychrotrophs and lactobacilli (Figure 11), the former microbial group not being detected in these cheeses at day 1. All microorganisms studied showed an increasing trend during the storage period, at both storage temperatures (4 and 8°C), except moulds and yeasts, *Enterobacteriaceae* and *Pseudomonas* spp. counts, which were not able to recover after the application of 500 MPa (data not shown). It is important to point out the relationship between *Enterobacteriaceae* and hygienic quality; therefore, the complete inactivation of this microbial group induced by HPP could be an important guaranty of food safety. Daryaei et al. (2008) working with fresh lactic curd cheese also observed that HPP effectively controlled the occurrence of spoilage yeast when 300 to 600 MPa were applied.

No significant reduction of aerobic mesophilic bacteria and lactococci counts were observed after high pressure treatment, and both counts showed a similar trend during storage, suggesting that (1) mesophilic bacteria consisted mainly of lactic acid bacteria, and (2) a particular selection of microbial strains (i.e. lactococci) could occur due to their bare-resistance, explaining the total aerobic mesophilic bacteria counts obtained after treatment. These results are in agreement with those from a previous study carried out

with the same cheese variety but smaller format (~80 g) and treated at 300 MPa and 400 MPa for 5 min in a lab high-pressure equipment (Evert-Arriagada et al., 2012). Initial lactobacilli counts in cheese made from pasteurised milk could be explained by two phenomena: (1) a heat resistance has been described for some strains which may survive pasteurisation and proliferate in the cheese (Jordan & Cogan, 2002), and (2) a post processing contamination from dairy environment has been suggested by other authors (McSweeney et al., 1994; Somers et al., 2001). It has been described that rods are more baro-sensitive than cocci (San Martín et al., 2002; Yuste et al., 2004; Patterson, 2005), possibly due to the rod-shape cell wall is more susceptible to stress than spherical bacteria (Koch, 1995).

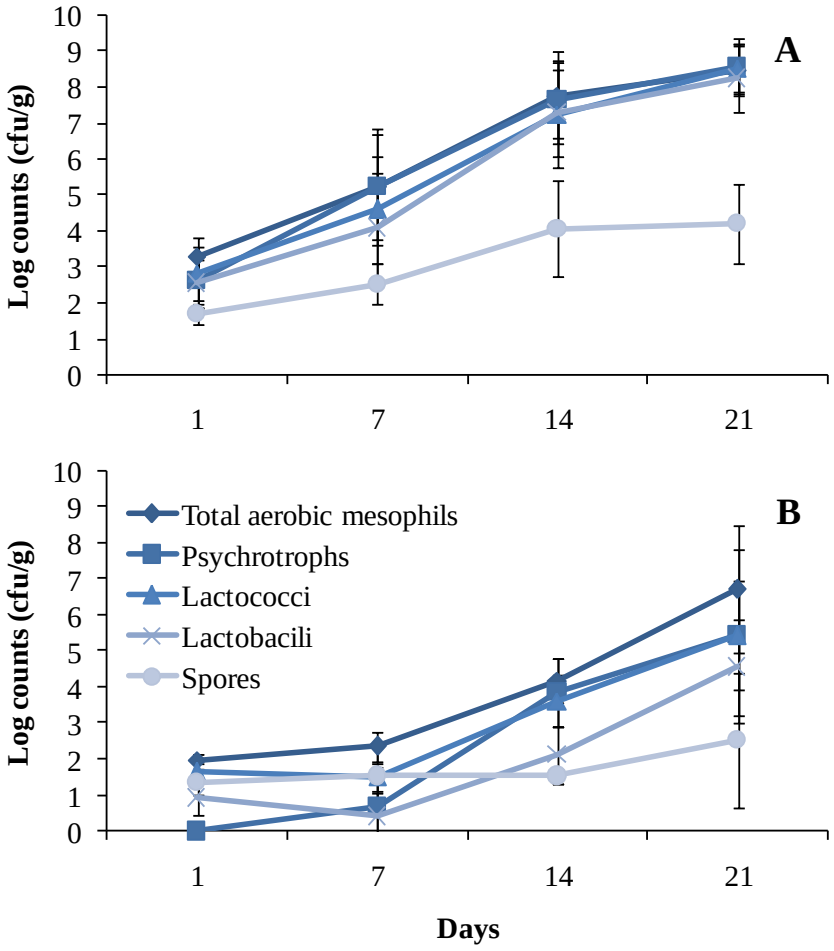

Figure 11. Microbiological counts of control (A) and HP-treated (500 MPa) (B) cheeses stored at 4°C

In this study, lactobacilli counts were about 1 log cfu g^{-1} lower than lactococci for all sampling days regardless of the storage temperature (Figures 11 and 12). After HPP, changes in the physiological state of bacteria cells and in the physicochemical environment resulted in an increasing of lag phase in aerobic mesophilic bacteria from 1 to 6 days in control and pressurised cheeses, respectively, in cheeses stored at 4°C.

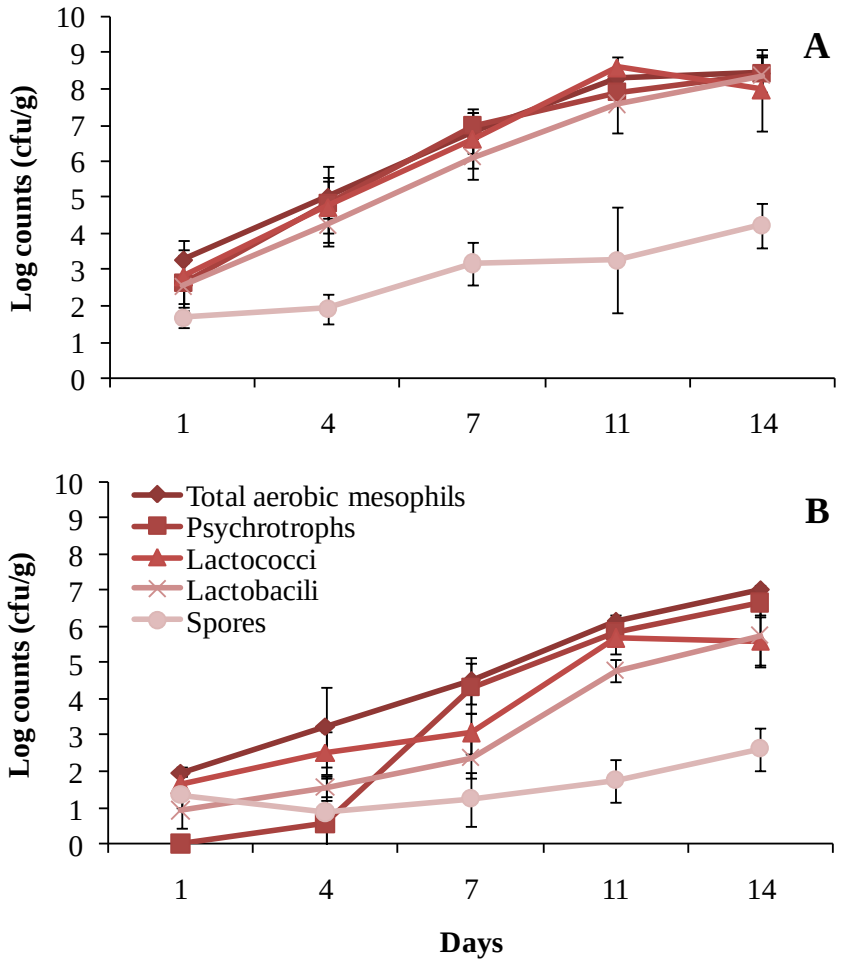

Figure 12. Microbiological counts of control (A) and HP-treated (500 MPa) (B) cheeses stored at 8°C

In this work, after using the DMFit tool for modelling aerobic mesophilic bacteria growth estimation of cheese shelf-life at 4°C was 8 and 19 days for control and pressurised cheese, respectively; while at 8°C the results of the shelf-life estimation for the cheese treated by HPP was 10 days and control cheeses became unsuitable for consumption after 5 days.

5.2.3 Texture

Uniaxial compression is a standard and simple test to evaluate texture in cheese. O'Callaghan & Guinee (2004) suggested that fracture stress describes strength of cheese matrix, fracture strain describes longness of cheese, and elastic modulus describes elasticity.

HPP induced significant ($p<0.05$) textural changes immediately after its application (Table 12). Pressurised cheeses were more resistance to deformation (higher modulus values), less fracturable and deformable (higher and lower numerical values of stress and strain, respectively) than control cheeses. Similar results were reported by Juan et al. (2008) at day 1 in ewe milk cheeses treated at 300 MPa. In addition, Capellas et al. (2001) also observed a lower fracturability at day 1 in Mató cheese (goat milk) treated at 500 MPa. Regarding cheeses treated by HPP, all textural parameters were affected by storage time. Pressurised cheeses became less deformable and less fracturable during storage. Creamer & Olson, (1982) described fracture strain decreases during ripening in Cheddar cheese, which was attributed to the loss of elastic structural elements and to the decrease in the amount of water available for solvation of protein. Saldo et al. (2000) proposed that textural changes induced by HPP are related to changes in calcium-caseinate complex. It seems to be that Ca-casein associations disrupt under high pressure, and although the equilibrium is re-established, the association between caseins is not the same as it was before pressurization. In addition, Juan et al. (2008) found in ewe milk cheeses treated at 300 MPa that HPP induces changes in the cheese protein network, forming a new more compact cheese structure. This could also explain the microstructural changes observed in pressurised cheeses. Stiffness

Table 12. Texture of control and HP-treated cheeses stored at 4°C

	Day	*Treatment*	
		Control	*500 MPa*
Modulus (×10 kPa)	1	2.20 ± 0.73^{b}	2.94 ± 0.90^{aC}
	7	2.55 ± 0.32	2.63 ± 0.34^{C}
	14		3.37 ± 0.55^{B}
	21		3.99 ± 0.41^{A}
Stress ($\sigma_{(t)}$; ×10 kPa)	1	1.15 ± 0.31^{b}	1.83 ± 0.49^{aAB}
	7	1.30 ± 0.12	1.38 ± 0.18^{C}
	14		1.68 ± 0.25^{B}
	21		1.91 ± 0.17^{A}
Strain (ε)	1	0.73 ± 0.10^{aA}	0.54 ± 0.25^{bA}
	7	0.69 ± 0.59^{aB}	0.47 ± 0.04^{bB}
	14		0.48 ± 0.04^{B}
	21		0.49 ± 0.08^{B}

Values represented by mean ± standard deviation.
[a, b] Different superscript in the same row indicates significant differences ($p<0.05$).
[A, B, C] Different superscript in the same column indicates significant differences ($p<0.05$).

5.2.4 Microstructure

Confocal scanning laser microscopy (CLSM) studies of cheese have produced invaluable information on their microstructure related to composition and processing (Blonk & Van Aalst, 1993). CSLM showed visual differences for the protein matrix and fat between pressurised and unpressurised cheeses (Figure 13). The appearance of protein matrix (stained in green) in control cheese was a sponge-like structure, which contained several fat globules and cavities of different shape and size distributed throughout the protein matrix. The microstructure of cheeses treated by HPP appeared with a uniform and continuous protein matrix with only few and small holes. These results agreed with those reported by other authors (Torres-Mora et al., 1996; Capellas et al., 1997; O'Reilly et al., 2001; Serrano et al., 2004) in different cheese matrices, where a more continuous protein matrix was described in pressurised cheeses. In addition, Koca et al. (2011) also reported a dense and continuous casein structure with few holes in white-brined cheeses treated at 200 and 400 MPa for 5 and 15 min. On the contrary, Capellas et al. (2001) did not observe any differences in Mató cheese treated at 500 MPa (5 min, 25°C); the authors

attributed these results in this high-moisture cheese variety to the slight pressure applied to cheeses during cheese-making before high-pressure treatment, and therefore the homogenization of the protein structure was not observed.

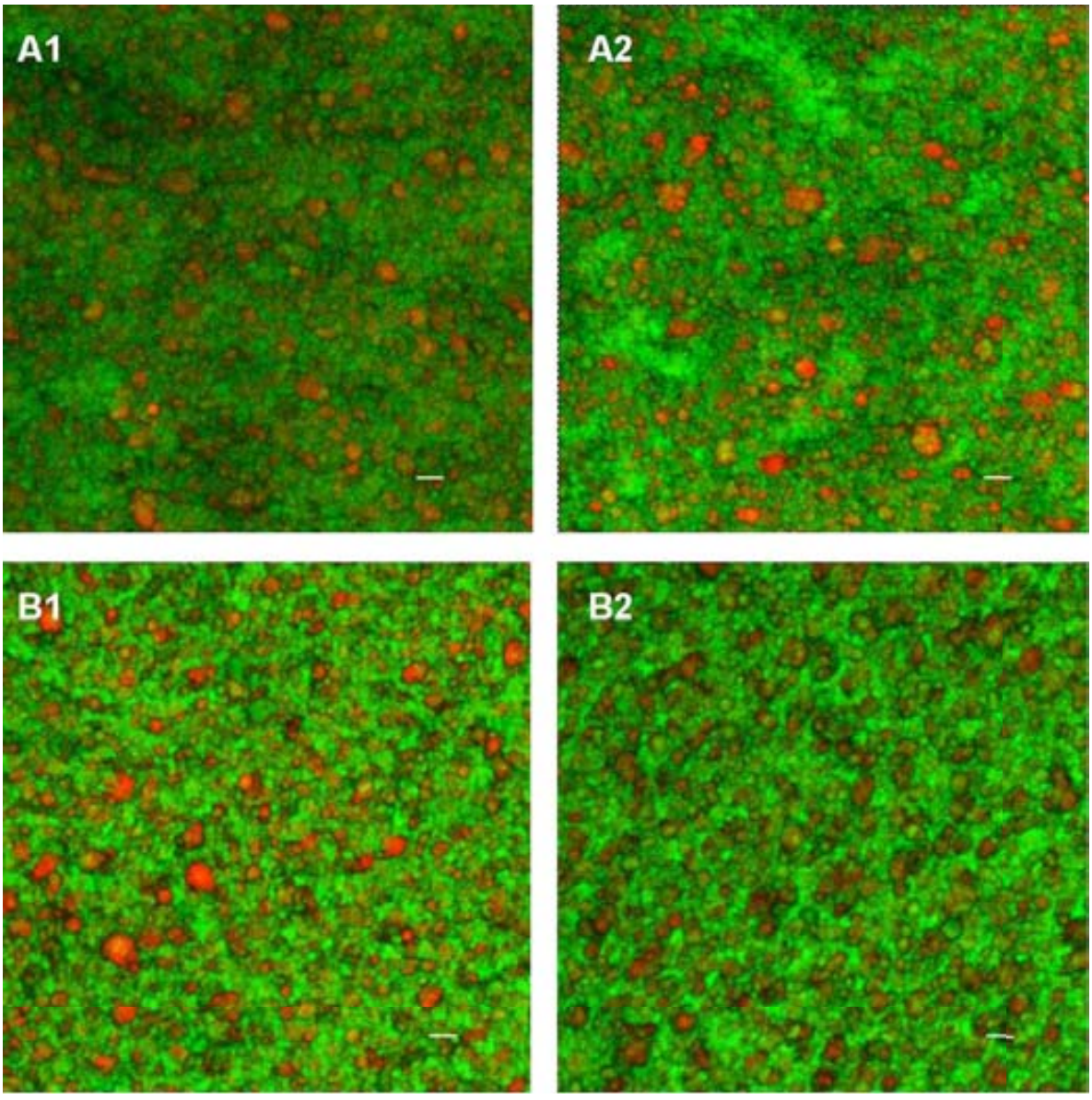

Figure 13. CLSM of control (A1, A2) and pressurised fresh cheeses (B1, B2) from different batches at day 4. Images obtained using 63 × objective lens. The Nile blue stained fat appears red and stained protein appears green in these images. The scale bars are 20 µm in length.

5.2.5 Colour

Table 13 shows the changes in colour of cheeses, measured with instrumental methods, during storage at 4°C after HPP. The application of 500 MPa for 5 min resulted in a significant ($p<0.05$) decrease in lightness and an increase in yellowness, for both 1 and 7 days of storage, with respect to control cheeses. Nevertheless, colour parameters were not affected by storage time.

Results for lightness and yellowness agree with those reported by Capellas et al. (2001) for goat milk fresh cheeses treated at 500 MPa for 5-30 min, and by Juan et al. (2008) for ewe milk cheeses pressurised at 300 MPa for 10 min. An increase in yellowness has also been found in other cheese varieties treated by high pressure (Okpala et al., 2010; Koca et al., 2011). Microstructural changes observed and described above (see Figure 13) may also explain the differences found in lightness.

Table 13. Colour of control and HP-treated cheeses stored at 4°C

	Day	*Treatment*	
		Control	*500 MPa*
L*	1	95.26 ± 0.35[a]	94.39 ± 0.37[b]
	7	95.36 ± 0.36[a]	94.39 ± 0.27[b]
	14		94.08 ± 0.78
	21		94.01 ± 0.75
a*	1	–0.66 ± 0.17	–0.76 ± 0.18
	7	–0.70 ± 0.11	–0.78 ± 0.22
	14		–0.79 ± 0.31
	21		–0.84 ± 0.24
b*	1	10.53 ± 0.99[b]	11.36 ± 0.64[a]
	7	10.47 ± 0.44[b]	11.55 ± 1.08[a]
	14		11.77 ± 1.21
	21		11.97 ± 1.22

Values represented by mean ± standard deviation.
[a, b] Different superscript in the same row indicates significant differences ($p<0.05$).

5.2.6 FFA (for methodology see 3.2.7)

Concentrations of free fatty acids (FFA; mg kg^{-1} of cheese) during shelf-life of control and cheeses treated by HPP are presented in Table 14. A total of 10 FFA were found in the cheeses studied. Generally, FFA levels increased during the shelf-life of control cheeses and decreased from day 14 in cheeses treated by HPP. However, the highest concentration of total FFA on days 1 and 7 was observed in pressurised cheeses. High pressure-treated cheeses, presented at day 1, had twice the level of total FFA in comparison to untreated cheeses mainly due to the high levels of C10, C18:1 and C18:2 observed; however, differences in FFA level (lipolysis) between cheeses were not perceived by the panel (see Sensory analysis section). Juan et al. (2007) working with ewe milk cheese treated by HPP also observed higher levels of FFA at day 1 in cheese pressurised at 300 MPa, which was attributed to the action of microbial lipases due to microbial lysis produced by HPP. The main changes caused by HPP application implicated in lipolysis would be inactivation of microorganisms and lysis accompanied by enzyme release, and modification of protein conformation which have repercussions in enzyme activity, accessibility to substrates and modifications in the curd network (O'Reilly et al., 2001).

As it has been previously reported, in cow milk (Collins et al., 2003), palmitic acid (C16:0) was the main FFA for both treated and untreated cheeses. In pressurised cheeses, total FFA decreased from day 14 especially due to the decrease of C8, C10, C18 and C18:1. There is very little information about the concentration of unsaturated fatty acids (C18:1 and C18:2) in fresh cheese. According to Certik & Shimizu (1999) oleic acid (C18:1) is generally desaturated to yield linoleic acid (C18:2), which may be further converted to α-linolenic acid (C18:3) or to conjugated linoleic acid (CLA) whose formation may be attributed to a free radical type oxidation of 18:2 by heat, aging, and protein quality (Ha & Lindsay, 1991). However, we cannot asseverate that the decrease in C18:2 over storage will end in the production of CLA due to this fatty acid was not evaluated in this assay, but we consider that this pathway could explain somehow the decrease of both C18:1 and C18:2 in high pressure-treated cheeses during storage. Nevertheless, the shelf-life increase in pressurised fresh cheeses may lead to the oxidation of certain FFA.

Table 14. Free fatty acid composition (mg kg^{-1} of cheese) of control and HP-treated cheeses

FFA	*Day*	*Treatment*	
		Control	*500 MPa*
C4:0	1	24.94 ± 6.31^{B}	30.18 ± 6.78
	7	41.21 ± 13.35^{A}	33.65 ± 12.46
	14		25.79 ± 5.03
	21		21.99 ± 0.78
C6:0	1	16.20 ± 4.05^{bB}	27.04 ± 9.87^{a}
	7	28.65 ± 5.62^{A}	26.27 ± 9.09
	14		17.56 ± 2.99
	21		18.21 ± 0.85
C8:0	1	9.98 ± 4.04	16.59 ± 4.15^{AB}
	7	15.21 ± 1.62	18.62 ± 6.67^{A}
	14		14.07 ± 4.29^{AB}
	21		10.14 ± 1.84^{B}
C10:0	1	33.58 ± 15.53^{b}	60.56 ± 16.98^{aA}
	7	50.77 ± 6.24	55.98 ± 22.36^{AB}
	14		39.19 ± 14.58^{AB}
	21		31.18 ± 6.28^{B}
C12:0	1	42.35 ± 18.19	61.89 ± 6.73
	7	56.95 ± 11.85	68.45 ± 22.11
	14		59.63 ± 14.46
	21		45.84 ± 6.36
C14:0	1	81.20 ± 37.49	118.50 ± 21.99
	7	89.53 ± 25.32	115.68 ± 41.83

	14		93.08 ± 19.99
	21		81.15 ± 15.38
C16:0	1	300.08. ± 142.25	457.85 ± 112.41
	7	338.19 ± 61.12	467.76 ± 162.73
	14		334.75 ± 65.53
	21		298.36 ± 23.21
C18:0	1	43.58 ± 29.01	35.18 ± 26.29^{A}
	7	34.18 ± 22.72	15.90 ± 6.64^{AB}
	14		N.D.^{B}
	21		N.D.^{B}
C18:1	1	49.43 ± 27.57^{bB}	343.93 ± 114.71^{aB}
	7	208.55 ± 101.87^{bA}	558.71 ± 95.04^{aA}
	14		233.52 ± 63.97^{B}
	21		231.46 ± 55.12^{B}
C18:2	1	72.88 ± 10.94^{bB}	220.24 ± 92.42^{a}
	7	250.67 ± 35.16^{A}	349.11 ± 200.89
	14		145.65 ± 21.72
	21		156.83 ± 40.63
Total FFA	1	674.23 ± 232.55^{bB}	1371.96 ± 270.43^{aB}
	7	1113.91 ± 194.59^{bA}	1710.16 ± 402.79^{aA}
	14		963.24 ± 115.40^{C}
	21		895.17 ± 82.27^{C}

Values represented by mean ± standard deviation.
$^{a,\ b}$ Different superscript in the same row indicates significant differences ($p<0.05$).
$^{A.\ B,\ C}$ Different superscript in the same column indicates significant differences ($p<0.05$).
N.D. = Not detected.

5.2.7 Sensory analysis

Microbiological control of cheese samples was performed to guarantee safety of the product that would be offered to the panellists. Scores for sensory characteristics during storage of pressurised cheeses are shown in Table 15. Panellists observed minimal differences (±1) in colour, pasty and watery attributes between freshly made control and pressurised cheeses, while flavour, aroma, elasticity and off-flavour parameters remained unchanged. An increase in firmness in high pressure-treated cheeses, described as a noticeable difference (+2), was reported by the panel. In general, as we reported for instrumental analysis for colour and firmness, pressurised cheeses were more yellow and firmer than the reference cheeses. The results for colour observed by the panel were in agreement with the works of Sandra et al. (2004) and Evert-Arriagada et al. (2012) who also observed an increase in yellowness. Nevertheless, Daryaei et al. (2006) reported that untrained panellists could not distinguish any differences between untreated and pressure-treated fresh lactic curd cheeses when 200 to 600 MPa were applied.

In general, high-pressure treatment of cheese did not affect panellists' preference ($p \geq 0.05$) for treated cheese over the non-treated cheese (Figure 14). Moreover, the preference mean score for the pressurised cheeses stored during 22 days and for the freshly made cheese was the same (mean score of 6.5); this finding is very positive for a possible application of this treatment at commercial level. Similarly, Trujillo et al. (2000) reported that pressurised fresh cheeses (500 MPa, 5-30 min, 25°C) were equally preferred to non-treated cheeses, although HPP caused hardening of the cheese surface.

Overall texture acceptability, taste and colour were the main attributes chosen by the panellists for both reference and pressurised cheeses when they were asked to identify the attributes perceived as a positive characteristic considering a global perception of the product.

Table 15. Sensory scores of cheeses pressurised at 500 MPa

Sensory attribute	*Day*			
	4	***8***	***15***	***22***
Colour	1	1	1	2
Firmness	2	2	2	1
Elasticity	0	1	0	0
Grainy	1	0	1	1
Pasty	-1	-1	-1	-1
Watery	-1	-1	-1	0
Aroma	0	0	0	1
Flavour	0	0	0	0
Off-flavour	0	0	0	0

Scoring scale: 0 = no differences with control; ±1 = minimal differences; ±2 = noticeable differences; ±3 = considerable differences; ±4: very considerable differences.

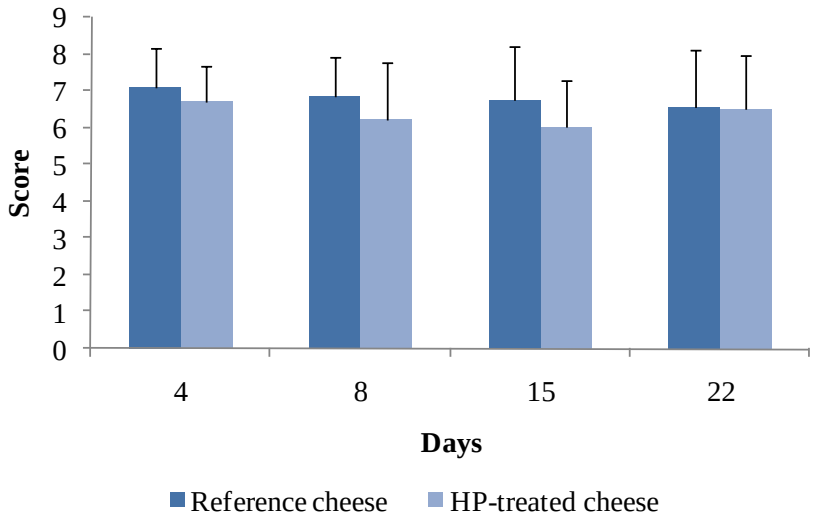

Figure 14. Preference scores during shelf-life of reference and pressurised cheeses.

5.3 Conclusion

An important advance in the commercial applications of HPP of foods over the last twenty years has been carried out. This study demonstrates that HPP under industrial conditions is able to extend the shelf-life of a commercial starter-free fresh cheese up to 19-21 days. Although, pressurised cheeses were firmer and more yellow than control cheeses, these changes did not affect the preference of the panel for pressurised cheese.

These results may lead to a window of opportunities for practical implementation of this technology in the food industry to produce microbiologically safe cheese, with high nutritional and sensory quality, considering that HPP is today more cost-effective than in the past.

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Chapter 6

Effect of HPP on the volatile profile compounds of starter-free fresh cheese

This chapter consists of the paper II

6.1 Introduction

It is well known that HPP is an efficient method to improve cheese quality and achieve microbial safety of food (Patterson, 2005; Rendueles et al., 2011). To date, extensive work has been carried out by several authors on the effect of high pressure on the flavour of different types of ripened cheese (Engels et al., 1997; Carbonell et al., 2002; Juan et al., 2007; Rynne et al., 2008). However, no detailed information is yet available on the effect of the application of this technology on the volatile profile during the shelf-life of starter-free fresh cheese. During cold storage of HP-treated fresh cheese, microbial and biochemical changes (e.g. lipolysis) could lead to a decrease in the quality of fresh cheeses since many of them are undesired (see Chapter 5). The question arises as to whether increasing fresh cheese shelf-life as a consequence of a significant reduction of spoilage microorganisms, will affect its flavour characteristics related to other biochemical reactions.

The aims of this study were to characterise the volatile compounds profile of a commercial starter-free fresh cheese made from pasteurised cow milk and treated by high-pressure (500 MPa, 5 min, 18ºC) under industrial conditions, to study the differences between this profile of volatiles with that of an unpressurised cheese, and to investigate the consequences of increasing the fresh cheese shelf-life by means of HPP on its volatile

compounds profile. For this purpose, extraction of volatile compounds was performed by SPME and identification by GC-MS (see 3.2.9).

6.2 Results and discussion

Forty-nine compounds were identified in the volatile fraction of untreated and high pressure-treated fresh cheese samples during their shelf-life in cold storage. The volatile compounds belonged to eight major groups: ketones, acids, sulphur compounds, terpenes, aldehydes, alcohols, lactones, and miscellaneous compounds. Figure 15 shows the main chemical groups of volatile compounds at day 1. Only minor changes were caused in the volatile fraction by the application of HPP. Ketones were by far the most abundant volatile compounds detected in the headspace of fresh cheeses, and showed a statistically significant decrease after pressurisation ($p<0.05$). On the other hand, aldehydes and alcohol levels were higher in pressurised than in control samples but these differences were not significant.

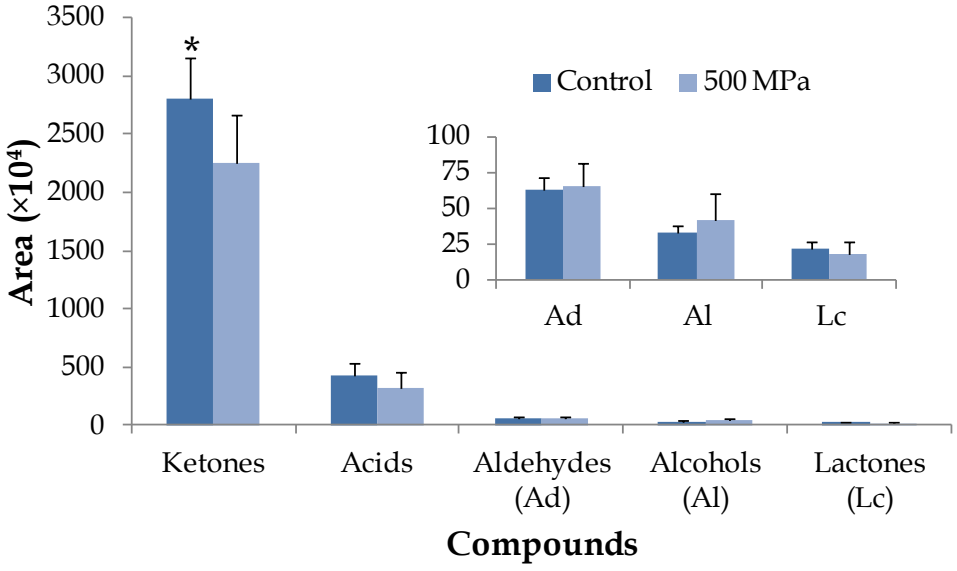

Figure 15. Total volatile compounds (Area x 104) grouped in the main chemical families detected at day 1 in control and pressurised fresh cheeses. * = $p<0.05$

Volatile compounds results are presented in Tables (16 to 24) and expressed as relative abundance of compounds, only at days 1 and 7 due to differences in shelf-life of both control and cheeses treated at 500 MPa. Untreated cheeses exhibited a shelf-life of about one week, while cheeses treated by HP showed a shelf-life close to 21 days. Furthermore, for PCA analyses only those volatile compounds which were significantly affected by HPP treatment and storage time were selected (Figure 16). It is important to point out that only minor changes were caused in the volatile profile by the application of HPP itself, showing a highly stability as the time goes by.

As far as we aware, volatile composition of starter-free fresh cheese has not been studied. However, most of the compounds identified in our study have been previously reported in other types of cheese and milk (Moio et al., 1993; Molimard & Spinnler, 1996; Carbonell et al., 2002; Curioni & Bosset, 2002; Vazquez-Landaverde et al., 2006; Juan et al., 2007).

6.2.3 Acids. Acids have a significant role in aroma development, but are also precursors for other flavour compounds such as methyl ketones, lactones, alcohols and esters (McSweeney & Sousa, 2000).

Seven acids (Table 16) were identified in the cheese samples. Hexanoic and octanoic acids were the most abundant acids in the headspace. However, only octanoic acid was affected by the application of HPP ($p<0.05$). Both acids are mainly produced by lipolysis, but can also be originated from breakdown of amino acids (Urbach, 1993). Nevertheless, as proteolysis is not extensive in this cheese type (short shelf-life and not starter added) these acids most probably originated from lipolysis.

Branched-chain fatty acids are related to an extensive breakdown of proteins (Curioni & Bosset, 2002). Only one branched-chain fatty acid (2-ethyl hexanoic acid) was found in the cheeses studied; this branched-chain fatty acid has been described as an important compound in the Minas cheese flavour with a fruity pleasant note (Corrëa Lelles Nogueira et al., 2005).

Table 16. Abundance (10^4) of Acids detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Butanoic acid	1640	**60**, 88, 73	MS, RI(a)	46.09	44.84	61.22	46.06	NS	NS	NS
Hexanoic acid	1855	**60**, 116, 73	ST, MS, RI(a)	138.23	111.17	151.05	117.49	NS	NS	NS
2-Ethyl hexanoic acid	1958	**88**, 144, 73	ST, MS, RI(b)	1.52	0.79	1.37	1.14	NS	NS	NS
Heptanoic acid	1962	**60**, 130, 73	ST, MS, RI(b)	5.54^{A}	4.11^{B}	5.01	3.91	NS	**	NS
Octanoic acid	2069	**60**, 144, 43	ST, MS, RI(a)	131.94	84.60	125.14	85.16	NS	*	NS
Nonanoic acid	2174	**60**, 158, 57	ST, MS, RI(a)	53.25	36.63	61.61	28.43	NS	NS	NS
Decanoic acid	2276	**60**, 172, 73	MS, RI(a)	55.89	32.55	61.30	26.50	NS	NS	NS
Total				432.46	314.79	466.70^{a}	308.85^{b}	NS	**	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). [A-B] differences between treatments at day 1. [a-b] differences between treatments at day 7. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. (b) Shiratsuchi, Shimoda, Imayoshi, Noda & Osajima, 1994. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; ** $p<0.01$; NS, not significant.

Table 17. Abundance (10^4) of Alcohols detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
2-Butanol	1035	**45**, 74, 59	MS, RI(b)	ND	ND	34.50	5.82	NS	NS	NS
1-Butanol	1160	**56**, 74, 31	ST, MS, RI(a)	ND	ND^{x}	5.48	7.64^{y}	*	NS	NS
3-Methyl 1-butanol	1206	**55**, 88, 70	ST, MS, RI(c)	8.98^{BX}	18.36^{A}	211.62^{aY}	14.36^{b}	*	*	*
1-Hexanol	1368	**56**, 102, 43	ST, MS, RI(a)	5.49	5.64	16.05	4.78	NS	NS	NS
2-Ethyl 1-hexanol	1499	**57**, 130, 82	MS, RI(a)	12.77	13.62	13.88	9.58	NS	NS	NS
1-Octanol	1563	**56**, 130, 55	ST, MS, RI(a)	2.31	3.57^{x}	2.00	1.00^{y}	*	NS	NS
Benzyl alcohol	1903	**79**, 108, 107	ST, MS, RI(a)	4.11^{A}	1.74^{B}	2.41	1.69	NS	*	NS
Total				33.67^{X}	42.92	285.95^{aY}	44.87^{b}	*	*	*

[a] Means within a row with different superscript differ significantly ($p<0.05$). $^{A-B}$ differences between treatments at day 1. $^{a-b}$ differences between treatments at day 7. $^{X-Y}$ differences between days for control cheeses. $^{x-y}$ differences between days for HP cheeses. ND not detected. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) Umano, Nakahara, Shoji & Shibamoto, 1999. (b) Umano, Hagi & Shibamoto, 2002. (c) Umano, Hagi, Nakahara, Shoji & Shibamoto, 1992. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; NS, not significant.

In general, the abundance of acids in control cheeses was higher than in those treated by HPP, although these difference were not significant ($p \geq 0.05$) in most compounds. Only on day 7 were total acids significantly ($p < 0.05$) higher in control cheeses.

6.2.4 Alcohols. Seven alcohols were identified in the cheese samples (Table 17). The synthesis of alcohols is produced by the reduction of their corresponding aldehydes or ketones, in the case of primary or secondary alcohols, respectively (Molimard & Spinnler, 1996). Primary alcohols impart alcoholic, fruity, and sweet notes in cheese (Corrëa Lelles Nogueira et al., 2005). 1-Butanol was not detected at day 1, showing a significant increase from day 1 to 7 in cheeses treated by HPP. On the other hand, levels of 1-octanol decreased by the application of HPP after 7 days of storage. According to Singh et al. (2003) secondary alcohols do not contribute to aroma of certain cheese varieties such as Cheddar cheese. Only one secondary alcohol (2-butanol) was detected in cheeses at day 7.

Branched-chain alcohols are the result of the catabolism of amino acids by the action of lactococci strains, and seem to be important contributors to cheese flavour (Morales et al., 2003). In this study, 3-methyl 1-butanol (isoamyl alcohol) significantly increased in control cheese from day 1 to 7, and also significant differences were observed between treatments; amounts of isoamyl alcohol at day 1 were higher in pressurised cheese, while on day 7, the important increase observed in control cheese, produced a change in this trend, showing higher level in untreated cheese. Isoamyl alcohol was also the major alcohol in cheeses, as in La Serena and Minas cheeses (Carbonell et al., 2002; Corrëa Lelles Nogueira et al., 2005) and it derivates from catabolism of leucine, through conversion of aldehydes (Engels et al., 1997), and confers fruity and alcoholic notes giving a fresh cheese aroma. 2- Ethyl-1-hexanol, another branched-chain alcohol, was found in the headspace, and it was the second most abundant alcohol observed, but its contribution to cheese flavour has not been reported as important (Corrëa Lelles Nogueira et al., 2005). Total alcohol was higher in control cheeses at 7 days due to the contribution of 3-methyl-1-butanol. On the other hand, total volatile alcohol concentration remained constant in pressurised cheeses during storage.

6.2.5 Ketones. Ketones were the largest group of volatile compounds identified in fresh cheese; 11 ketones were identified in the samples analysed (Table 18). These compounds are lipid degradation products and are formed by β-oxidation and their subsequent decarboxylation to methyl-ketones with one less carbon atom (McSweeney & Sousa, 2000). Ketones are primarily recognised to contribute to cheese flavour, being correlated with floral, fruity, and musty and Blue cheese notes (Curioni & Bosset, 2002). Of all the ketones, acetone was the most abundant, and higher levels ($p<0.05$) were observed in untreated cheeses on days 1 and 7. This compound, as well as 2-butanone, probably derives from cow's feed (Gordon & Morgan, 1972). In the present study, the 2-methyl ketones observed were 2-butanone, 2-pentanone, 2-heptanone, 2- octanone and 2-decanone, but only the latter was affected by HPP ($p<0.05$). Acetophenone, which has a floral aroma, was another ketone found in cheeses, and its biogenesis is the result of phenylalanine degradation (Sieber et al., 1995). Diacetyl (2, 3-butanedione) has been described as one of the most important ketones, and identified as a key volatile component of different types of cheeses such as Cheddar, Emmental and Camembert varieties (Curioni & Bosset, 2002) with a buttery and nut-like flavour (Molimard & Spinnler, 1996; Engels et al., 1997). In dairy products, diacetyl and also 3-hydroxy 2-butanone (acetoine) are products of citrate metabolism by lactococci and *Leuconostoc* spp. In a previous study, we observed that, at day 7, lactococci counts were significantly lower ($p<0.05$) in cheeses treated at 400 MPa as compared with control samples (Evert-Arriagada et al., 2012), which could explain the lower levels ($p<0.05$) of 2, 3-butanedione and 3-hydroxy 2-butanone in cheeses treated by high pressure. Moreover, the significant increase of 3-hydroxy 2-butanone during shelf-life of control cheeses could also be explained since acetoine can derivate from diacetyl reduction (McSweeney & Sousa, 2000).

Table 18. Abundance (10^4) of Ketones detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Acetone	822	**43**, 58, 27	ST, MS, RI(a)	1508.37^{A}	1092.22^{B}	1318.69^{a}	1153.66^{b}	NS	*	NS
2-Butanone	908	**43**, 72, 29	MS, RI(b)	865.11	724.61	711.06	820.45	NS	NS	NS
2-Pentanone	984	**43**, 86, 41	ST, MS, RI(c)	277.29	271.05	380.89	303.98	*	NS	NS
2, 3 Butanedione	989	**43**, 86	ST, MS, RI(c)	18.98	11.82	29.01^{a}	14.24^{b}	NS	**	NS
2-Heptanone	1188	**43**, 114, 58	ST, MS, RI(b)	75.92	96.43	132.46	191.34	*	NS	NS
2-Octanone	1293	**58(43)**, 128, 71	MS, RI(b)	2.49	4.41^{x}	3.22^{a}	1.53^{by}	NS	NS	*
Cyclohexanone	1296	**55**, 98, 42	MS, RI(b)	1.83	2.58	2.61	2.94	NS	NS	NS
3-Hydroxy 2-butanone	1298	**45**, 88, 43	ST, MS, RI(a)	2.15^{X}	1.04	23.65^{Ya}	ND^{b}	*	*	*
2-Nonenone	1398	**58**, 142, 43	ST, MS, RI(b)	36.9	29.99	36.4	29.84	NS	NS	NS
2-Decanone	1612	**58**, 156, 43	MS, RI(b)	8.05	5.88	6.79	4.67	NS	*	NS
Acetophenone	1685	**105**, 120, 77	ST, MS, RI(d)	5.72	11.35	7.06	6.02	NS	NS	NS

Total	2802.39^{A}	2251.05^{B}	2651.54	2528.52	NS	*	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). [A-B] differences between treatments at day 1. [a-b] differences between treatments at day 7. [X-Y] differences between days for control cheeses. [x-y] differences between days for HP cheeses. ND not detected. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) Umano, Nakahara, Shoji & Shibamoto, 1999. (b) Umano & Shibamoto, 1987. (c) El-Sayed, 2012. (d) Shiratsuchi et al., 1994. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; ** $p<0.01$; NS, not significant.

6.2.6 Aldehydes. The aldehydes detected in this study are shown in Table 19. These compounds do not accumulate in cheese due to them being transformed to alcohols or to the corresponding acids (Dunn & Lindsay, 1985). Straight-chain aldehydes (e.g. hexanal, octanal) are produced by β-oxidation of unsaturated fatty acids, and are characterised by green-grass like and herbaceous aromas (Moio et al., 1993). Similar total levels of aldehydes for both cheeses were found.

Hexanal, the main aldehyde found in our cheese, does not seem to be affected by HPP, although control cheese experimented a significant reduction from day 1 to 7. Hexanal, has been described to confer a green note of immature fruit, and octanal to contribute with orange notes (Molimard & Spinnler, 1996). However, no octanal was found in cheeses treated by HPP, with a significant effect of this technology being observed ($p<0.01$). On the other hand, benzaldehyde and 2-pentenal were not affected by HPP presenting similar values over time without any significant displacement for both cheeses. Benzaldehyde gives an aromatic note of bitter almond (Molimard & Spinnler, 1996) and may be produced from breakdown of tryptophan or phenylalanine amino acids (McSweeney & Sousa, 2000) as well as from oxidation of phenylacetic acid (Sieber et al., 1995).

6.2.7 Lactones. Lactones play an important role in cheese aroma. They are cyclic esters formed by intramolecular esterification of hydroxy fatty acids (McSweeney & Sousa, 2000). According to Molimard & Spinnler (1996), the forming ring structure occurs by the action of pH, microorganisms or both. On the other hand, Urbach (1995) suggested that the lipase responsible for their production is more likely to originate from the milk itself than from lactic acid bacteria. Nevertheless, it was recently shown that the mechanism of lactone formation in Gouda cheese was a one-step, non-enzymatic reaction, where a hydroxy fatty acid, esterified in a triglyceride undergoes trans-esterification to release the lactone directly (Alewijn et al., 2007).

Principal lactones in cheese are γ- and δ- lactones with 5- and 6- sided rings, respectively (McSweeney & Sousa, 2000). However, in this study only δ-lactones were identified. Tables 20 and 21, show the volatile profile of lactone compounds.

Table 19. Abundance (10^4) of Aldehydes detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Hexanal	1088	**56**, 100, 44	ST, MS, RI(a)	31.11^{X}	33.38	21.92^{Y}	29.77	*	NS	NS
2-Pentenal	1140	**55**, 86, 83	MS, RI(a)	17.59	22.73	16.77	18.06	NS	NS	NS
Octanal	1296	**56 (43)**, 128, 44	ST, MS, RI(a)	5.29^{A}	ND^{B}	2.21	ND	NS	**	NS
Benzaldehyde	1560	**105**, 106, 77	ST, MS, RI(a)	9.91	9.64	7.66	7.06	NS	NS	NS
Total				63.83^{X}	65.75	48.56^{Y}	54.82	*	NS	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). $^{A-B}$ differences between treatments at day 1. $^{X-Y}$ differences between days for control cheeses. ND not detected. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; ** $p<0.01$; NS, not significant.

Table 20. Abundance (10^4) of Lactones detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Butyrolactone	1672	**42**, 86, 56	MS, RI(a)	ND	1.71	1.02	4.45	NS	NS	NS
δ-Hexalactone	1843	**42**, 114, 70	MS, RI(a)	4.72^X	4.60	7.79^Y	6.49	***	NS	NS
δ-Octalactone	2023	**99**, 142, 71	MS, RI(a)	3.25^X	2.57	5.06^Y	4.08	**	NS	NS
δ-Decalactone	2251	**99**, 170, 42	ST, MS, RI(a)	12.06	8.54	17.19	12.29	*	*	NS
δ-Undecalactone	2477	**99**, 184, 43	ST, MS, RI(b)	2.75	1.73	3.43	2.29	*	NS	NS
Total				22.78^X	19.18	34.42^Y	29.53	**	NS	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). $^{X\text{-}Y}$ differences between days for control cheeses. ND not detected. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. (b) Shiratsuchi et al., 1994. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS, not significant.

Table 21. Evolution of Lactones during shelf life of fresh cheeses treated at 500 MPa[a]

				Days			
	***RI*[b]**	***QI*[c]**	***ID*[d]**	***1***	***7***	***14***	***21***
δ-Hexalactone	1843	**42**, 114, 70	MS, RI(a)	4.60[a]	6.49[ab]	9.52[bc]	12.30[c]
δ-Octalactone	2023	**99**, 142, 71	MS, RI(a)	2.57[a]	4.08[ab]	5.89[b]	8.68[c]
δ-Decalactone	2251	**99**, 170, 42	ST, MS, RI(a)	8.54[a]	12.29[ab]	19.42[b]	29.12[c]
δ-Undecalactone	2477	**99**, 184, 43	ST, MS, RI(b)	1.73[a]	2.29[a]	3.75[ab]	5.29[b]

[a] Integrated area counts (10^4). Means within a row with different superscript differ significantly ($p<0.05$). [a-c] differences between days of shelf life. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra. ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. (b) Shiratsuchi et al., 1994.

In Table 20 it can be observed that total and individual lactone levels were significant affected by time in control cheeses, in particular δ-hexalactone ($p<0.001$). On the other hand, Table 21 shows the evolution of lactones during storage of cheese treated by HPP, showing an increase of their levels with time. This fact could be due to the longer product shelf-life that allows the non-enzymatic formation of these compounds, in agreement with the mechanism described by Alewijn et al. (2007).

δ-Decalactone appeared as the main lactone found in fresh cheese, and although its level was higher in control than pressurised cheeses during the first 7 days of storage, these differences were not significant. This compound has been described as one of the most important lactones in cheese (Curioni & Bosset, 2002). The characteristic smell of lactones has been correlated to fruity notes (peach, coconut, apricot). A significant increase ($p<0.05$) of δ-hexalactone and δ-octalactone levels was observed from day 1 to 7 in control cheeses.

6.2.8 Sulphur compounds. Six sulphur compounds were identified in the cheese samples and no differences ($p\geq0.05$) were observed comparing untreated cheeses with those treated at 500 MPa during storage (Table 22). Sulphur compounds are mainly produced by degradation of methionine. The possible pathway suggested for the catabolism of methionine includes the further degradation of methanethiol to dimethyl disulphide (McSweeney & Sousa, 2000). Sulphur amino acid products from their catabolism seem to be implicated as major contributors to the flavour of many cheese varieties (Molimard & Spinnler, 1996; Engels et al., 1997; McSweeney & Sousa, 2000). However, dimethyl sulfone, the major sulphur substance observed in this study, has a very high threshold to be considered an important contributor to milk aroma (Vazquez-Landaverde et al., 2006). Methanethiol and dimethyl disulphide have a strong and unpleasant cabbage, sulphur-like aroma; the study of their importance to flavour has been limited due to their high reactivity and volatility (Vazquez-Landaverde et al., 2006).

Table 22. Abundance (10^4) of Sulphur compounds detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Carbon disulfide	<800	***76***, 32	MS	13.68	32.19	32.49	40.39	NS	NS	NS
Ethanethiol	<800	***62***, 29	ST, MS	10.57	6.85	6.45	5.82	NS	NS	NS
Methanethiol	<800	**47**, 48, 45	MS	2.92	1.69	2.84	1.41	NS	NS	NS
Dimethyl disulphide	1080	***94***, 45	ST, MS, RI(a)	3.46	3.11	4.41	2.94	NS	NS	NS
3-Methylthiophene	1095	**97**, 98, 45	MS, RI(b)	5.54	5.78	9.50	13.96	NS	NS	NS
Dimethyl sulfone	1943	**79**, 94, 63	ST, MS, RI(c)	96.38	82.89	78.60	76.02	NS	NS	NS
Total				132.55	132.53	134.32	140.54	NS	NS	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion (in italic), and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) Shimoda & Shibamoto, 1990. (b) El-Sayed, 2012. (c) Almela, Jordán, Martínez, Sotomayor, Bedia & Bañón, 2010. [e] Statistical significance: T, time; HP, high pressure treatment; NS, not significant.

6.2.9 Terpenes. Presence of terpenes has been attributed to animal feed (Carbonell et al., 2002). Viallon et al. (1999) reported that most abundant terpenes in forages (e.g. limonene, pinene) were also the most abundant in Saint Nectarine-type cheeses. Levels of these compounds are shown in Table 23. No significant differences were observed between control and high pressure-treated cheeses; their content either increased or decreased over time without any significant change. Although the role of terpenes in cheese flavour has not yet been clarified, they seem to have an indirect effect on cheese flavour by modifying the microbial ecosystem due to their antimicrobial action (Martin et al., 2005).

6.2.10 Miscellaneous compounds. Furfural, ethyl acetate, naphthalene, toluene and phenol were also found in fresh cheese (Table 24). Of all these compounds only ethyl acetate was affected by time ($p<0.05$), showing an increase in its level after one week under cold storage in high pressure-treated cheeses. Ethyl esters are responsible for fruity notes in cheese and reduce the sharpness of fatty acids by reducing harsh flavours (Molimard & Spinnler, 1996; Curioni & Bosset, 2002). Esterification occurs when fatty acids and alcohols react (McSweeney & Sousa, 2000).

6.2.11 Principal component analysis

PCA was performed on the data set of cheeses treated by HPP (days 1, 7, 14 and 21) to investigate the relationship between the application of this technology and the development of volatile compounds during storage. The results of PCA analysis showed that storage time influenced the production of volatile compounds in HPP treated cheeses (Figure 16). Among all volatile compounds (49) found in the headspace of cheese samples, only lactones (except butyrolactone), heptanoic acid, and ethyl acetate were investigated by PCA due to their behaviour differing significantly during pressurised cheese shelf-life.

Table 23. Abundance (10^4) of Terpenes detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
α-Pinene	1025	**93**, 136, 91	ST, MS, RI(a)	23.95	23.98	25.29	23.09	NS	NS	NS
Camphene	1068	**93**, 136, 121	ST, MS, RI(a)	1.18	1.32	1.76	1.00	NS	NS	NS
β-Pinene	1097	**93**, 136, 41	MS, RI(a)	31.43	34.89	34.85	29.17	NS	NS	NS
Limonene	1195	**68**, 136, 93	ST, MS, RI(a)	32.55	30.23	35.76	41.31	NS	NS	NS
Total				89.04	90.14	97.39	94.30	NS	NS	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. [e] Statistical significance: T, time; HP, high pressure treatment; NS, not significant.

Table 24. Abundance (10^4) of miscellaneous compounds detected in the headspace of volatile fraction in fresh cheese[a]

				Days						
				1		*7*		*Effects (P)*[e]		
	RI[b]	*QI*[c]	*ID*[d]	*Control*	*500 MPa*	*Control*	*500 MPa*	*T*	*HP*	*T × HP*
Ethylacetate	896	**43**, 88, 29	ST, MS, RI(a)	111.68	95.66[x]	174.52	168.97[y]	*	NS	NS
Toluene	1050	**91**, 92, 65	ST, MS, RI(c)	122.72	150.82	136.27	144.95	NS	NS	NS
Furfural	1491	**95**, 96, 39	MS, RI(a)	1.29	1.51	1.10	1.16	NS	NS	NS
Naphthalene	1791	**128**, 321, 64	MS, RI(b)	16.09	20.55	19.17	9.27	NS	NS	NS
Phenol	2032	***94***, 66	ST, MS, RI(d)	7.22	7.35	6.88	6.59	NS	NS	NS

[a] Means within a row with different superscript differ significantly ($p<0.05$). [x-y] differences between days for HP cheeses. [b] Retention index. [c] Quantification ions. Main ion (in bold), molecular ion, and identification ion. [d] Identification. MS = mass spectra.ST = Positive identification by comparing retention times and MS of authentic standards. RI = retention index. (a) El-Sayed, 2012. (b) Shiratsuchi et al., 1994. (c) Umano & Shibamoto, 1987. (d) Almela et al., 2010. [e] Statistical significance: T, time; HP, high pressure treatment; * $p<0.05$; NS, not significant.

Figure 2 shows that the two first principal components account for 91.87% of the total variability. From this plot we can see that PC1, which explains 70.83% of the total variability, is related to lactone behaviour. Heptanoic acid showed positive loading with PC2, whereas ethyl acetate showed negative loading with this factor. Sampling dates are separated and situated along the PC1 axis, showing four separate groups of points corresponding to the different days of storage. A sequential distribution from the first (1) to the last (21) day of storage, located in the negative and positive area of PC1, respectively. All volatile compounds analysed are situated on the right part of the plot, and they correlated strongly with later sampling dates (days 14 and 21), suggesting storage time had a big influence on volatile production.

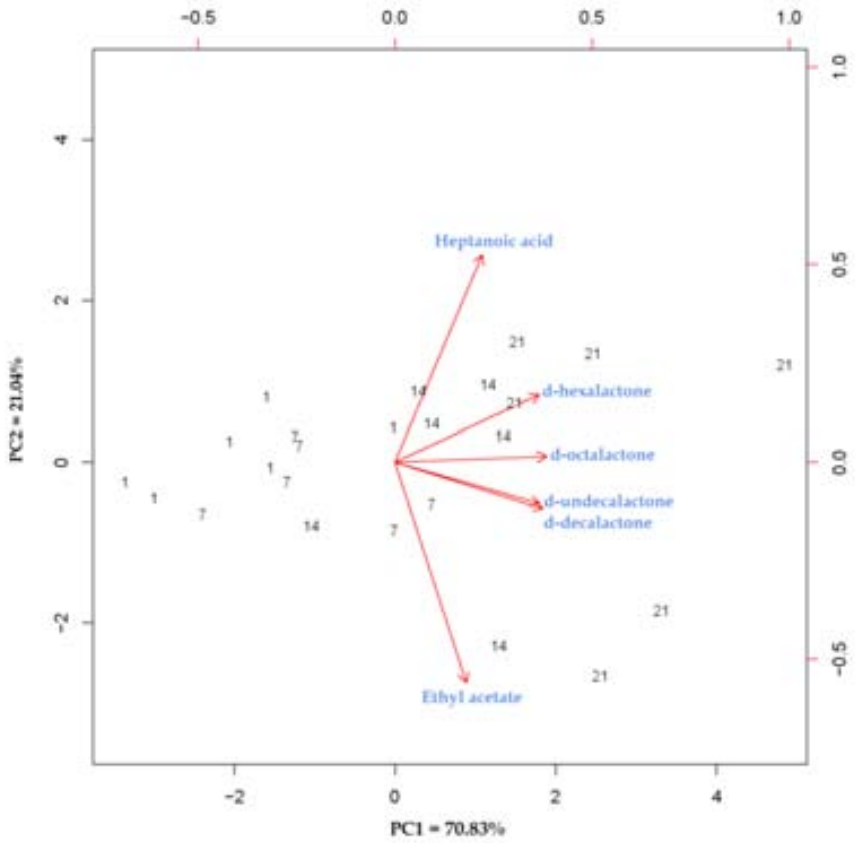

Figure 16. Principal component analysis of the volatile compounds during the shelf-life of cheeses treated by HPP. Compounds loading are shown as vectors.

6.3 Conclusion

High pressure processing (500 MPa for 5 min) of fresh cheeses, produced and treated in industrial conditions, did not seem to affect markedly the volatile fraction when stored at 4°C. The increase in shelf-life of pressurised cheese (from 7 to 21 days) did not change significantly its volatile compounds profile, leading to a delay in the formation of volatile compounds responsible for off-flavours. Fewer compounds, mainly lactones, and other compounds such as isoamyl alcohol, 2-octanone, acetoine, heptanoic acid, and ethyl acetate changed in their levels after processing, or during shelf-life, compared to control cheeses. In conclusion, HPP is a useful tool, able to maintain aroma characteristics of cheese while extending its shelf-life.

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Chapter 7

Fate of Listeria spp. inoculated in a commercial starter-free fresh cheese by means of HPP

This chapter consists of the paper IV

7.1 Introduction

L. monocytogenes is of major concern to the food industry and several outbreaks of listeriosis have been often related to the consumption of dairy products, including pasteurised products, and products that have long shelf-life at refrigerating temperatures (Linnan et al., 1988; Lundén et al, 2004; Swaminathan & Gerner-Smidt, 2007; Ryser, 2007; Koch et al., 2010). The main source of food product contamination with *L. monocytogenes* might be cross contamination, which occurs in the environment of the processing plants. The pathogen may re-contaminate milk cheese subsequent to heat treatment from processing environment or food-contact surfaces, as well as during cheese making steps (especially during molding and curd pressing), and packaging. In addition, studies have shown that *L. monocytogenes* is able to survive for prolonged periods in food production plants through biofilm production (Chmielewski & Frank, 2003; Lado & Yousef, 2007; Swaminathan & Gerner-Smidt; 2007; Cruz & Fletcher, 2012).

Several reports have documented the efficacy of HPP to inactivate both pathogenic and spoilage microbes (Patterson et al., 1995; O'Reilly et al., 2000; Trujillo et al., 2002). Therefore, the reduction of pathogens from packed fresh cheese could be a potential application of HPP to assure food safety.

The main aims of this study were:

(1) To evaluate the inactivation and generation of sublethal injury of three strains of *Listeria* spp. inoculated into commercial starter-free fresh cheese treated by HPP.
(2) To study the fate of inoculated *Listeria* strains during 15 days of storage at 4°C.
(3) To investigate potential differences in behaviour among the *Listeria* strains used.

The methodology applied was as follow: selection and inoculation of *Listeria* strains (see 3.3.1 and 3.2.2), selection of HPP conditions (see 3.2.3) and microbiological analysis (see 3.2.4).

7.2 Results and discussion

7.2.1 Inactivation of *Listeria* strains

Considering that contamination by *L. monocytogenes* during cheese production it is unlikely to occur at high level as 6 or more log cfu g^{-1} and since the Institute of Food Technologists (Heldman and Newsome, 2003) has recommended using lower loads of bacteria to test the efficacy of the treatments. In this study, a more realistic load of approximately 3 log cfu g^{-1}, as starting inocula, were inoculated to simulate environmental contamination of the cheese during manufacturing and to confirm the real efficiency of the treatment in cheese samples. However, to be able to compare our results with previous studies and to evaluate the lethality caused by HPP, we also used a high load of *L. monocytogenes* of ~6 log cfu g^{-1} of cheese.

As expected, the fate of *L. innocua* and *L. monocytogenes* in fresh cheeses was affected by the pressure level applied (Figures 17 and 18). Different behaviours were observed among *Listeria* strains, being *L. monocytogenes* CECT 4031 more barosensitive than the other strains, in particular when 6 log cfu g^{-1} were inoculated. Counts below quantification limit (1 log) were only observed for *L innocua* and *L. monocytogenes* CECT 4031 when 500 MPa and 600 MPa were applied and 3 log cfu g^{-1} were inoculated. For cheeses inoculated with 6 log cfu g^{-1}, the treatment performed at 500 MPa was not able to inactivate completely *L*

innocua, observing a reduction of ~3.5 log cfu g^{-1}. On the other hand, *L. monocytogenes* Scott A was the most pressure resistant strain; only 1.5 log cfu g^{-1} of the pathogen was inactivated at 500 MPa for both loads. Better results were observed for 600 MPa, reaching an inactivation of 2.9 and 4.3 log cfu g^{-1} for low and high inocula levels, respectively. Many authors have shown that HPP is able to reach great inactivation of *Listeria* spp. (Patterson, 1995; Simpson & Gilmour, 1997; Szczawinski et al., 1997; Chen & Hoover, 2004; López-Pedemonte et al., 2007; Linton et al., 2008; Jofré et al., 2010; Hnosko et al., 2012). In fact, López-Pedemonte et al. (2007) who studied the effect of HPP (300, 400 or 500 MPa at 5 or 20°C) on the survival of *L. monocytogenes* in model cheese system inoculated with approximately 7.5 log cfu g^{-1} observed a reduction about 5-6 log cfu g^{-1} after 500 MPa treatment; however, for 300 and 400 MPa reduction obtained was approximately 0.7 log cfu g^{-1}.

Despite the significant reduction of counts, total inactivation was not achieved since *L. monocytogenes* was able to grow after the incubation (37°C) of the initial dilution in buffer peptone water for 24 h. A recent study on Queso Fresco cheese made from pasteurised milk (Hnosko et al., 2012) has shown that, although greater than 5 log reductions of *L. innocua* occurred when 500, 550, or 600 MPa for 15, 3, or 1 min were applied, the inactivation was not complete. They attributed these results due to the fat composition of the cheese used, since a baroprotective effect of fat content had been described elsewhere (Gervilla et al., 2000; Kheadr et al., 2002). In this sense, Gervilla et al. (2000) found in inoculated ewe milk that *L. innocua* showed greater pressure resistance as the fat content increased from 0% to 50%. Nevertheless, the protective effect of fat on foodborne pathogens subjected to HPP remains unclear since dissimilar results have been reported (Gervilla et al., 2000; Kheadr et al., 2002; Mor-Mur & Yuste, 2005; Escriu & Mor-Mur, 2009).

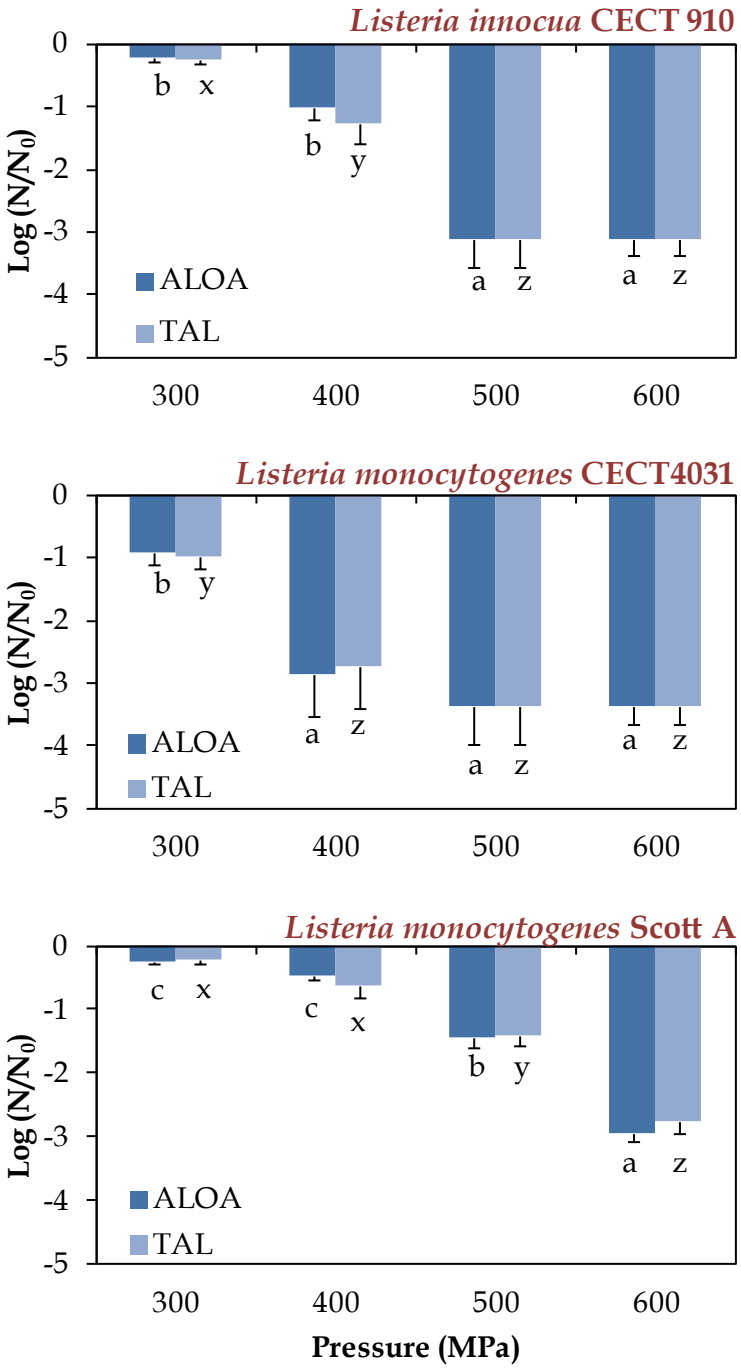

Figure 17. Lethality of *Listeria* strains after pressurisation of fresh cheeses inoculated at an initial load of ~3 log cfu g^{-1}. For each pressure, bar means with different letters (a, b, c for ALOA and x, y, z for TAL) differ significantly ($p<0.05$).

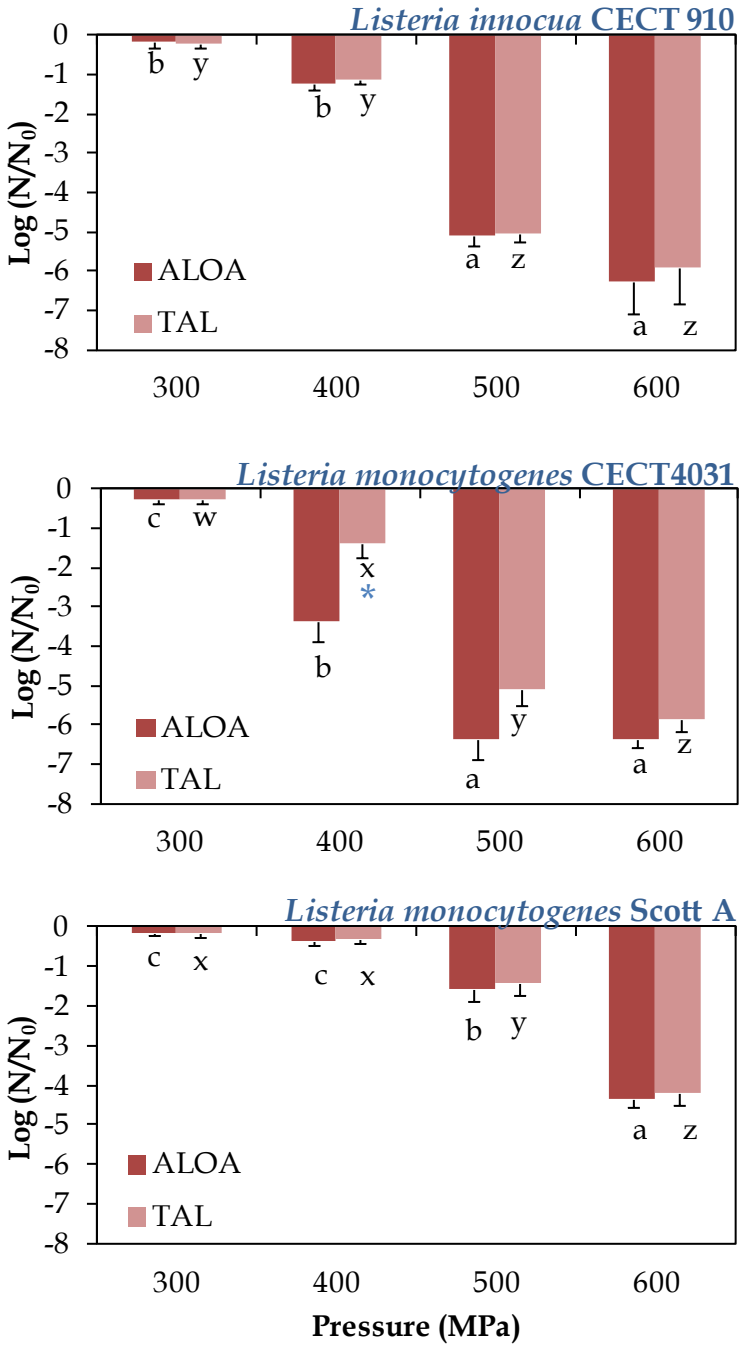

Figure 18. Lethality of *Listeria* strains after pressurisation of fresh cheeses inoculated at an initial load of ~6 log cfu g^{-1}. For each pressure, bar means with different letters (a, b, c for ALOA and w, x, y, z for TAL) differ significantly ($p<0.05$). Differences ($p<0.05$) between ALOA and TAL are represented by *

7.2.2 Sublethal injury of *Listeria* strains by High Pressure Processing

In order to evaluate sublethal injury caused by high-pressure treatment, the thin agar layer (TAL) method was used. TAL method consists on selective medium (ALOA) overlaid with non-selective medium (TSAYE); injured cells will grow on the non-selective layer while the selective medium agents spread to the top layer. Thus, the microorganism is able to perform most reactions that normally does on selective medium (Kang & Fung, 1999). Recovery of injured cells is an important issue due to the number of cells of a food-borne pathogen might be underestimated if injured cells cannot repair and grow, especially in food samples, where background microbiota interfere with the counts onto non-selective media, and therefore is not a useful tool to assess sublethal injury. In previous studies carried out with *L. monocytogenes* strains in different food matrixes exposed to different stressful conditions, such as heat, freeze, acid and pressure injured, significant value of TAL method recovering injured cells were observed (Wu & Fung, 2001; Chang et al., 2003; Yuste et al., 2004; López-Pedemonte et al., 2007; Miller et al., 2010). However, in the present study no significant differences were observed between ALOA and TAL methods in cheeses inoculated with 3 log cfu g^{-1} (Figure 17). On the other hand, when 6 log cfu g^{-1} inoculum was used (Figure 18) a different behaviour was observed in cheeses inoculated with *L. monocytogenes* CECT 4031 and treated at 400 MPa. It seems that TAL was effective to recover injured cells (~ 2 log cfu g^{-1}) of this strain. This difference could be attributed to difference in sensitivity to HPP among *Listeria* strains. Similar results were reported by López-Pedemonte et al. (2007) in model cheeses inoculated with *L. monocytogenes* Scott A and NCTC 11994 strains and pressurised at 20ºC. These authors observed higher amount of injured cells for *L. monocytogenes* NCTC 11994 when 300 MPa were applied. In any case, sublethally injured cells were able to recover and develop during the storage at 4°C of pressurised cheeses.

7.2.3 Evolution of *Listeria monocytogenes* and *L. innocua* during cold storage

In order to evaluate the number of viable cells that might remain and grow in fresh cheese after the application of HPP, the evolution of *Listeria* strains during 15 days of storage at

4ºC was monitored. Different behaviour was observed among the three strains of *Listeria* tested (Figures 19 and 20). Populations of *L. innocua* and *L. monocytogenes* CECT 4031 into non treated commercial fresh cheese gradually increased during the storage period, and reached final counts about 5.5 and 6.6 log cfu g^{-1}, respectively at 15 days of storage. Counts on days 1 and 15 were not significantly ($p \geq 0.05$) different between control and cheeses treated at 300 MPa, suggesting that this pressure level was inefficient to inhibit the growth of *Listeria* strains and therefore changes on its physiological state were not significant. However, when 3 log cfu g^{-1} were inoculated, and 500 and 600 MPa were applied both, *L. innocua* and *L. monocytogenes* CECT 4031 were not able to recover, showing not significant differences ($p \geq 0.05$) between these treatments. For low inoculum level, *L. innocua* and *L. monocytogenes* CECT 4031 presented μ max-values of 0.16 and 0.23 log cfu day^{-1}, respectively in cheeses treated at 400 MPa. For high inocula level (ca. 6 log cfu g^{-1}) the maximum rates of growth were 0.06 and 0.14 log cfu day^{-1} under the same pressure level, respectively, indicating that *L. monocytogenes* CECT 4031 recovered faster than the other strain did, although a considerable higher lethality was achieved for this strain (Figures 17 and 18). On the other hand, Scott A strain showed the slower rate of growth, usually less than 0.1 log cfu day^{-1} for all treatments, including untreated samples.

The different behaviour showed by *L. monocytogenes* Scott A, i.e. its population remained stable in samples inoculated at initial load of ~ 3 log cfu g^{-1} during cold storage, could be explained firstly by its particular long lag phase. Several authors have reported lag phase duration (LPD) as long as 8-9 days in different matrices such as broth, milk, buffer, fresh cheese and meat (Buchanan & Klawitter, 1991; Begot et al., 1997; Cornu et al., 2002; Barbosa et al., 1994; Mendoza-Yepes et al., 1999; Uhlich et al., 2006). In addition, the presence of competing microorganisms in control cheese, such as lactococci, lactobacilli, moulds and yeast, psychrotrophs, *Pseudomonas* spp., and spores (see Chapters 4 and 5), might be capable of producing antimicrobial agents that affect this strain growth. Mendoza-Yepes et al. (1999) reported an inhibitory effect of the starter culture Fargo 763 (*Lactococcus lactis* subsp. *diacetylactis*) in fresh cheese (pH 6.5) against a cocktail of three *L. monocytogenes* strains, including Scott A. In that study the pathogen was not able to grow

during a storage period of 22 days at 3 and 7ºC. Moreover, a study carried out in Alentejo cheese (Guerra & Bernardo, 2001) suggested an anti-*Listeria* effect against Scott A strain by *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Staphylococcus* Genera through organic acids and hydrogen peroxide production. Regarding pressurised cheeses, it has been reported the resistance of Scott A strain to HPP (Mussa et al., 1998; Karatzas & Bennik, 2002; Chung et al., 2005; López-Pedemonte, et al. 2007).

In this study, only 600 MPa ($p < 0.05$) were able to reduce initial counts ~ 3 and 4 log cfu g^{-1} for low and high loads, respectively. Since Scott A is a pathogen strain (serotype 4b) and is one of the most extensively *Listeria* strain used in research studies should be important to keep in mind the difference in growth parameters among strains, specially at refrigeration temperatures. The choice of *L. monocytogenes* strains for use in challenge studies may affect the results and conclusions regarding food safety. Greater safety margins will be obtained if the hardiest *L. monocytogenes* strains are used in such studies (Lado & Yousef, 2007).

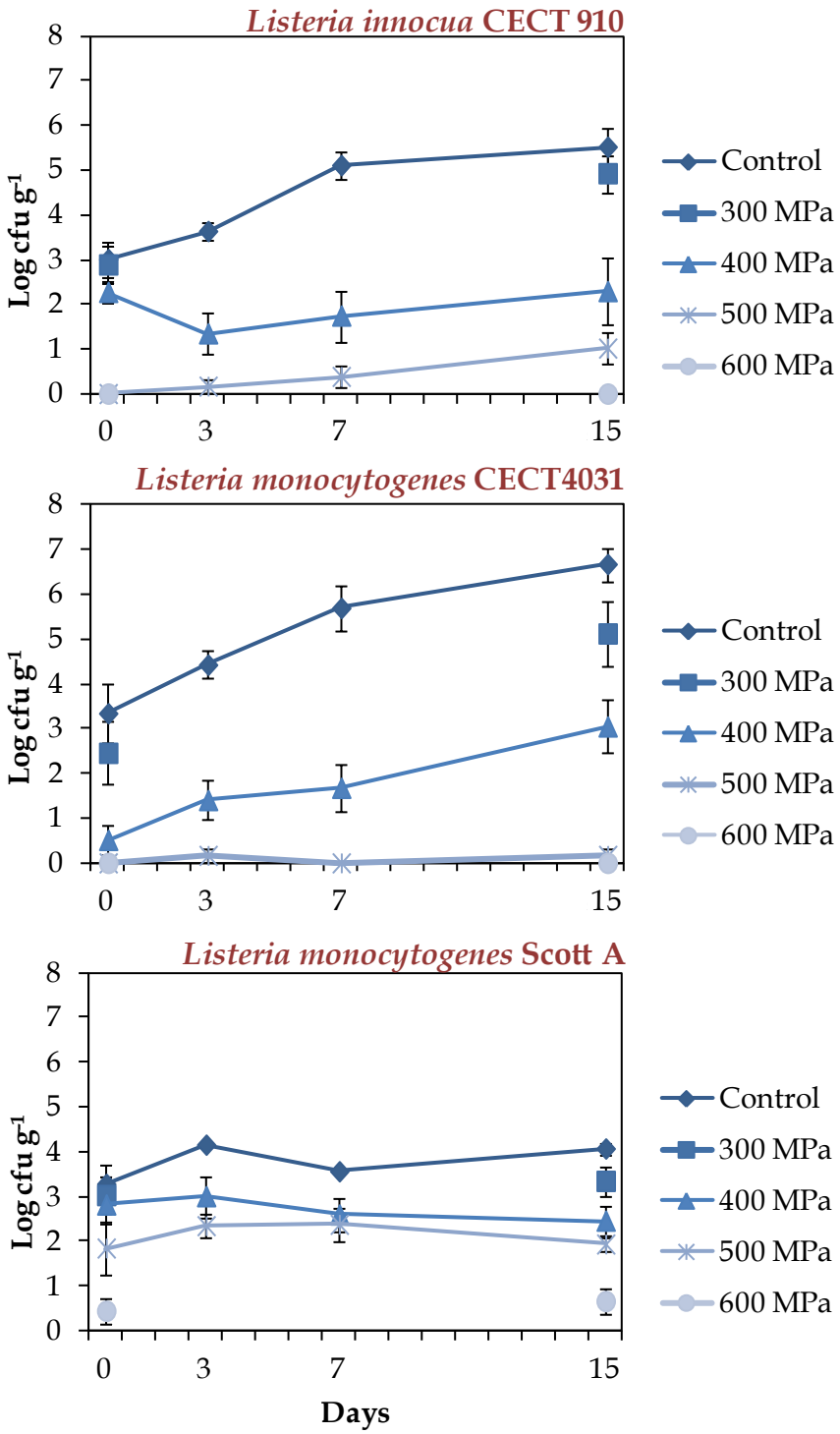

Figure 19. Evolution of *Listeria* strains during storage at 4ºC in fresh cheeses inoculated at an initial load of ~ 3 log cfu g-1 and high pressure treated.

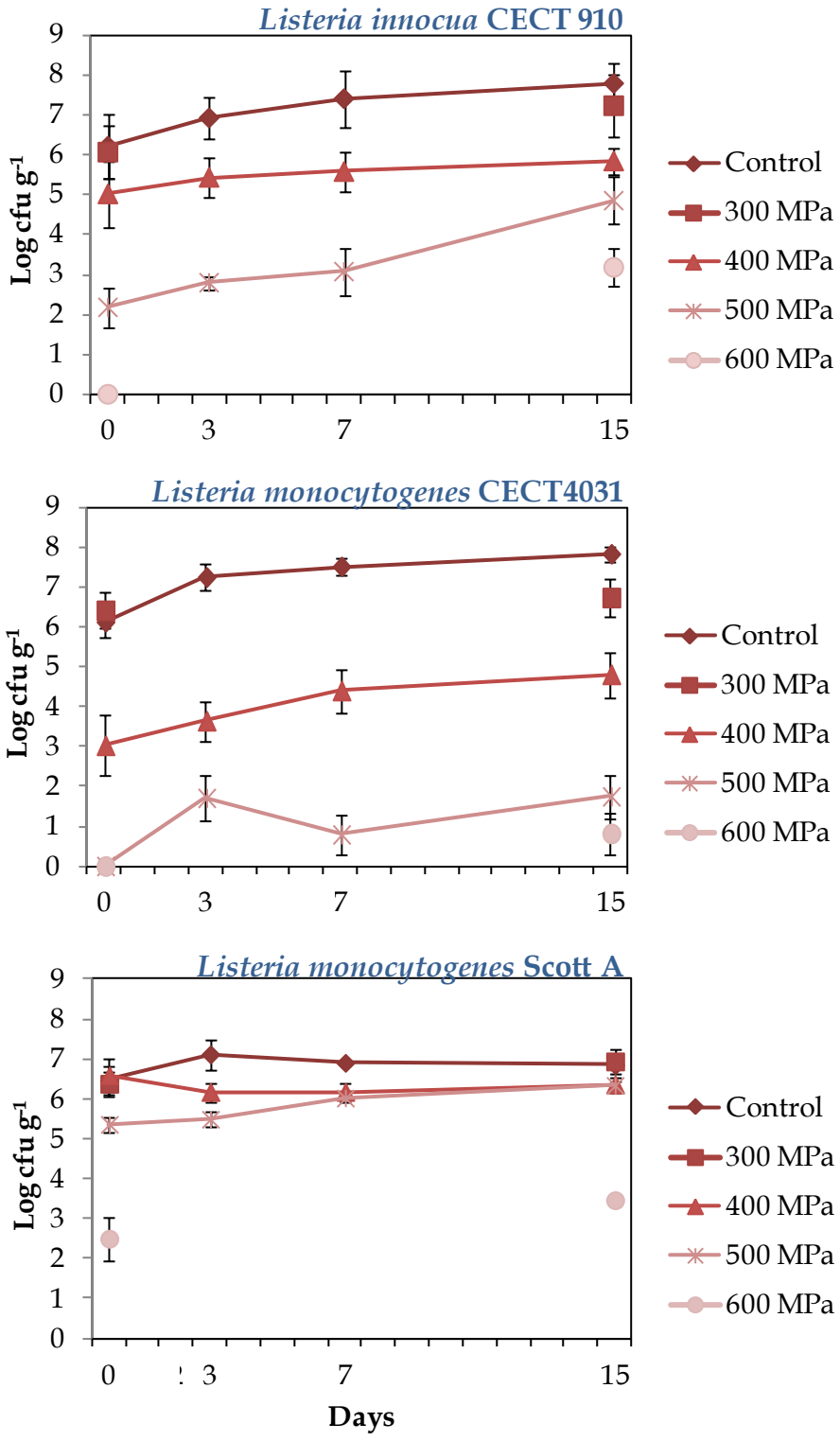

Figure 20. Evolution of *Listeria* strains during storage at 4ºC in fresh cheeses inoculated at an initial load of ~ 6 log cfu g^{-1} and high pressure treated.

7.3 Conclusion

The efficacy of HPP against foodborne pathogens like *L. monocytogenes*, would strongly depend on the pressure level applied and on the strain found in the samples. Treatment at 300 MPa were not enough to achieve this goal in fresh cheese. However, the application of 500 and 600 MPa were effective to achieve significant reductions of pathogen counts but not enough to assure food safety. Counts of *L. monocytogenes* Scott A remained higher than 2 log cfu g^{-1}, level that could present a risk to consumers according to the Commission Regulation on microbiological criteria (Anonymous, 2005).

Growth during storage at 4°C was not prevented since viable cells remain after the treatment, confirming that fresh cheese, due to their characteristics of pH and a_w, is able to support growth of *Listeria* strains.

Different behaviour among strains was observed, being *L. monocytogenes* CECT 4031 the most sensitive to HPP. Therefore, microbial challenge studies of *L. monocytogenes* on fresh cheese that use non-pathogenic strains as target may overestimate the effect of HPP on bacterial cells under real conditions.

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Chapter 8

Final conclusions

To summarize, the main conclusions of this thesis are:

1. High pressure treatment at pilot plant-scale of starter-free fresh cheese (pH 6.72) at 300 and 400 MPa (5 min at 6ºC) is an effective method to extend its shelf-life from 7 to up to 21 days by controlling the growth of contaminating microorganisms such as *Enterobacteriaceae*, psychrotrophs and moulds and yeasts.

2. HP treatment did not affect cheese composition and provoked only little changes in colour and textural properties of fresh cheeses treated at pilot plant-scale, as measured by both instrumental and sensory methods. The sensory attributes of fresh cheese during storage were described as more yellow and firmer.

3. High pressure processing (500 MPa for 5 min) of fresh cheeses, produced and treated in industrial conditions was able to extend the shelf-life of starter-free fresh cheese by up to 19-21 days. The growth of mesophilic bacteria, lactococci, lactobacilli and psychrotrophs was inhibited to some extent, resulting in lower counts at the end of the storage period. Finally, *Enterobacteriaceae*, moulds and yeasts, and *Pseudomonas* spp. were not detected throughout the cold storage.

4. As in pilot plant-scale experiment, colour and texture of fresh cheeses treated in industrial conditions were significantly affected by HPP, observed by both instrumental and sensory analysis. HPP produced minimal differences in

colour, pasty and watery attributes, and for texture, noticeable differences were observed. Such changes could be explained by modifying the cheese matrix as was showed by confocal laser scanning microscopy. Nevertheless, *the preference* of the panel for pressuri*sed cheese* was not affected.

5. Forty nine volatile compounds were identified in starter-free fresh cheeses. HPP did not affect markedly the volatile fraction of fresh cheese when stored at 4°C, maintaining the aroma characteristics of cheese while extending its shelf-life, leading to a delay in the formation of volatile compounds responsible for off-flavours. Only few compounds, mainly lactones, changed in their levels after processing, or during shelf-life, compared to control cheeses.

6. The efficacy of HPP against *L. monocytogenes*, strongly depends on the pressure level applied and on the strain inoculated. Treatment at 300 MPa were not effective to achieve significant reductions of pathogen counts in fresh cheese. However, 500 and 600 MPa were able to reduce noticeably *Listeria* strains counts. Different behaviour among strains was observed, being *L. monocytogenes* CECT 4031 the most sensitive to HPP. Counts of *L. monocytogenes* Scott A remained higher than 2 log cfu g^{-1}, level that could present a risk to consumers according to the Commission Regulation on microbiological criteria.

7. Microbial challenge studies of *L. monocytogenes* on fresh cheese that use non-pathogenic strains as target, such as *L. monocytogenes* CECT 4031, may overestimate the effect of HPP on bacterial cells when more realistic conditions are applied.

8. Finally, the results obtained in this study show that HPP is able to produce fresh cheese with extended shelf-life and good sensory quality when stored at 4°C for 21 days, leading a *window of opportunities* for practical implementation of this technology in the food industry.

Annex

Sensory analysis form

FECHA: NOMBRE DEL CATADOR:

Cátese atentamente las muestras de queso y señálese la valoración que se considere para cada carácter (comparando con la muestra control).
Para la notación de cada atributo, la siguiente escala da la magnitud de la desviación (nota hacia la parte positiva o negativa de la escala según es mayor o menor la desviación apreciada respecto a la muestra C):

Puntos

C desviación no apreciable respecto al Control

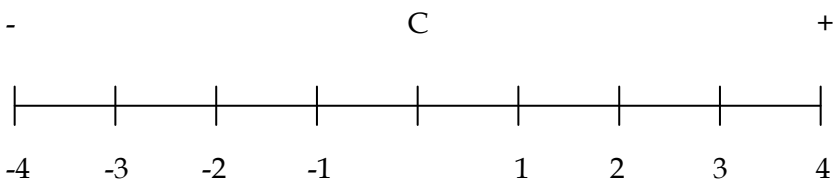
-1 y 1 desviación mínima respecto al Control

-2 y 2 desviación notable respecto al Control

-3 y 3 desviación considerable respecto al Control

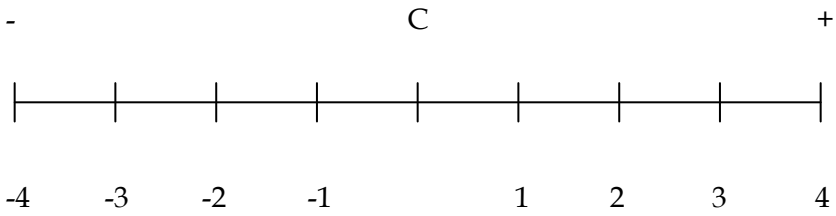
-4 y 4 desviación muy considerable respecto al Control

COLOR (Si nota diferencias, por favor indique amarillo)

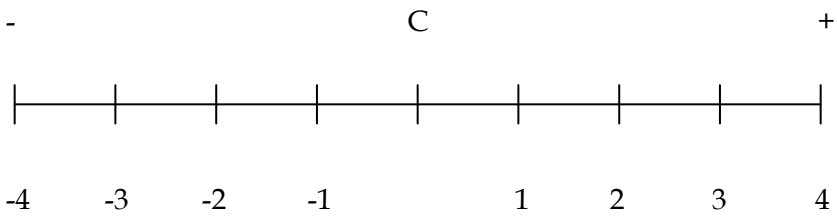


TEXTURA

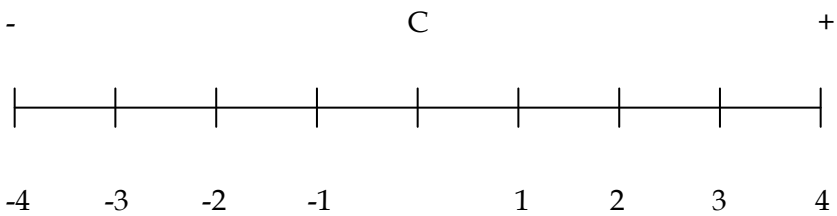
Firmeza



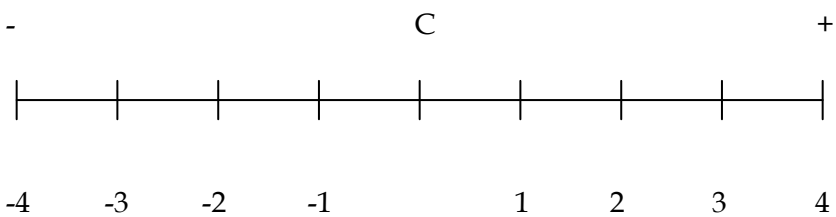
Elasticidad



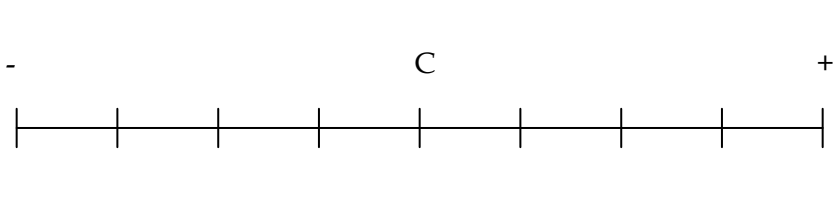
Granulosidad



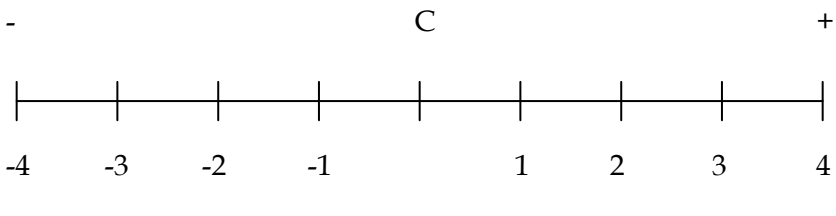
Sensación pastosa



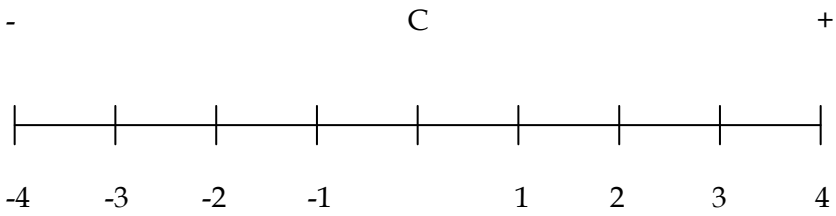
Sensación acuosa



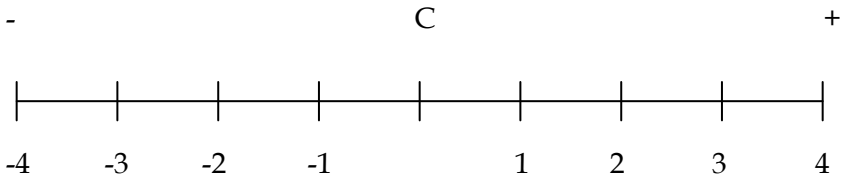
AROMA (intensidad)



FLAVOR (intensidad)



SABORES EXTRAÑOS



Observaciones (por favor describir todo aquello que creáis conveniente de los parámetros o atributos analizados):

PREFERENCIA/ACEPTACIÓN

En la siguiente escala de puntuación marque con el código asociado a cada queso el comentario que mejor describe la muestra que ha probado. Tenga presente que usted es el juez y el único que puede decir lo que le gusta. Nadie sabe si este alimento debe ser considerado bueno, malo o indiferente. La sincera expresión de su valoración personal nos ayudará a decidir.

_____ Me gusta muchísimo (9)

_____ Me gusta mucho (8)

_____ Me gusta moderadamente (7)

_____ Me gusta ligeramente (6)

_____ Ni me gusta ni disgusta (5)

_____ Me desagrada ligeramente (4)

_____ Me desagrada moderadamente (3)

_____ Me desagrada mucho (2)

_____ Me desagrada muchísimo (1)

Describir cuáles son los aspectos (atributos o parámetros) más y menos valorados de cada tipo de queso.

Código queso	**Aspectos mejores**	**Aspectos peores**