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Effect of high hydrostatic pressure processing on biogenic amines formation in artisan caprine and ovine raw milk cheeses

Tesis Doctoral

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HACEN CONSTAR: Que la Licenciada en Química de los Alimentos Diana Maricela Espinosa Pesqueira ha realizado, bajo nuestra dirección, el trabajo titulado **"Efecto de la aplicación de la Alta Presión Hidrostática sobre la formación de aminas biogenas en dos quesos artesanos elaborados de leche cruda de cabra y oveja"** que presenta para optar al grado de Doctor en Ciencia y Tecnología de los Alimentos.

Y para que así conste, firmamos el presente documento en Bellaterra (Cerdanyola del Vallès), el 20 de Diciembre de 2010.

Dra. Ma. Manuela Hernádez Herrero Dr. Artur Xavier Roig Sagués

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AQAEE	Asociación de Queseros Artesanos del Estado Español
ATCC	American Type Culture Collection
AUC	Area under the ROC curve
BA	Biogenic Amines
BP-RPF	Bair Parker agar supplemented with rabbit plasma fibrinogen
CA	Cadaverine
EC	European Community
EC	Control ovine milk cheese
ECF	Ovine milk cheese factory
EHHP1	Ovine milk cheese pressurized between 3dr and 7th day of ripening
EHHP15	Ovine milk cheese pressurized on 15th day of ripening
EU	European Union
FAA	Free amino acids
FN	False Negative
FP	False Positive
GC	Control caprine milk cheese
GCF	Caprine milk cheese factory
GHHP1	Caprine cheese pressurized between 5th and 7th day of ripening
HACCP	Hazard analysis critical control point
HHP	High Hydrostatic Pressure
HI	Histamine
HPLC	High Performance Liquid Chromatography
KF	KF Streptococcus agar
LAB	Lactic acid bacteria

MRS	Man Rogose Sharpe agar
NPV	Negative predictive values
NSLAB	Non starter lactic acid bacteria
PHE	β-pheniletilamine
PPV	Positive predictive values
PU	Putrescine
RH	Relative humidity
ROC	Receiver Operating Characteristic curve
SD	Spermidine
SM	Spermine
TN	Total nitrogen
TNR	True negative
TNBS	Amino group content measure by trinitrobenzensulphonic acid
TPR	True positive
TR	Tryptamine
TY	Tyramine
UAB	Universitat Autònoma de Barcelona
USFDA	The USA Federal Food Drugs Administration
VRBG	violet red bile glucose agar
WSE	Water soluble extracts
WSN	pH 4.6 water-soluble nitrogen

RESUMEN

El objetivo comprendido en esta tesis doctoral fue evaluar el efecto de la aplicación de la Alta Presión Hidrostática (APH) en la formación de Aminas Biogenas (AB) en quesos elaborados a partir de leche cruda. Para este propósito fue necesaria la participación de dos queserías artesanas como proveedoras de las dos variedades de queso elaboradas a partir de leche cruda utilizadas en este estudio, una de leche de oveja y otra de de leche de cabra. La visita a cada una de las queserías se realizó previamente con el fin de conocer las instalaciones, las condiciones de elaboración de los quesos y el grado de cumplimiento de la gestión del autocontrol.

Los quesos fueron ajustados a las dimensiones de la canasta del equipo de APH, envasados al vacío y tratados por APH a 400 MPa de presión, durante 10 min, y 2 ºC de temperatura. Estas condiciones fueron aplicadas a los quesos entre los días 3 y 7 (APH1) y al día 15 (APH15) después de la elaboración. En cada caso se realizaron, durante la maduración, los análisis necesarios para conocer el efecto de cada tratamiento en la microbiología, los parámetros fisicoquímicos, el contenido de AB y las características sensoriales y de textura de los quesos.

Asímismo, se estudió la capacidad formadora de AB en la microbiota presente en cada una de las variedades de queso. Para este propósito se realizó la validación de un método cualitativo para la detección de bacterias con actividad aminoácido decarboxilasa y se evaluó la frecuencia de las diferentes especies y cepas bacterianas encontradas con esta capacidad.

El sistema de higiene y calidad basado en los principios del análisis de peligros y puntos críticos de control (APPCC) observado en cada quesería participante no está propiamente implantado. Sin embargo en ambas queserías se aplica, de diferentes maneras, el programa de pre-requisitos, aunque en algunos casos estos procedimientos y controles no estén por escrito y/o registrados. Los principales puntos débiles encontrados en ambas queserías fueron: El mantenimiento preventivo de instalaciones y equipos; los procedimientos de limpieza y desinfección; y la gestión y eliminación del lactosuero. Adicionalmente, en una de las queserías se observó que el control de la calidad del agua suministrada era inadecuado.

En ambos tipos de queso se observó que la aplicación de APH influyó en el contenido de AB, mostrando una reducción, en comparación con la muestra control, alrededor del 75 y 35% en los quesos con tratamiento APH1 y APH15, respectivamente.La tiramina (TY) y la putrescina (PU) fueron las aminas más afectadas en los quesos elaborados de leche cruda de cabra, mientras que la TY y la cadaverina (CA) lo fueron en los quesos de leche cruda de oveja.

La disminución en la concentración AB en los quesos presurizados al inicio de la maduración pudo deberse a la reducción observada en los recuentos microbianos un día después de la aplicación del tratamiento (principalmente lactococos, lactobacilos, enterococos y enterobacterias), junto con en el descenso del contenido de aminoácidos libres percibido, 34 y 48% menor que el obtenido en los quesos control de leche de cabra y oveja al final de la maduración, respectivamente. Por otro lado, la aplicación del tratamiento APH15 mostró también una reducción significativa en los recuentos microbianos. Sin embargo el contenido de aminoácidos libres observado fue similar a los quesos control.

Los dos medios decarboxilantes, bajo en nitrógeno (LND) y bajo en glucosa (LGD), utilizados en el método cualitativo para la detección de bacterias con actividad aminoácido decarboxilasa mostraron parámetros de diagnostico satisfactorios para la identificación de bacterias con la capacidad de formar PU, CA y TY, siendo su optimo punto de corte fijado entre 10-25 mg L^{-1}, con un área bajo la curva ROC superior al 0,90 y unos valores de sensibilidad y especificidad superiores al 84 y 92%, respectivamente. No obstante, este método mostró menor capacidad en la detección de bacterias con la habilidad de producir HI, siendo considerada, de acuerdo con los parámetros de diagnostico evaluados, como aceptable y deficiente, para los medios LND y LGD, respectivamente.

La mayor actividad aminoácido decarboxilasa observada entre las bacterias aisladas de los quesos de leche de cabra y oveja fue la tirosina decarboxilasa, con una capacidad de producción de TY superior a 100 mg L^{-1}, seguida por la lisina y la ornitina decarboxilasa, con una habilidad de formación de CA y PU en concentraciones de 100-1000 mg L^{-1} y superiores a 1000 mg L^{-1}, respectivamente. Los principales microorganismos productores de TY mostraron ser aquellos pertenecientes a los grupos de *Lactococcus*, *Lactobacillus*, *Enterococcus* y *Leuconostoc*, mientras que lass cepas bacterianas de *Enterobactericeae* y *Staphylococcus* fueron las principales formadoras de CA y PU, aunque algunas cepas de *Enterococcus*, *Lactococcus*, *Leuconostoc*, y *Pediococcus* mostraton tener capacidad de producir diaminas a niveles superiores de 100 mg L^{-1}.

En lo referente a las características sensoriales y de textura, los quesos tratados con APH muestran una reducción de la fracturabilidad. Este hecho pudo ser debido a los cambios en la microestructura causados por la aplicación de APH y, en el caso de los quesos presurizados, al inicio de la maduración posiblemente también a la disminución en la velocidad de proteólisis producida. Por otro lado, en la evaluación sensorial de la textura, las diferencias significativas percibidas fueron únicamente encontradas en la firmeza de los quesos, siendo consideradas las muestras presurizadas como más firmes que los quesos control. La determinación de color

mostró que la diferencia total *(ΔE*) de las muestras tratadas en comparación con los controles fue mayor después de la aplicación de APH (alrededor de 4.4), sin embargo esta diferencia fue disminuyendo a medida que la maduración avanzaba. Asímismo, la diferencia de color percibida por el panel de evaluación sensorial, fue significativa únicamente en las muestras de leche de oveja con tratamiento APH1.

SUMMARY

The aim of this work was to evaluate the effect of the High Hydrostatic Pressure (HHP) on the formation of Biogenic Amines (BA) in raw milk cheeses. For this purpose, two Spanish artisan cheese factories were selected to provide the cheeses to be used in the survey. These factories were previously visited to check the compliance with the hygienic standards required by European Regulations. One variety of cheese was chosen from each factory, both made from raw milk. One of them was made from ovine milk and the other from caprine milk. A HHP treatment of 400 MPa during 10 min at 2 ºC was chosen as the most suitable. These conditions were applied at the begging of the ripening between 3rd and 7th day (HHP1) or on the 15th day (HHP15), in each case the effect of the treatments on the microbial, physicochemical, textural, and sensory parameters, as well as on the formation of different BA were assessed during the maturity period.

The BA forming capacity of the microbiota present in the cheeses was also evaluated. To this purpose a qualitative screening method to detect the amino acid decarboxylase activity of baceria was validated and the frequency of the different species and strains with this capacity was determined in either the ovine and caprine raw milk cheeses studied.

The hygienic quality system based on the HACCP principles of the two visited artisan cheese factories was not strictly stablished. However, the prerequisites program was applied according to their necessities, although in most cases the procedures and results of the controls were not appropriately written or registered. The preventive maintenance of the facilities and equipments, cleaning and sanitation procedures, and the whey waste disposal were the main weaknesses found in both cheese factories. Besides, the water supply control was also unsuitable in one of them.

Biogenic amine content in both types of cheeses was greatly influenced by HHP. The treatments applied on the beginning and on the 15th day of ripening displayed 75 and 35% lower amounts of BA, respectively, than those obtained in the control untreated samples, being TY and PU the most affected amines in caprine milk cheeses, while TY and CA were so in ovine milk cheeses. The BA reduction in the caprine and ovine HHP1-samples can be explained as a result of a significant decrease of microbiological counts observed one day after the HHP-treatment (specially in the lactoccocci, lactobacilli, enteroccocci and enterobacteria groups) and the slower proteolysis detected in these samples, which showed 34 and 48% less free amino acids than the control caprine and ovine milk cheese samples at

the 60th day of ripening, respectively. On the other hand, the HHP treatment applied on the 15th day also resulted in the decrease of microbiological counts, although in this case the liberation of amino acids was not different than the control samples.

The amino acid decarboxylase microplate screening method using low nitrogen decaboxylase (LND) and low glucose decaboxylase (LGD) broths had satisfying diagnostic parameters to detect the PU, CA and TY- forming bacteria, being their optimal cut off between 10-25 mg L^{-1}, with an area under ROC curve above 0.90 and a sensitivity and specificity values above 84 and 92%, respectively. Nevertheless, the test had less capacity to detect the HI-forming bacteria. According to the diagnosis parameters evaluated, this test was considered only as acceptable and poor, for LND snd LGD media, respectively.

Among the isolates obtained from caprine and ovine milk cheeses with amino acid decarboxylase activity, the TY forming bacteria were the most frequent, showing a strong production (exceeding 100 mg L^{-1}), followed by those with CA and PU-forming ability with strong and prolific production (100-1000 and over 1000 mg L^{-1}, respectively). In the first case, strains belonging to the *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Leuconostoc* groups were found as the main TY producers, whereas *Enterobactericeae* and *Staphylococcus* strains were the main PU and/or CA forming bacteria, although some *Enterococcus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* strains showed an ability to produce diamines at levels above 100 mg L^{-1}.

The decelerated rate of proteolysis in HHP1 samples, combined with the structural changes caused by the pressure, could contribute to reduce the fracturability of cheeses. However, this decrease could be only due to the formation of a more homogeneous microstructure in the ovine milk cheeses with the HHP15 treatment. The sensory panel noticed that the firmness in the HHP1 and HHP15 samples was significantly different than in control samples. The total color differences *(ΔE)* in the HHP samples was higher during the first stages of the ripening (around 4.4 in HHP1 and HHP15 samples), decreasing as the sample aged. In addition, differences in color were also perceived by the sensory panel, being significant only in ovine milk cheeses with the HHP1-treatment.

SECTION I

INTRODUCTION

INTRODUCTION

1. CHEESE AND ITS CONSUMPTION IN SPAIN

According to the Codex Alimentarius, (Anonymous, 2007a) 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. 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.

The cheese is a form of milk preservation, which is largely achieved by controlling the pH and water activity (Little et al., 2008). The chemical composition of cheese depends on the composition of the milk and on the manufacture process. Besides, is known that climate, season, animal feed, age and breed, stage of lactation and health state of the animal could influenced the chemical composition of milk (Farkye, 2004). The conversion of milk into cheese curd is carried out by clotting the main proteins of milk, the caseins, at acid pH to form a gel in which the fat is entrapped. When this gel is cut or broken, it separates in cheese curd releasing the whey that contains most of the water, lactose, proteins and minerals that are soluble at cheese-making pH. When curd is drained fat and casein in milk is concentrated between 6-12 fold times (Fox and McSweeney, 2004). This phase depends on diverse factors like temperature, protein amount in the milk, pH, ion calcium content and agitation (Eck, 1990). The method used to clot milk for cheese-making influences the overall structure, characteristics and firmness of cheese (Farkye, 2004), and this process can be induced by: enzymes (animal, vegetable or microbial acid dependent proteases), acidification (chemical or biological lactic acid bacteria) or by increasing the temperature, 80-90º C, approx pH 5.2 (Eck, 1990). Depending on the type of cheese, mold and/or press are practiced, helping to expel whey and regulate moisture. During all these phases the production of lactic acid by lactic acid bacteria (LAB) follows and contributes to acidification of curd.

Curd salting contributes to decrease water activity, control enzymatic activity and microorganism growth, affect biochemical and physicochemical changes and enhance the flavor (Guinee, 2004). The salt content in cheeses is best expressed as salt in moisture, which controls the extent to which starter activity continues after salting. Beyond 6% of salt in moisture the activity of most starter bacteria is inhibited (Farkye, 2004).

Ripening is the phase in which cheese must be held for such time, at such temperature, and under such other conditions as to result in the necessary biochemical and physical changes characterizing the cheese in question (Anonymous, 2007a). In addition, the chemical composition of milk, moisture, salt, pH and the cheese microbiota regulate and control the biochemical changes that occur during the ripening and hence determine the flavor, aroma, and texture of the finished product (Fox and McSweeney, 2004). The main biochemical events occurring during this stage include the glycolysis of residual lactose and catabolism of lactate and citrate, lipolysis and proteolysis (McSweeney, 2004).

Milk and dairy products are some of the principal elements on Spanish diet, representing 17.16% of the food basket. Cheese is the second most consumed dairy product (20.66 %) in this country, with a yearly production of approximately 313000 tons and a consumption per capita of about 6.7 kg per year (2.7 kg ripened or semi ripened cheese, 2.14 kg fresh cheese and 1.9 kg other type) (Anonymous, 2010a). Depending on the kind of milk used, 39.5% of cheeses are manufactured from cow milk, 40% are from mixed milk, 14 % are made from ovine milk, and 7% from caprine milk (Anonymous, 2010c).

In Spain, the geography, climate and environmental diversity in combination with the variability of bovine, ovine and caprine species and socio-cultural aspects have given place to many kinds cheese. The last count performed has found more than 600 types of cheese. However, only 84 types are recognized or cataloged (25 made of capine milk, 19 of bovine milk, 14 of ovine milk and 26 of mixed milks) (Anonymous, 1990; Franco et al., 2001). Only 26 of those cheeses are protected with Protected Designation of Origin (PDO) and one with Protected Geographical Indication (PGI) (Anonymous, 2010b). The increment in consumer demand in the last years increased the presence of products with unique characteristics, principally artisan and/or PDO or PGI products, in many food distribution and promotional channels (supermarket, gourmet stores, restaurants, artisan fairs, informative and professional taste meetings, etc.), and also boosted the research and development of this kind of products. According with the European agricultural statistics (Anonymous, 2009b) in Spain the 75% of the enterprises that manufacture dairy products are small companies with 1-19 persons employed.

The diversity in cheese reflected the variability of tradition and manufacture methods. The majority of artisan cheeses have limited production and some of them are made from raw milk, with great microbial diversity, sometimes with intentional addition of starter culture, and also in some cases natural cave, cellar and/or wooden shelves are used during ripening stage, all of these help to confer their qualitative and sensory unique characteristics. However,

these dairy products, like other traditional fermented foods, are sometimes manufactured under poor or uncontrolled hygiene conditions. In addition, they are produced following different protocols, which can vary from one cheese maker to another. Therefore, particular attention has to be devoted to improve their safety quality production preserving their singular and diversity characteristics (Martuscelli et al., 2005).

2. CHEESE SAFETY

Many cheese-makers used raw milk or add raw milk to the cheese milk considering this essential for assuring the good flavor of the product, primarily due to the greater proteolysis and lipolysis activity of the raw milk microorganisms (Little et al., 2008). However, raw-milk cheeses have often been categorized as a "risky" food products since they have been frequently associated with diseases caused by *Mycobacterium tuberculosis*, *Escherichia coli, Salmonella spp, Brucella melitensis, Staphylococcus aureus, Listeria monocytogenes* and *Campylobacter spp.*(West, 2008). According to the data available in the Community Summary Report on Food-borne Outbreaks in the European Union in 2007 (Anonymous, 2009a), From the total of 2025 verified food-borne outbreaks reported, cheese was involved in 1.7% of them, being, *S. aureus*, *E. coli* , *Bacillus cereus*, *Salmonella spp, L. monocytogenes* and other agents, such as histamine, the main etiologic agents involved.

De Buyser et al., (2001) estimated the proportion of diseases due to milk and dairy products in France and other countries such as USA, Finland, The Netherlands, England, Germany and Poland. They reported that milk and milk products were implicated in 5% of the total bacteria outbreaks reported in France from 1988 to 1997 and in 1-5% of the total bacterial outbreaks in the other six countries. Overall, 37.5% of the food vehicle transmission was made from raw milk, 32.8% from pasteurized milk and the other 29.7% did not provide any type of milk treatment. Among the milk and dairy products confirmed or suspected cheese was the major vehicle implicated (53%). *Salmonella spp., E. coli, L. monocytogenes* and *S. aureus* were the principal etiologic agents responsible of these food-borne outbreaks. However, the occurrence of pathogens such as *L. monocytogenes* is not solely a problem for raw milk cheeses, in cases of cheese made from pasteurized milk, post-contamination is also involved. Little et al., (2008) found in UK that unripened cheeses made from raw milk or thermized milk were microbiological unsatisfactory principally due to *S aureus, E. coli* and *L. monocytogenes*, while in semi-hard types cheeses made from pasteurized milk were mainly due to *S. aureus* and *E. coli*. It has been said that many hard cheeses made from raw milk and aged for 60 days

or longer have an excellent food safety record because of the interaction of factors such as pH, salt content and water activity that render these cheeses microbiologically safe (Donnelly, 2001). Neaves, (2000), indicate that *E. coli* and *Salmonella spp.* die slowly during maturation of hard cheeses and for these products the use of a minimum maturation time forms a requirement of Hazard Analysis Critical Control Point (HACCP) plans. However, it has been described that different pathogens inoculated in raw milk could survive in aging cheese more than 60 days (Kummer, 2000). The current European regulation permits manufacturing cheeses from raw milk with less than 60 days of ripening provided that certain requirements are covered: the milk must come from animals officially free or free from brucellosis and/or tuberculosis, there must be a low count of somatic cells and germs in milk, among other requirements. If the milk does not satisfy the required levels of germs and somatic cells it can be used to manufacture cheese provided that the cheese is aged 60 days as a minimum. In this case, the regulation demands a ripening control, with their corresponding records, to ensure this minimum period (Anonymous, 2004b; Anonymous, 2006).

In an attempt to formalize the procedures for in-factory inspection in the USA, the Federal Food Drugs Administration (USFDA) made an effort to define and delineate their inspection system by adopting the Hazard Analysis Critical Control Point (HACCP) concept (Harrigan and Park, 1991). Nowadays the HACCP has international recognition and it is one of the main guidance devices that set up an effective preventive system which leads to safe 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 manufacture 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, 2009c). These conditions and practices are now considered to be prerequisite for the development and implementation of effective HACCP plans.

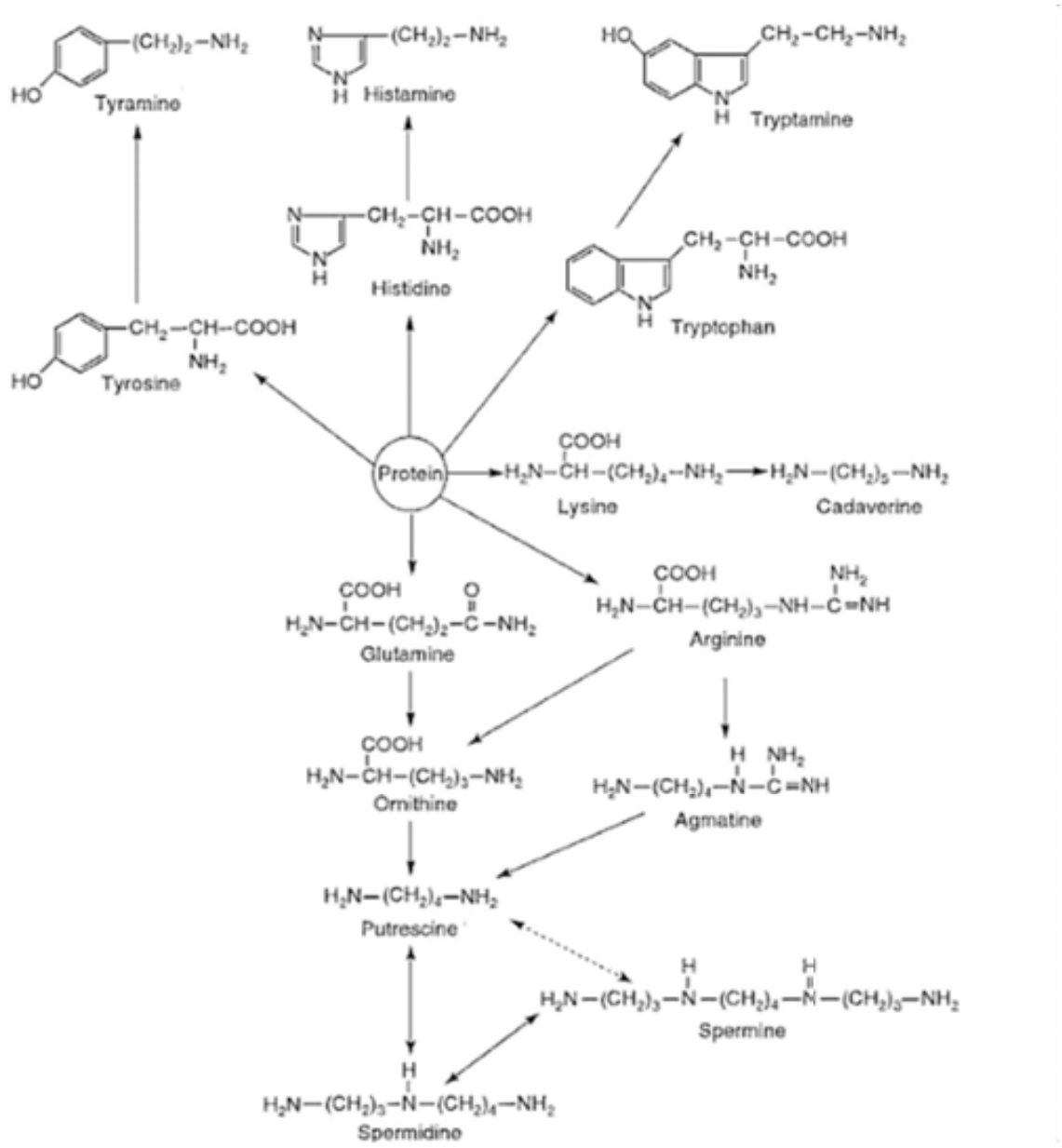
The European Community (*EC*) regulation on the hygiene of foodstuffs established that food-industry operators shall ensure that all stages of the production, processing and distribution of food, under their control, satisfy the general hygiene requirements and shall put in place, implement and maintain a permanent procedure based on the HACCP principles (Anonymous, 2004a). By the other side, the Commission to the Council and the European Parliament reported that most industrial food businesses have HACCP based procedures in place but delays in implementation were noted in former small capacity. Moreover,

difficulties with such procedures were identified in small food businesses and more particularly, in micro retail establishments. Provision of generic systems from outside consultants has been reported in some cases not to reflect the reality of the establishment. Moreover, this commission noted that record-keeping is sometimes perceived as an administrative burden by small food businesses, and that different member states have taken initiatives to simplify this step and minimize the documentation to be kept (Anonymous, 2009d).

3. BIOGENIC AMINES

Biogenic amines (BA) are basic nitrogenous compounds with low molecular weight and according with their chemical structure are classify in aromatic monoamines (tyramine, TY; histamine, HI, β-phenylethylamine, PHE; and tryptamine, TR); aliphatic diamines (putrescine, PU; and cadaverine, CA) and aliphatic polyamines (agmatine, AGM; spermine, SPM; spermidine, SPD) (Bardócz, 1995; Mariné Font, 2005). Histamine, putrescine, cadaverine and tyramine are synthesized by microbial decarboxylation of its amino acid precursors (histidine, ornithine, lysine and tyrosine, respectively). While the spermidine and spermine result from cellular metabolism in living animals and therefore their presence has an endogenous origin (Ten et al., 1990; Halázs et al., 1994; Bardócz, 1995; Silla Santos, 1996) (Figure 1).

Figure 1. Metabolic pathway for the formation of biogenic amines (Halázs et al., 1994)



3.1. Toxicological effects of biogenic amines

Some BA have psychoactive and/or vasoactive properties that may cause food poisoning (histamine, tyramine, β- phenylethylamine and tryptamine) and some others can favor their toxic action (putrescine and cadaverine) (Roig Sagués et al., 1998; Mariné Font, 2005). HI is involved in local immune responses as well as regulating physiological functions (Mariné Font, 2005). However, it is well known the effect related to human toxicity main associated in an intoxication named "Scombroid food poisoning" because of the frequent association of this illness as a result of scombroid fish consumption, such as tuna, bonito, or mackerel (Niven et al., 1981; Behling and Taylor, 1982; Sumner and Taylor, 1989). The symptoms relate to effects on blood vessels and smooth muscles and include skin flushing, headache,

oral burning, abdominal cramps, nausea, diarrhea, palpitations, and, rarely, prostration or loss of vision (Stratton et al., 1991; Bardócz, 1995; Anonymous, 2002b; Jarisch, 2004). This intoxication is characterized by an incubation period ranging from a few minutes to hours (Jarisch, 2004).

TY has been implicated in the toxicity known as "cheese reaction", usually caused by the consumption of cheese (Shalaby, 1996). This aromatic amine show vasoconstrictor action and provoke diverse symptoms such migraine, headache and, increased blood pressure, (Taylor, 1986; Stratton et al., 1991; Anonymous, 2002b).

Under normal circumstances there are intestinal and hepatic enzymes which are capable of metabolizing the BA ingested by oxidative deamination process. These systems include the activity of the enzymes monoaminooxidase (MAO), diaminooxidase (DAO) and poliaminooxidase (PAO) (Stratton et al., 1991; Bardócz, 1995; Holt et al., 2004; Mariné Font, 2005). Health risk commonly appears when these systems are engaged or affected, whether by a genetic deficiency of these enzymes, gastrointestinal illnesses or other factors which boost the BA toxic action, such as MAO inhibitor drugs (IMAO), ethanol, and high levels of diamines (Halázs et al., 1994; Bodmer et al., 1999; Kalac and Abreu Gloria, 2009). The diamines, PU and CA, favor the intestinal absorption of HI and TY by decreasing their metabolic detoxification pathways (Joosten, 1988b; Stratton et al., 1991; Bardócz, 1995).

Table 1. Toxicological risks associated with the consumption of BA in foodstuff (Mariné Font, 2005).

	Histamine poisoning	Migraine	Interaction with MAO inhibitor drugs	Formation of nitrosamine	Other effects
Histamine	+++	++	+++	+	Hepatic toxicity
Tyramine	+	+++	+++	+	Neurologic
β-Pheniletilamine	+	++	+++	+	Neurologic
Triptamine	+	-	+	+	
Putrescine	+	-	-	+	
Cadaverine	+	-	-	+	

(-) No effect (+) Low effect (++) Medium effect (+++) Strong effect

Outbreaks caused by BA are frequent, although not always correctly diagnosed or declared. According to the Community Summary Report on Food-borne Outbreaks in the European Union in 2007 (Anonymous, 2009a), 69 outbreaks caused by histamine were reported, being France (50 cases) and Spain (12 cases) the countries with higher incidence, and fish products, cheese and canned food products the main foodstuff implicated. Likewise, 7 histamine poisoning outbreaks were reported in Catalonia in 2006 (Anonymous, 2007b) and cheese was involved as vehicle of transmission in two of them. The seriousness of BA poisoning depends of the ingested dose and the sensitiveness of the consumer (genetic or acquired) (Roig Sagués et al., 1998; Novella-Rodríguez et al., 2003; Mariné Font, 2005). It has been reported that concentrations between 500-1000 mg kg^{-1} of HI are considered potentially dangerous to human health (Ten et al., 1990) and TY content between 100-125 mg could provoke migraine in susceptible individuals (Hanington, 1967; Millichap and Yee, 2003; Mariné Font, 2005). Likewise, food with TY content over 6 mg kg^{-1} (McCabe, 1986), 4-5 mg of PHE (Mariné Font, 2005), or 20 mg of HI (Vind et al., 1991) would be toxic if it is consumed together with monoamineoxidase inhibitor drugs. On the other hand, threshold values of BA in food have been suggested between 100-800 mg kg^{-1} of TY, 30 mg kg^{-1} of PHE (Ten et al., 1990), HI levels below 500 and 400 mg kg^{-1} in fermented sausages and cheese, respectively (Rauscher-Gabernig et al., 2009) or 100-200 mg kg^{-1} of total BA content (Mariné Font, 2005). Even though, there are not established legal limits on the toxicity of BA in food, and most authors have used as a reference the European Union (EU) and/or the USFDA regulation. According with EU the average content of HI in fish and fish products, specially for certain fish families associated with high contents of this amine, should not exceed a level of 100 mg kg^{-1} and 200 mg kg^{-1} if the fish product is cured and have undergone enzymatic maturation in salt brine (Anonymous, 2005). The USFDA set the HI limits to 50 mg kg^{-1} and recommends the use of microbiological, chemical and sensory indexes to judge fish freshness (Anonymous, 2001). In addition, The Nutritional codex of the Slovak Republic had determined the maximal tolerable limit of HI 20 mg kg^{-1} in beer and 200 mg kg^{-1} in fish and fish products and 200 mg kg^{-1} of TY in cheese (Karovicova and Kohajdova, 2005).

3.2. Conditions allowing the formation of biogenic amines in cheese

Significant BA amounts have been reported in a large variety of food, mainly in those which contain high levels of protein or have been submitted to maturing processes, such as meat, fish, cheese, fermented vegetables, beer and wine (Roig-Sagués et al., 1998; Suzzi and Gardini, 2003; Kalac and Abreu Gloria, 2009; Roig-Sagués et al., 2009). The occurrence of

these amines depends of several factors, the most important are the presence of amino acid decarboxylating microorganisms, availability of substrate (free amino acid precursors), presence of co-factors (pyridoxal 5'- phosphate), and an appropriate microenvironment conditions (pH, water activity, salt amount and temperature) (Stratton et al., 1991; Schneller et al., 1997; Roig-Sagués et al., 1998). Landete Iranzo, (2005), found that 0.025 g L^{-1} of pyridoxal 5'-phosphate increased two fold times the histidine decarboxylase activity of three strains isolated from Spanish wines *Lactobacillus hilgardii* 464, *Pediococcus parvoulus* P270 and *Oenococcus oeni* 4042, and the addition of 1 g L^{-1} of this co-factor raised it in 3 fold times. Some studies have described that acidic environments cause a more strong amino acid decarboxylase activity (Halázs et al., 1994), being the optimum enzyme activity at pH range of 4-7 (Eerola, 1997; Kalac and Abreu Gloria, 2009). Joosten, (1988a) observed that the histamine content in Gouda cheese, made with *Lactobacillus buchneri,* with pH 5.39 at two weeks of ripening was almost twice as the histamine content in the same type of cheese with pH 5.19 after one year. Moreover, the same author described that reducing the salt in moisture ratio results in higher amine content, a fact which is worth considering due to the current tendency to reduce the salt amount in food for health reasons.

Other factors, such as the quality of milk, thermal treatment of milk, addition of starters, time and temperature of ripening and also storage may influence the formation of amines (Joosten, 1988a; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2002a; Novella-Rodríguez et al., 2004b). Microbiological diversity and quality of milk for cheese making depend on hygienic conditions during milking, storage conditions and thermal treatments, thus it has an active contribution to the formation of BA in cheese. Cheese which is elaborated with inadequate hygienic quality contains higher amounts of BA (Joosten, 1988a; Novella-Rodríguez et al., 2004b; Pintado et al., 2008; Marino et al., 2008). Novella-Rodríguez et al., (2004a) observed that the storage of raw milk for 48 h at 4 ºC before cheese making resulted in higher microbial counts and biogenic amine levels compared with the cheese made of fresh milk. The main differences in amine contents between batches were found for PU, HI and PHE, whose levels were more than two fold higher in samples from stored than from fresh milk. The preliminary thermal treatments applied on milk, such as the thermization or pasteurization, could affect the microbiological quality of milk and, consequently, the accumulation of BA in cheese. Generally, heat treatments play a critical role in controlling bacterial counts of processed milk products. The effectiveness of these treatments depend on three main factors: temperature to which milk is raised, length of time during milk is held at this temperature and resistance of the microorganisms of milk to thermal destruction (López-Pedemonte, 2006). Marino et al.,

(2008) observed that the Italian Montasio cheese made from raw milk contained lower amounts of BA than cheeses made from thermized milk (67 ºC, for 20 s). This thermal treatment was considered not enough to significantly reduce the decarboxylase bacteria in cheese. However, when the thermization was applied with the subsequent addition of commercial starter bacteria a wide decrease of BA amounts during ripening was observed. Sumner et al., (1990) found that a HI-forming strain of *L. buchneri* survived after heating at 49 to 80 °C for 10 min, suggesting that this organism would easily survive the normal heating process applied to raw milk used prior to cheese making. Cheeses elaborated from raw milk tend to have more flavor than those made from pasteurized milk, however pasteurized milk cheese usually contain lower amounts of BA. These differences could be due to varied native microorganism in raw milk and to higher proteolytic activity and pyridoxal 5'-phosphate availability in raw milk than in pasteurized milk (Novella-Rodriguez et al., 2003). Novella-Rodríguez et al., (2004b) observed that microbial counts during ripening of cheese elaborated from raw caprine milk were 2-3 log cfu g^{-1} higher than those made from pasteurized milk, specially *Enterobacteriaceae* and enterococci. Consequently, BA content in raw milk cheeses was higher, being TY the main amine found. Likewise Martuscelli et al., (2005) found that Pecorino Abruzzese cheeses, at the end of ripening, elaborated from raw milk without starter culture contained significantly higher amounts of BA than cheeses elaborated from pasteurized milk with added starter. HI was the main amine in the cheeses elaborated from raw milk while in cheeses made from pasteurized milk was PHE Some microorganisms may resist the thermal treatments of milk (thermization or pasteurization) or can contaminate the cheese during the subsequent elaboration procedures if the hygienic conditions are not adequate (Hull et al., 1992).

Wide differences have been reported in the type of BA and in the amounts formed depending on the type of cheese and on the length of the ripening period. Cheeses made from raw milk and with longer ripening periods usually contain more and larger amounts of BA, than less matured cheeses (Ordoñez et al., 1997; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2003). This could be related to the fact that the intensity of proteolysis process during the maturing phase increases the free amino acids level that can be decarboxylated by BA-forming bacteria (Joosten, 1988a; Ordoñez et al., 1997; Durlu-Özkaya et al., 2001). Moreover, BA contents not only vary among cheese types but also within the same type (Roig-Sagués et al., 2002; Kalac and Abreu Gloria, 2009).

In Swiss type cheese, temperatures up to 25 ºC were required during 2-7 weeks of ripening. These conditions favor the histidine-decarboxylase activity (Taylor et al., 1982), while in other kind of cheeses with lower ripening temperatures, such as Cheddar, the amounts of BA usually found are low (Stratton et al., 1991). Díaz-Cinco et al., (1992) found that HI and TY amounts in Chihuahua cheese increased with the time and storage temperature. Pinho et al., (2001) observed that 4 weeks storage at 25 °C of Azeitão cheese promote significantly the contents of TY and PU. Joosten, (1988a) in Gouda cheese, inoculated with *L. buchneri,* observed that the combination of pH 5.39 and storage temperature of 21 ºC resulted in a further increase of HI accumulation.

Pure or mixed starter cultures in fermented and/or mature food products have been used to decrease BA accumulation. The efficiency of this practice depends on microbiological quality of raw material and their characteristics, especially amino acid decarboxylase organisms (Suzzi and Gardini, 2003). Joosten, (1988a) made pasteurized milk Gouda cheeses contaminated with *L. buchneri* (St2A) using six different starters commonly used for Dutch cheese and observed that the choice of these starter cultures has a minor effect on HI formation. Some studies have described that some starter cultures may increase the proteolysis and, in consequence, the amounts of free amino acids available for the active amino acid decarboxylase organisms (Joosten and Northolt, 1989; Eerola et al., 1996; Leuschner et al., 1998). By the other side, some surveys have been found some starter culture strains with decarboxylation activity (Maijala, 1993; Bover-Cid and Holzapfel, 1999; Burdychova and Komprda, 2007). In addition, the great difference in BA forming ability within a group of microorganisms seems to be strain dependent rather than species dependent (Bover-Cid et al., 2001; Novella-Rodriguez et al., 2004a).

3.3. Biogenic amine forming bacteria in cheese

Amino acid decarboxylase activity has been described for several groups of microorganisms, such as enterobacteria, *Pseudomona*s spp, enteroccocci, micrococci and lactic acid bacteria (LAB). These amine-producing organisms may form part of the microbiota of food or may be introduced by contamination before, during or after processing of the product (Silla Santos, 1996; Roig-Sagués et al., 1997a; Hernández-Herrero et al., 1999b; Bover-Cid et al., 2001; Roig-Sagués et al., 2002; Kalac and Abreu Gloria, 2009). LAB have an important role in cheese elaboration, however they are also the most important bacterial group that build-up of BA in cheese (Joosten and Northolt, 1987). Amine forming capacity has been reported for

diverse strains of lactobacilli, lactococci, enterococci and Leuconostoc. (Edwards and Sandine, 1981; Joosten and Núñez, 1996; Roig-Sagués et al., 2002; Pircher et al., 2007). Sumner et al., (1985) isolated a strain of *Lactobacillus buchneri* (strain St2A) from a Swiss cheese involved in an outbreak of HIS-poisoning occurred in USA in 1980 which was able to form high amounts of HIS. Roig-Sagués et al., (2002) isolated strains of *Lactobacillus brevis* and *Lactobacillus casei* subsp. *casei* in Spanish cheeses with tirosine decarboxylase activity and others such as *Lactobacillus delbrueckii* and *Lactococcus lactis* subsp *lactis,* with histidine decarboxylase activity. Likewise, other surveys have also described HI-forming strains of lactobacilli isolated from cheese such as *L. curvatus,* and *L. helveticus* (Burdychova and Komprda, 2007).

Enteroccoci are commonly associated with unhygienic conditions during the production and processing of milk, as well as they could be gain through the water supply. On the other hand these bacteria are important for maturing and developing the aroma and flavor of certain cheeses, especially traditional cheeses produced in the Mediterranean area (Foulquié Moreno et al., 2006). There is evidence that some enterococci strains like *Enterococcus. faecalis*, *Enterococcus. faecium* and *Enterococcus. durans* could produce great amount of TY in food (Leuschner et al., 1999; Galgano et al., 2001; Roig-Sagués et al., 2002; Rea et al., 2004; Pircher et al., 2007; Burdychova and Komprda, 2007; Landete et al., 2007) , and also have been registered as capable to produce PU, CA and/or HI (Tham, 1988; Stratton et al., 1991; Leuschner et al., 1999; Galgano et al., 2001; Pircher et al., 2007). Joosten and Northolt, (1987) suggested that their counts in cheese should reach, at least, 10^7 cfu g^{-1} to increase significantly the amount of TY formed and also PHE. *Enterobacteriaceae* are common contaminants of milk and milk products and numerous species have been mainly related with the accumulation of diamines in cheese, among them *Havnia alvei*, *Escherichia coli*, (Joosten and Northolt, 1987; Pircher et al., 2007), *Klebsiella oxytoca* and *Citrobacter freundii* (Roig-Sagués et al., 2002; Pircher et al., 2007) have been mainly reported as PU and CA producers but also as HI and/ or TY formers depending on the strain. In addition species from other microbiological groups such as *Bacillus macerans* isolated from Italian cheese have been reported with the ability to decarboxylate histidine (Rodríguez-Jerez et al., 1994).

The most important BA reported in cheeses are TY, HI, CA, PU, and PHE, while the amounts of SPD and SPM remain low (Roig-Sagués et al., 1998; Novella-Rodríguez et al., 2003). Aliphatic polyamines are synthesized by vegetable and animal cells in a more complex process and not by microorganism action (Bardócz, 1995; Kalac and Abreu Gloria, 2009). The presence of HI, PU and CA in food is generally considered as non lactic bacterial

contamination, while tyramine is related with a fermentative type of bacteria and spermine and spermidine content are linked with raw material (Mariné Font, 2005). For this reason, it is suggested that BA levels could be indicators of hygienic quality in raw material, manufacturing process and final product (Vale and Gloria, 1997; Roig-Sagués et al., 1998; Anonymous, 2002b; Mariné Font, 2005).

3.4. Methods to evaluate amino acid decarboxylase activity

Diverse qualitative and quantitative methods have been described to evaluate the amino acid decarboxylase activity of microorganisms isolated from different food products. Qualitative methods have been proposed as screening procedures and most are formulated in a basal composition that include sources of carbon (glucose), nitrogen (peptone, yeast or meat extract), vitamins and salt, with BA precursor amino acids and a pH indicator, such as bromocresol purple. Results in these media are based on the response of the pH indicator, to a pH shift when the accumulation of BA raises the pH of the medium (Niven et al., 1981; Marcobal et al., 2006b). To adapt these media to different proposes, diverse modifications have been described and some of them are displayed in the Table 2. However, some reports have described the occurrence of false-positive reactions in synthetic decarboxylase media probably due to the formation of different alkaline compounds that increase the pH of the medium (Rodríguez- Jerez et al., 1994; Roig-Sagués et al., 1997a; Hernández-Herrero et al., 1999b). False-negative responses have also been observed as a result of the fermentative activity of some bacteria, such as lactic acid bacteria, on sugars that reduces the pH of the medium neutralizing the BA influence on it (Joosten and Northolt, 1989; Maijala, 1993; Bover-Cid and Holzapfel, 1999). Although these diverse screening methods have been employed in fish products, cheese and meat, most of them have not reported their validation data.

Quantitative procedures have been proposed by some authors to detect or confirm decarboxylating activity. Sumner and Taylor, (1989) developed an enzymatic method for detecting histamine-producing lactic acid bacteria using leucocrystal violet and diamine oxidase. However, it presented interference from the culture broth. A modification proposed by López-Sabater et al.,(1993) proved to be more simple, fast, and low-cost technique. They developed a broth (NB medium) without dextrose to avoid the interference caused by reducing sugars in this enzymatic method to evaluate HI-forming capacity in bacteria isolated from fish. Other confirming methods proposed were thin-layer chromatography (Tham, 1988;

Guraya and Koehler, 1991) and high-performance liquid chromatography (Bover-Cid and Holzapfel, 1999; Landete et al., 2005; Pircher et al., 2007), being the last method the most frequently used due to its precision and sensitivity. Moreover, recent methods for detection of genes encoding microbial amino acid decarboxylases on polymerase chain reaction (PCR) have been developed (Marcobal et al., 2004; de las Rivas et al., 2008).

Table 2. Basal formula (%) of decarboxylase media according with the literature

	Moeller., (1954)	Niven et al., (1981)	Joosten and Northolt., (1989)	Maijala.,(1993)	Bover-Cid and Holzapfel., (1999)	Landete., et al (2005a)	Landete et al., (2005b)	Pircher et al., (2007)
Tryptone	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
Yeast extract	0.5	0.5	0.5	0.4	0.5	0.4	0.4	0.3
Meat extract	-	-	-	0.8	0.5	0.8	0.8	-
Sodium chloride	0.5	0.5	0.5	-	0.25	-	-	-
Glucose	-	-	0.1	-	0.05	0.15	0.15	0.1
Fructose	-	-	-	-	-	0.1	0.1	-
Tween 80 ®	-	-	0.05	0.05	0.1	0.05	0.05	-
$MgSO_4$	-	-	0.02	0.02	0.02	0.02	0.02	0.1
$MnSO_4$	-	-	0.005	0.005	0.005	0.005	0.005	00.5
$FeSO_4$	-	-	0.004	0.004	0.004	0.004	0.004	-
Ammonium citrate	-	-	-	-	0.2	-	-	-
Thiamine	-	-	-	-	0.001	-	-	-
K_2HPO_4	-	-	-	-	0.2	-	-	-
$CaCO_3$	0.1	0.1	0.01	0.01	0.01	0.01	0.01	-
Pyridoxal 5'-phosphate	0.005	-	-	-	0.005	0.025	0.025	-
Amino acid	0.5–1.0	2.7	2.0	2.0	1.0	0.3	1.2	0.3
L-Histidine	-	*	*	*	*	-	* (2.0)	*
L-Tyrosine	-	-	*	*	*	*	*	*
L-Phenylalanine	-	-	-	-	-	-	*	-
L-Ornithine	-	-	*	-	*	-	*	*
L-Lysine	-	-	*	-	*	-	*	*
L-Tryptophan	-	-	-	-	-	-	*	-
Bromocresol purple	0.001	0.006	0.006	0.006	0.006	-	-	-
Cresol red	0.0005	-	-	-	-	-	-	-
Agar	-	2.0	2.0	2.0	2.0	2.0	-	-
pH	6.0	5.3	5.0	5.3	5.3	5.5	5.2-5.5	6.7 and 5.2
Application	Enterobacteria	Enterobacteria from fish	Lactobacilli from cheese	LAB from meat products	LAB and enterobacteria	LAB from wine	LAB from wine	Bacteria from cheese, meat and fermented sausages

LAB: lactic acid bacteria *: amino acid added * (): amino acid eventually added

4. HIGH HYDROSTATIC PRESSURE (HHP)

New preservation treatments have received considerable attention in the last 30 years in response to consumer demands for more “natural” and “fresh like” foods, but also safety and almost intact nutritional quality. With this aim, new physical non-thermal treatments, such as high hydrostatic pressure (HHP) have been tested in different foods as an alternative to thermal treatments to avoid the presence of undesirable microorganisms, including the amino acid decarboxylating bacteria (Roig Sagues et al., 2009)

Hite, (1899) investigated the effects of HHP on milk and noticed that pressures around 460 or 1400 MPa for 1 h extended its shelf life at 48h or 4 days, respectively, at room temperature. However, in those days, the technical impossibility of working at industrial level reduced interest in such a treatment until ceramic and metallurgical advances allowed for the application of this technology in food during the 70's and 80's. Later, in 1990, the first HHP food product was marketed in Japan and gradually other products have been appearing in the market extending the use of this technology in other countries of Europe and America. Nowadays, there are diverse food products treated by HHP available in the market, like fruit juices; York ham; fruits and vegetables; sauces; desserts; milk products; oysters and guacamole, among others.

The HHP processing is based on two physical principles: a) Principle of Pascal: “The pressure at any point of a fluid is the same in every direction, exerting equal force on equal surfaces”. According to this, HHP acts immediately and it is applied by a transmitter fluid to the product, independently of its size and shape (Knorr, 1993; Smelt et al., 1994; Cheftel, 1995).

b) The principle of Le Chatelier: “If a system at equilibrium is disturbed, this system shifts to counteract the change, reaching a new position of equilibrium”. During HHP processing any phenomenon (transition phase, chemical reaction and change in molecular configuration) accompanied by the decrease in volume will be enhanced. On the other hand HHP can inhibit or retard reactions that lead to a volume increase (Cheftel, 1995).

Many changes are carried out as a result of a volume change caused by pressure. However these changes depend on the treatment intensity; length of the process; pressure build-up and decompression; temperature and food matrix involved among others (Patterson et al., 1995; Smelt, 1998). HHP acts on non-covalent interactive forces which stabilize the structure of biopolymers such proteins and polysaccharides. As a consequence, pressure may induce denaturation, aggregation and gel formation of food macro-components (Heremans, 1995). In the case of enzymes, the HHP processing could result in an activation or inactivation,

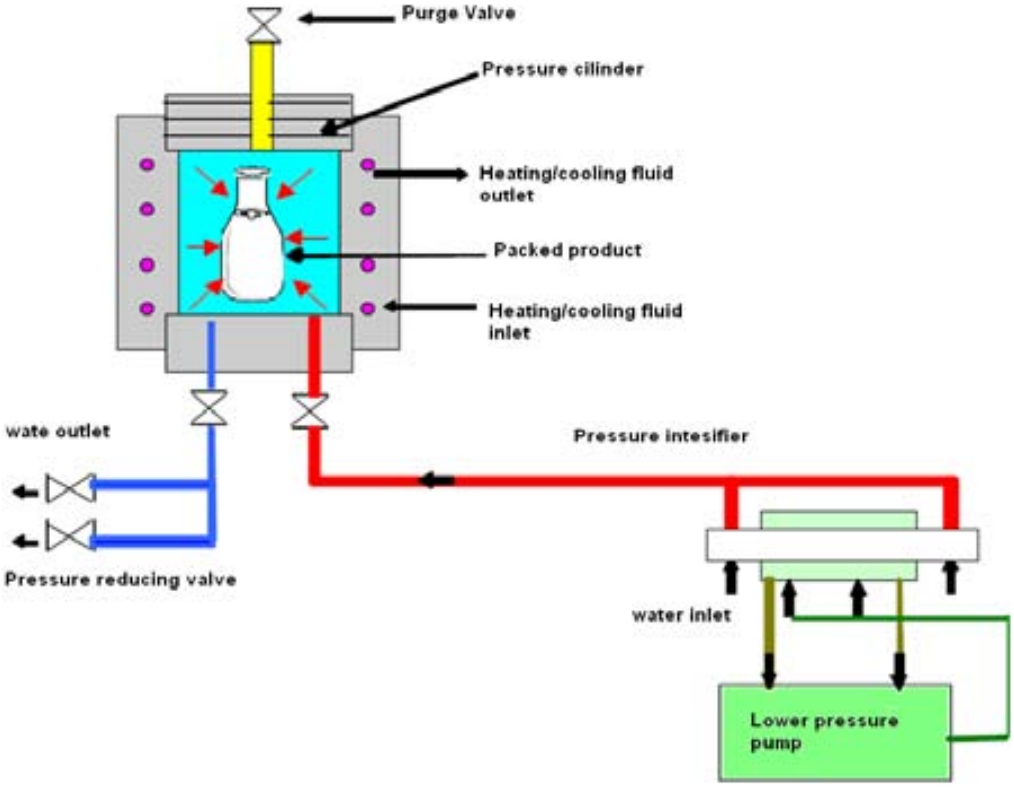
depending of the kind of enzyme and the nature of substrates (Trujillo et al., 1997). HHP affects microorganisms as it induces changes to their morphology, biochemical reactions, genetic mechanisms and cell membranes and walls (Hoover et al., 1989).

4.1. HHP equipments

HHP processing is traditionally a batch process. Industrial pressure vessels have a capacity of 35 to 350 L and reach up to 600-900 MPa, although 1400 MPa pressure vessels with models of 0.5 L have been developed for research purposes (Stanted Fluid Power, Inc). Depending on the product, the treatment is carried out in discontinuous (solid or liquid packed products) or semi-continuous (unpacked liquid products) equipments. In the first case, the food product is put in the pressurization basket 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, pumping is stopped, valves are closed and pressure is maintained without further necessary energy input (Figure 2). Pressure is transmitted rapidly and uniformly throughout the pressure fluid to the food (López-Pedemonte, 2006). 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 the product safety under these conditions. The presence or air pockets reduces the efficacy of treatments, thus, the product has to occupy the entire container (Mertens, 1993).

Figure 2. Diagram of high pressure equipment from Planta Piloto de Tecnología dels Aliments, Universitat Autònoma de Barcelona (by courtesy of ACB-GEC ALSTHOM, Nantes, France)



4.2. HHP on cheese

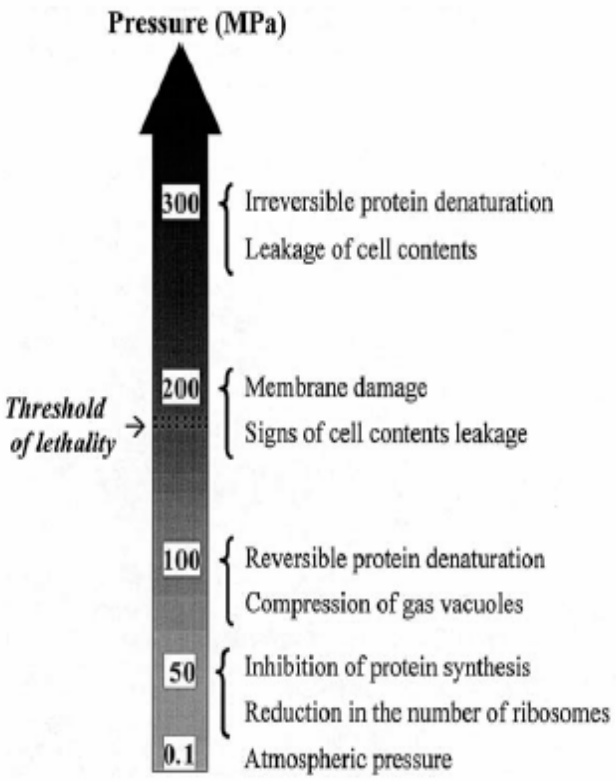
Since high pressure has been pointed out as a technology suitable for food processing, some studies have focused on it for cheese treatment to inactivate or reduce pathogenic and spoilage microorganisms, cheese ripening acceleration or arresting, and reduction of biogenic amines, among others.

4.3. Effect of HHP on microbiological inactivation

The resistance of microorganisms to pressure is very variable, the cells in exponential growing phase are more sensitive than cells in stationary phase and bacterial spores are highly resistant to pressure, more so than yeast or moulds. Vegetative cells are usually inactivate by HHP, however differences in pressure resistance have been observed between bacteria, being Gram negative bacteria generally more sensitive to pressure than Gram positive, and small size coccoid forms are generally more resistant than large rod-shaped bacteria. HHP processing induces changes in the morphology, wall and cell membrane, modifies

biochemical reactions, genetic mechanisms, and interferes with the cellular transcription mechanisms, thus microorganisms are inactivated by failure on their cellular structure and physiological functions (Hoover et al., 1989; Smelt et al., 1994; Cheftel, 1995; Patterson, 2005) (Figure 3). The range of microbial inactivation depends on diverse factors such as HHP conditions (pressure, time, temperature); food constituents and their properties, the type of microorganisms and their physiological state (Patterson et al., 1995; Smelt, 1998).

Figure 3. Structural and functional changes in microorganisms at pressure (Lado and Yousef, 2002)



HHP is a useful tool for the inactivation or reduction of pathogenic and spoilage microorganisms in cheese (O'Reilly et al., 2000; Trujillo et al., 2002b). Casal and Gómez, (1999) studied the effect of HHP (100–400 MPa for 20 min at 20 ºC) on the viability, acidifying and peptidolytic activities of lactococci and lactobacilli isolated from goat's milk cheese. They found that lactoccoci were more sensitive than lactobacilli to pressures of 100 to 350 MPa. Likewise, pressure-treated cells in 10% of reconstituted skim milk exhibited lower acidification rates, even at pressures that did not affect its viability. Reps et al., (1998) reported a significant decrease in the total microbial counts at pressures above 400 MPa in Gouda and Camembert cheeses, while spore counts was unaffected even at 1000 MPa. Kolakowski et al. (1998) found that the decrease in the number of total microbial count was

higher when the same HHP treatment was applied (200, 400 or 600 MPa, for 15 min) on two-weeks than in 6-weeks old Gouda cheese. Similar results have been reported by Juan et al., (2007a) in ovine milk cheeses, observing that the total count decreased 3.6 and 2.3 log units after HHP treatment (400 MPa, 10 min) on 1 and 15 days-old cheeses, respectively. Likewise, Arques et al., (2006) observed that HHP treatments at 400 MPa, for 10 min reduced significantly the total and undesirable microbial counts improving the microbiological quality and safety of "La Serena" cheese. However higher microbial decrease was obtained when the HHP was applied on 2-day old cheese than on 50-day old cheese.

Several studies have reported the efficacy of HHP treatment on pathogenic microoganisms in different cheese varieties. Fresh caprine milk cheeses inoculated with *Escherichia coli* CETC 405 (initial count of 10^8 cfu g^{-1}) were HHP treated by combinations of pressure (400-500 MPa), temperature (2, 10, or 25 ºC) and time (5-15 min) and later stored 60 days, at 2-4 ºC. No detected levels of *E. coli* were found one day after pressurization, except in samples treated at 400-500 MPa for 5 min at 25 ºC, while *E. coli* in control cheeses remained at levels of 10^8 cfu g^{-1}. In addition, no detected levels of this bacterium were found throughout the storage period (Capellas et al., 1996). The inactivation of *Escherichia coli* K-12 and *Staphylococcus aureus* ATCC 6538, inoculated in cheese slurry and cheddar cheese, by HHP treatments of 50 to 800 MPa for 20 min at temperatures of 10, 20, and 30 °C was studied by O'Reilly et al., (2000). The viability of *S. aureus* and *E. coli* was not detected after the application of pressures above 600 MPa at 20 °C and over 400 MPa at 30 °C, respectively, in cheese slurry. The sensitivity of these microorganisms in inoculated Cheddar cheese treated at 100 to 500 MPa for 20 min at 20 °C was higher than in cheese slurry, especially for *E. coli*. Moreover, Linton et al., (2008) reported undetectable levels of *L. monocytogenes* in Camembert type cheese elaborated from raw bovine milk inoculated with this pathogen (2 or 4 log cfu mL^{-1}) and HHP treated (500 MPa for 10 min at 20 °C) during 14 days of ripening, whereas curd and cheese made from inoculated and untreated milk presented higher counts.

HHP treatment might not always completely inactivate microorganisms, rather, it may injure a proportion of the population, and the recovery of the injured cells will depend on the conditions after treatment (Patterson, 2005). The recovery of injured cells could be a serious risk in the case of pathogenic microorganisms and shortens shelf life of HHP processed food. Model cheese inoculated with *L. monocytogenes* NTC 11994 or Scott A (7.5 log cfu g^{-1} of cheese), HHP treated at 400 MPa, for 10 min, at 20 º or 5 ºC, and stored during 30 days at 8 ºC showed sub-lethal injured cells only on days 1 and 2 (around 0.8 log cfu g^{-1}) and a significant reduction of the pathogen strains counts without excessive decrease of starter

counts (López-Pedemonte et al., 2007a). In the same way, De lamo-Castellvi et al.,(2006), in model cheeses elaborated with starter culture, observed a maximum lethality on *E. coli* O59:H21 CECT 405 at 400 and 500 MPa, for 10 min at 20 ºC. The authors suggested that low pH and the presence of starter culture contributed negatively on the survival cells that were not able to repair and grow after 15 days of storage at 8 ºC, remaining at counts below the level of detection.

4.4. Effect of HHP on cheese ripening index

Most rennet cheeses are ripened before consumption to achieve desirable organoleptic qualities. The extent and type of ripening depends on storage time and temperature, cheese composition, especially moisture and salt levels, and the types and activities of rennet, indigenous milk enzymes and micro-organisms present (Farkye and Fox, 1990). Proteolysis is the single most important event during the ripening of most cheese varieties with major impact on flavor and texture (Fox, 1989). 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).

Characterization of proteolysis is most commonly used as an index of maturity. Its procedure involves separating, quantifying and characterizing nitrogenous compounds from cheese during cheese ripening. Nitrogen can be separated into solvent-soluble, and solvent-insoluble components followed by fractionation of the soluble components (Farkye and Fox, 1990). Depending on the type of cheese, and the method used different amounts of nitrogen are extracted within diverse fractions and its amount increases with the ageing of the cheese. Proteolysis products soluble in the aqueous phase, extractable as the water-soluble fraction and commonly quantified by Kjendahl nitrogen assay could include those peptides, proteins, and amino acids of cheese resulting from rennet action, indigenous milk proteases and by lactic starter metabolism. Likewise, the soluble extraction-fraction at pH 4.6 is commonly used as an index of cheese ripening. This procedure at pH values about isoelectric point of casein is used to isolate small and medium size peptides (Rank et al., 1985; McSweeney and Fox, 1999). The components in pH 4.6 soluble fraction is heterogeneous and could be include whey proteins, proteose peptones and a variety of peptones (O'Keeffe et al., 1978).

There are varied methods to quantify proteolysis in cheese. One of them is the determination

of amino group by the reaction with 2,4,6-trinitrobenzensulphonic acid (TNBS), which reacts quantitatively with the α-amino groups at an alkaline pH producing a chromophore which absorbs maximally at 420 nm, enabling detection of all the amino groups released by hydrolysis of peptide bond (Polychroniadou, 1988). However, the disadvantage of the TNBS is that it reacts slowly with the hydroxyl ion, a reaction which is catalyzed by light, and also with ammoniacal nitrogen. Other method is the estimation of the chromophore formed when Cd-ninhidrin reacts with free amino groups. It was found that Cd-ninhydrin reagent was more selective for amino group of free amino acids (FAA) than the amino group of peptides or proteins (McSweeney and Fox, 1999).

Some surveys have indicated that high-pressure may be used to accelerate or arrest cheese ripening. (Kolakowski et al., (1998) found in Camembert cheese a significant increase of proteolysis when HHP treatment for 50 MPa during 4 h was applied. However, when Gouda cheese was subjected to pressures from 50-500 MPa a slight increase was observed. Saldo et al., (2000) noticed that goat's milk cheese HHP-treated at 400 MPa for 5 min exhibited faster proteolysis and higher pH values than untreated cheeses, although bitterness was detected in HHP-treated cheeses. They suggested that HHP accelerates ripening by increasing water retention and pH, by releasing bacterial enzymes, and by increasing enzyme activity under pressure. Most of lactoccoci enzymes are located intra-cellularly (Tan et al., 1992; Law and Haandrikman, 1997) and during cheese ripening starter bacteria die slowly and lyse, suggesting that the release of this intracellular enzymes may have an important role in proteolysis (Law and Haandrikman, 1997; Fox et al., 1999). After applying 50 MPa for 3 days at 25 ºC on two days old Cheddar cheese, O'Reilly et al., (2000) noticed an increase in pH 4.6 soluble nitrogen index and FAA, but this effect decreased with the age of the cheese. They concluded that the improvement of proteolysis may be attributed to a combination of the temperature and pressure used during the treatment. Combined HHP processing has been reported to accelerate the ripening of goat milk cheese. This treatment includes an initial treatment at 400 MPa for 5 min to release microbial enzymes followed by the application of 50 MPa for 72 h, to increase the activity of these enzymes (Saldo et al., 2000). On the contrary, (Messens et al., (1999) indicated that in Gouda cheese the possible lysis of the starter bacterial cells resulting from the damage suffered at 400 MPa did not lead to an influence on pH 4.6 soluble nitrogen, free amino acid content or SDS-PAGE profiles indicating proteolysis due to chymosin, plasmin and proteinases of starter was neither influenced. Casal and Gomez, (1999) observed that pressures 300-400 MPa did not reduced aminopeptidase or dipeptidase activity of *Lactoccoccus lactis* subsp. *lactis* and was partially

inhibited in L*actobacillus casei* subsp. *casei* after 400 MPa-treatment. Wick et al., (2004) found that 1 and 4 month-old Cheddar cheese treated with 400, 500 or 800 MPa for 5 min at 25 ºC, significantly arrested the development rate of FAA, being the most decrease observed at 800 MPa treated samples. Pressures below 400 MPa did not affect FAA level. They suggested that the fewer viable lactic acid bacteria, enzyme inactivation, substrate alteration or a combination of these factors cause HHP decreased proteolysis in these cheeses. Juan et al., (2007a) noticed that water soluble nitrogen in ewe cheese was influenced by the moment that the HHP treatment (200-500 MPa, for 10 min, at12 ºC) was applied; being the values obtained in samples processed at day 15 higher than those treated the 1st day. On the other hand they observed an increase of peptidolytic activity and FAA content when 300 MPa were applied at the first day, suggesting that this HHP treatment favored the lysis of starter bacteria and enhanced the release of intracellular aminopeptidases into the cheese matrix. In the contrast, pressures above 400 MPa seem to delay FAA development, being the lowest amounts of peptides and FAA observed in samples with 500 MPa applied the first day, probably due to the reduction of starter bacteria counts and inactivation of enzymes. These changes in cheese ripening provoked by the HHP treatments depend on various factors such as type and age of cheese, conditions of HHP processing and type and count of starter and no-starter microorganisms.

4.5. Effect of HHP on biogenic amine accumulation in cheese

Previously, it has been described that HHP affects microbial growth, and enzyme inactivation or activation depending on treatment intensity, temperature and length of process, the kind of enzymes, and the nature of substrates. For all these reasons, HHP treatments can influence on BA profiles of food. Some surveys have evaluated the effect of the HHP on the BA accumulation, most of them in seafood and meat, reporting changes on BA profiles including decrease and/or increase in some of these amines. However there are few data about the effect of HHP on biogenic amines in cheese.

Novella-Rodríguez et al., (2002c) observed higher FAA content in cheeses made from HHP treated (500 MPa, for 15 min, at 20 ºC) caprine milk than those elaborated with pasteurized milk, however no significant differences in BA content were observed throughout the ripening. With independence of the applied treatments, they also found differences within batches, resulting on much higher BA content in the first one (1056 and 900 mg kg^{-1} dry matter to HHP-milk cheese and pressurized respectively) than in the second (136 and 129 mg

kg^{-1} dry matter to HHP- milk cheese and pressurized respectively), specially in TY and CA amounts. They suggested that the initial quality of milk could be more responsible of the biogenic amine accumulation in ripened cheeses than the kind of treatment applied to milk. In a later survey, Novella-Rodríguez et al., (2002b) tested three HHP treatments applied to one-day old goat milk cheese. They found that TY was the main amine affected by pressure and its content in goat milk cheese depended on the treatment conditions. The application of 50 MPa for 72 h on cheese resulted in 2.7 times higher TY content than in control samples. On the contrary, the TY amounts decreased significantly when treatments of 400 MPa for 5 min and 400 MPa for 5 min followed by 50 MPa for 72 h were applied, although the higher proteolysis, induced by HHP, was not correlated with higher amine production in cheeses. These differences could be provoked by the incidence of 400 MPa treatments on non starter LAB, whose counts remained lower than control samples throughout the ripening. Espinosa et al., (2006) evaluated the effect of HHP treatments on BA content in a raw milk cheese along the ripening. The application of the HHP treatments on the 3rd day of ripening resulted in a reduction of about 49 % in the total amount of BA, and close to 80 % in the amount of TY and PU. However, HHP treatments applied on the 15th day of ripening hardly influenced the amount of BA formed.

4.6. Effect of HHP on cheese texture and sensory characteristics

The International Organization for Standardization (ISO) defines food texture as “all rheological and structural (geometric and surface) attributes of the product perceptible by means of mechanical, tactile and, where applicable, visual and auditory receptors (Anonymous, 1992).Texture is complex and includes a great amount of characteristics which can be evaluated by instrumental and sensory methods. Uniaxial compression is a standard and simple test to evaluate texture in cheese. The parameters that could be extracted from the force-displacement data obtained from the compression test gives information about the mechanical and fracture properties of cheese at large-scale deformation, which is relevant for comparison with data from sensory texture where cheese is degraded during mastication (Zoon, 1991; Wium and Qvist, 1998; González Viñas et al., 2007). The mechanical properties of cheese are related to cheese composition (moisture, protein, fat, salt and pH), matrix structure, interactions among molecules within the cheese network and their changes during ripening (Visser, 1991; Lucey et al., 2003) Therefore structure and hence the rheology and fracture behavior of cheese would be determinant for overall quality and preference in a given type of cheese (Foegeding et al., 2003).

The solid nature of cheese is mainly determined by the para-casein matrix whose three-dimensional structure consists of chains of aggregated and partly fused para-casein micelles. The maturation of cheese leads to a gradual reduction in the extent of internal matrix structure as reflected by the formation of a more homogeneous mass. The proteolysis process during ripening results in part of the matrix loosing contact with the main para-casein network and giving discontinuities or breaks in the matrix (de Jong, 1978). These structural discontinuities may result in the lack of tensile strength in cheese and it may be reflected in crumbliness, shortness and fracturability (O'Callaghan and Guinne, 2004).

The level of aqueous phase in cheese is directly related to the firmness. During the ripening of cheese, water near the surface evaporates and uneven water distribution may arise and a gradient of firmness may be found, consequently the network becomes brittle. Likewise at high water levels, the smaller number of inter particle bonds between casein would result in a more deformable matrix (Visser, 1991).

Besides, pH effects on the properties of casein micelles are related directly to chemical changes in the protein network of the cheese curd (Lawrence et al., 1987). The changes in the cheese pH induce changes in the amount of calcium-induced cross-linking in the caseins which, being the main load-bearing components in cheese, induce changes in rheological and fracture properties (Watkinson et al., 2001). At about pH 5.2 the salvation of casein micelles are at maximum, therefore the protein-protein bonds in cheese are fewer and weaker. Consequently the casein particles in the cheese matrix are more deformable and available for fracture (Visser, 1991).

Salt plays an important role in moisture content, lactose fermentation, para-casein hydration/solubility and conformation and proteolysis. Thus the salt may have and indirect effect on the rheological properties (Guinee, 2004). In some cheeses the increase in salt in moisture at range of 0.4-12% resulted in increased of firmness, fracture stress, and sensory hardness attributed in part to the influence of salt in cheese composition (Visser, 1991; Guinee and Fox, 1999)

Several surveys have been reported different changes in rheological, textural and sensory cheese properties induced by HHP treatments. Buffa et al., (2001b) found that cheeses made from raw or HHP treated milk (500 MPa, 15 min) were firmer and less fracturable than those made from pasteurized milk. However, these differences became less notable throughout the end of the maturity. Although the cheeses exhibited a loss of elasticity during the time, HHP samples were initially the most elastic. Likewise, these authors observed color differences

depending on the milk treatment and ripening time. Garde et al., (2007) reported that the fracturability, hardness and elasticity were higher throughout ripening in cheeses treated by HHP (400 MPa, 10 min) on day 2 than in control, whereas the application of HHP treatment on day 50 had no significant effect on texture of 60 day old *La Serena* Spanish cheese. In addition, the HHP applied on day 2 had negative effect on taste quality preference. Besides, Juan et al., (2007b) observed in ovine milk cheeses that moderate pressures (200-300 MPa) applied on day 1 enhanced the firming of cheeses, while the highest conditions of pressure applied, especially 500 MPa, showed highest deformability and lowest fracturability and rigidity. On the other hand the HHP treatments applied on day 15 led cheese characteristics more similar than control and were also sensory preferred. Sheehan and Guinee, (2004) in reduced-fat Mozzarella cheese, observed that HHP treatments at 400 MPa, 5 min, 21 ºC did not significantly affect the rheology or cooking properties along 35 day storage period. However the HHP samples showed a decrease of lightness, yellowness and redness values one day after the treatment, but the differences were reduced after 75 days storage. Saldo et al., (2000) found that HHP cheeses were less crumbly and more elastic than control goat milk cheeses. The HHP-treatment at 400 MPa, 5 min did not show any differences in fracture properties and deformability modulus compared with control. However, the 400 MPa plus 50 MPa showed an increment of fracture stress and deformability modulus while fracture strain decreased. Wick et al.,(2004) observed that HHP treatments up to 300 MPa applied in one month-old cheddar cheeses did not affect fracture stress, but increased fracture strain compared with untreated cheeses.

SECTION II

OBJECTIVES, MATERIAL AND METHODS

OBJECTIVES

In recent years different methods and technologies have been proposed to control and/ or inactivate the presence of undesirable microorganisms in food, including amino acid decarboxylase bacteria. Therefore, knowledge of BA formation and its possible reduction in raw milk cheeses is necessary, in addition to good hygienic practices during manufacture processing and subsequent manipulation, to obtain safer products without losing their special and unique characteristics.

High Hydrostatic Pressure (HHP) technology has been applied satisfactorily in food products and its effects in milk and dairy products has been studied in a considerable amount of surveys, especially in aspects related to safety improvement and physicochemical changes. In some of these studies HHP treatments of 400 MPa between 5-15 min were related to the decrease of undesirable microorganisms, increase or decrease of proteolysis and improvement of texture, among other consequences. For this reason, HHP treatment could influence in BA profiles and its final content in cheese. However, there is few data on the effect of HHP on biogenic amines in raw milk cheese.

The general objective of this study was:

- To evaluate the effect of HHP processing in two artisan caprine and ovine raw milk cheeses, in order to reduce their BA content and improving their safety, without detriment of its sensorial characteristics.

The specific objectives of this work were described as follows:

- To evaluate two Spanish artisan cheese factories, wich would the suppliers of cheese samples used in this work, under a hygienic point of view.
- To evaluate the influence of HHP application on the growth of microorganisms, on the physicochemical characteristics (moisture, pH, salt, total nitrogen and fat), on the proteolysis, and on the textural, color, and sensory properties during the ripening of two artisan caprine and ovine raw milk cheeses.
- To improve and validate a screening method to detect the lysine, ornithine, histidine and tyrosine decarboxylase capacity of bacteria.

- To evaluate and identify the incidence of lysine, ornithine, histidine and tyrosine decarboxylase bacteria in goat and ewe raw milk cheeses with and without HHP treatments.
- To study the BA profile of the two artisan goat and ewe raw milk cheeses, and the consequence of HHP treatments applied during different phases of ripening.

MATERIAL AND METHODS

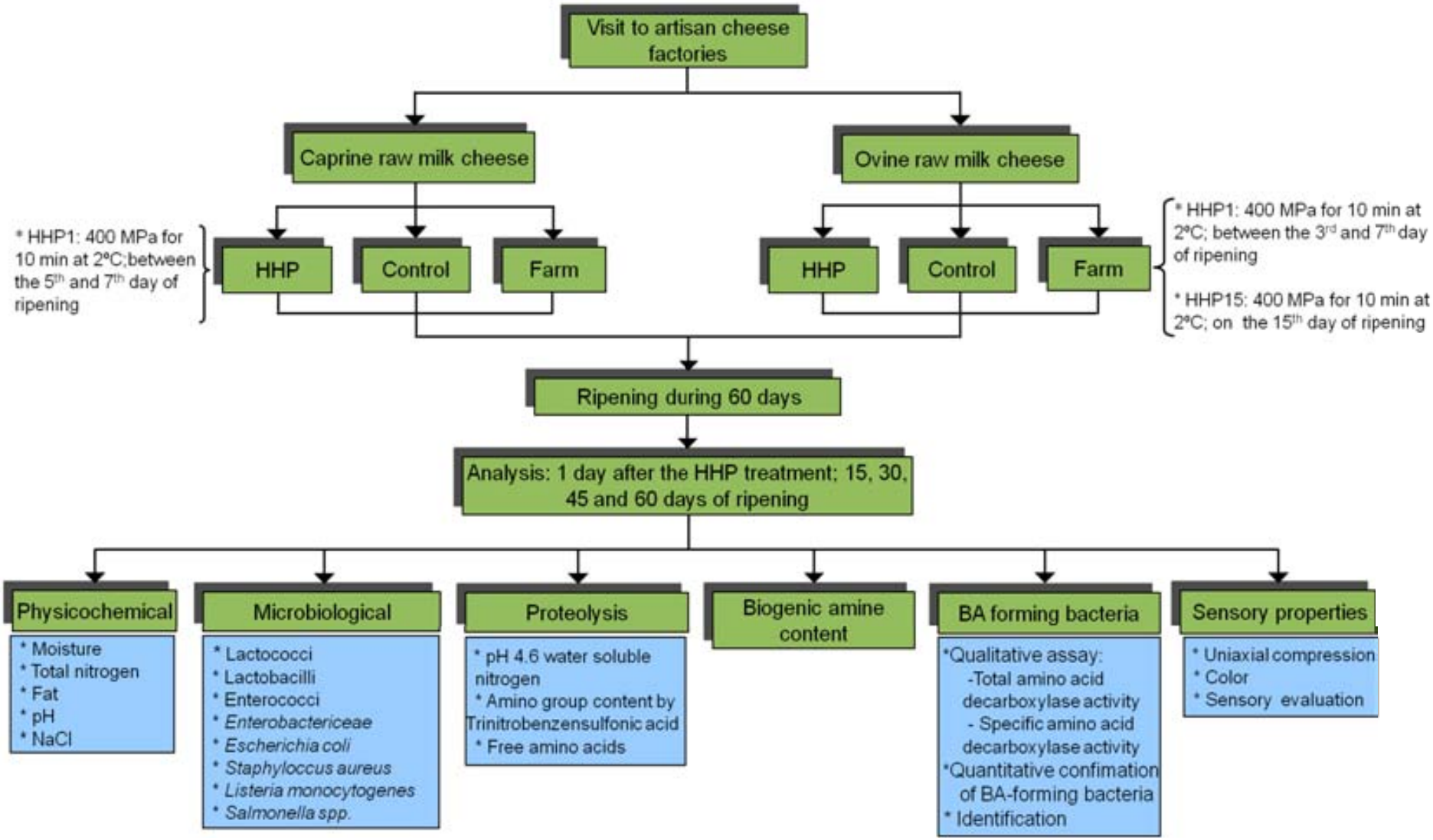
The development of this work was possible within the framework of a PETRI project (Spanish abbreviation of the research projects to incentive the transferring research results) (Proyectos de Investigación destinados a Estimular la Transferencia de Resultados de Investigación), accorded between the Asociación de Queseros Artesanos del Estado Español (AQAEE) and the Universitat Autònoma de Barcelona (UAB).

The AQAEE association was established in 1985 as a consequence of different meetings between cheese makers, in order to discuss the problems and concerns of the artisan cheese sector and with the objective to improve and preserve the diversity of cheese heritage.

In this PETRI project, 61 artisan cheese factories were visited and the cheese makers interviewed with the purpose to know the situation and characteristics of their facilities, operations and manufacturing practices. With this purpose, a questionnaire was elaborated based on the AQAEE and EU regulations EC Nº 853/2004, EC Nº 852/2004 and EC Nº 2073/2005 (Anonymous, 2004a; Anonymous, 2004b; Anonymous, 2005). The different sections included in this poll were: general data of the cheese factory and production; quality control methodology; origin of milk and/or conditions of reception and storage; traceability; cleaning and sanitation; pest control; facilities and production equipment; good manufacture practices; personal hygiene and training; water supply and waste and by-products management.

Among all the cheese producers visited, two were agreed to be involved in this study and support it with the supply of the cheeses to be used in the experiments. The work plan followed is displayed in Figure 4:

Figure 4. Working Plan



1. CHEESE MANUFACTURE

Two types of artisan ripened cheeses elaborated in Spain, both made of enzymatic curd and pressed paste, were studied. The first one was produced from caprine raw milk by a manufacturer located in the Autonomous Community of Catalonia, while the second one was made from ovine raw milk and manufactured at the Autonomous Community of Castilla y León. Three and four independent batches from the caprine and ovine raw milk cheese, respectively, were produced in the artisan cheese factories following their usual manufacturing practices.

The cheeses arrived to the laboratory after the salting stage, and each sample was adjusted to a diameter of 8.0 cm in order to adequate it to the HHP equipment basket, and then vacuum packaged individually in sterile polyethylene bags to avoid the direct contact between product and pressure transmission media.

2. HIGH HYDROSTATIC PRESSURE TREATMENT

The treatment of the caprine raw milk cheeses were performed at 400 MPa for 10 min at a temperature of 2 ºC, by using a discontinuous high hydrostatic pressure food processor equipment (EPSI NV, Temse, Belgium) at the pilot plant of the *Instituto de Agroquímica y Tecnología de Alimentos* (IATA), with a pressure volume chamber of 2.35 L and an operation pressure vessel of 689 MPa. The pressurization liquid was a mixture of water and glycol. The pressure level, pressurization time and temperature were controlled automatically. The pressurization rate was 350 MPa min^{-1} and the depressurization time was around 30 seconds.

The ovine raw milk cheeses were pressure-treated at 400 MPa for 10 min at temperature of 2 ºC, using discontinuous HHP equipment ALSTHOM (Nantes, France) (Figure 5) with a pressure chamber of 2 L capacity and able to reach 500 MPa. The pressure fluid medium was a mixture of alcohol and demineralized water (1:9). The temperature of the fluid was measured by a thermocouple. The pressure level, pressurization time and temperature were controlled automatically. The pressurization rate and depressurization time were 268 MPa min^{-1} and 55 seconds, respectively.

After the HHP treatments, all cheeses were kept into the ripening chamber during 60 days in regular conditions of 14 ºC of temperature and 88 % relative humidity.

Figure 5. HHP equipment from ALSTHOM (Nantes, France)

3. SAMPLING

Samples of each batch of caprine raw milk cheeses were separated in untreated cheeses (GC) as control and HHP treated cheeses at 400 MPa for10 min at 2ºC, applied between the 5th and the 7th day of ripening (GHHP1).

The samples of the four batches of ovine raw milk cheeses were also separated in untreated cheeses (EC) as control; cheeses in which the HHP treatment was applied between the 3rd and the 7th day (EHHP1); and cheeses that were HHP treated on the 15th day of ripening (EHHP15).

For both types of cheeses, and for each batch, there was a group of samples which remained in the ripening chamber of the artisan cheese factory of origin, that were named as "Farm cheeses", to control the influence of the different ripening conditions existing between the original manufacturer and our laboratory.

The analysis of cheeses was performed one day after HHP treatments and also after 15, 30, 45 and 60 days of ripening.

4. PHYSICOCHEMICAL ANALYSIS

Determination of moisture in cheeses was assayed in triplicate according to the International Dairy Federation IDF Standard 4A (Anonymous, 1982). The fat content was tested in triplicate using the Van Gulik method ISO 1735 (Anonymous, 1975). Total nitrogen (TN) was measured in duplicate by the Dumas combustion method IDF Standard 185 (Anonymous, 2002a). The pH was performed by five measures with a penetration electrode pHmeter

(micropH 2000; Crison Instruments, Barcelona, Spain). The sodium chloride was determined by four measures in 0.5 mL aliquots of 1% cheese filtered solutions using chloride analysis (Corning 926 Chloride Analyzer, Sherwood Scientific Ltd. Cambridge, UK).

5. MICROBIOLOGICAL ANALYSIS

Ten grams of each cheese sample were homogenized for around 90 s in 90 mL of sterile buffered peptone water (Oxoid, Basingstoke, Hampshire, UK) in a stomacher blender (BagMixer, Interscience, France). Decimal dilutions were prepared in sterile peptone water and pour-plated with media. Counts of *Lactococcus spp.* were made on M-17 agar (Oxoid) supplemented with bacteriological grade lactose solution (5 g L^{-1}, Oxoid) incubated at 30 ºC, 48 h; lactobacilli were plated on MRS agar (Man Rogose Sharpe, Oxoid) and incubated at 30 ºC, 48 h; enterococci were enumerated using KF streptococcus agar (oxoid) supplemented with 2,3,5-trifeniltetrazolium chloride solution 1% (Oxoid) after incubation at 37 ºC, 48 h; enumeration of *Enterobactericeae* was made on violet red bile glucose agar, VRBG (Oxoid), and for *Escherichia coli* chromogenic selective media Coli ID (BioMérieux, Marcy l'Etoile, France) was used, both media were incubated at 37 ºC, 24 h; 0.1 mL of decimal dilutions were surface spread on Bair Parker agar supplemented with rabbit plasma fribrinogen, BP-RPF agar (BioMérieux) and incubated at 37 ºC for 24-48 h to *Staphylococcus aureus* counts. Presence of *Listeria monocytogenes* was assessed by a 2-stage enrichment procedure using half-fraser broth and fraser broth (Biomeriux) as preenrichment and enrichment media, respectively, and Agar Listeria selon Ottaviani & Agosti (ALOA agar; AES- Chemunex, Bruz, France) as chromogenic media to detect *L. monocytogenes.* Presence of *Salmonella* spp was determined using buffered peptone water (Oxoid) as a preenrichment media, Muller-Kauffman broth and Rappaport-Vassiliadis (BioMérieux) as enrichment media, and, Xylose-Lysine-Deoxycholate Agar, XLD agar (Oxoid) and SM ID 2 agar media to detect *Salmonella* spp. colonies (Biomeriux).

All microbiological analysis were performed in duplicate, the results are shown as means of the count of batches and expressed as logarithm of colony forming units per gram.

6. ASSESSMENT OF PROTEOLYSIS

6.1. Nitrogen fractions

Water soluble extracts (WSE) of cheese were prepared according to the method described by (Kuchroo and Fox, 1982). Briefly, a grated cheese sample was added to 60 mL distilled water and homogenized with an Ultraturrax (Diax 900, Heidolph, Germany) for 5 min. Then the mix was warmed at 40 ºC for 1 h and centrifuged at 5697 g during 30 min at 10 ºC. The supernatant was filtered thorough glass wool. From the water soluble extracts a pH 4.6 water-soluble nitrogen (WSN) fraction was obtained and the nitrogen was determined by Dumas combustion method (Anonymous, 2002a). The nitrogen content of WSN fraction was expressed as a percentage of total nitrogen (WSN/TN, %) which is described as the ripening index. The determination was performed in duplicate.

6.2. Amino group content by Trinitrobenzensulphonic acid (TNBS)

The measurement was determined on WSE, diluted 10 or 100 times in 2 % sodium dodecyl sulphate (SDS; Sigma Chemical, St. Louis, Mo, USA) depending on the concentration of amino groups expected, according to the TNBS method described by Hernández-Herrero et al., (1999a). Aliquots of 10 µL of samples were put separately in a microtiter plate, adding to this 80 µL of phosphate buffer pH 8.2 and 80 µL of a 0.1% fresh and light protected solution of TNBS (Sigma, Chemical). The absorbance at 405 nm was determined by using a microtiter plate reader 340 ATTC (STL lab instruments, Austria) after the incubation of the plate at 42ºC during 1 h. The calibration curve was made of L-Leucine (Sigma chemical) solution (concentrations between 0.067 and 0.52 mg mL^{-1}) regularly prepared and measured, as described above, at the same time than samples. The results shown are expressed as mg of L-Leucine per g of cheese. Each analysis was made in duplicate

6.3. Total free amino acids

The assessment of the total free amino acids (FAA) was determined on WSE by the cadmium-ninhydrin method described by Folkertsmaa and Fox, (1992). The calibration curve was prepared using a 0.2 mg mL^{-1} solution of L-Leucine (Sigma chemical) which was diluted to obtain concentrations between 0.002–0.08 mg mL^{-1}. The analyses were made in duplicate. The results shown are expressed as mg L-Leucine per g of cheese.

7. TEXTURE AND SENSORY ANALYSIS

7.1. Uniaxial compression test

After removing the external part of the cheeses, a total of 6 cubes were cut from each cheese sample and held at 20 ºC for 2 h before the assay. The uniaxial compression test was carried out with a TA-TX2 Texture Analyzer (State Microsystem, Surrey, UK), using a 245 N load cell and a compression cylinder of 36 mm of diameter. Cheese cube samples were compressed to 80% of their original height at a constant temperature of 20 ºC, under lubricate conditions, with a crosshead speed of 80 mm min^{-1} as described by Juan et al., (2007b). True stress and true strain were calculated according to Calzada and Peleg, (1978) by the following Equations:

$$\text{True stresss: } \sigma_{(t)} = \frac{F_{(t)}}{A_{(t)}}$$

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).

$$\text{True strain: } \varepsilon = \ln \frac{H_0}{H_0 - \Delta H}$$

Where ε is the true strain; H_0 (m) is the original height; and ΔH is the change in height.

From the true stress-true strain curves resulted, the fracture stress (σ_f) and fracture strain (ε_f) values were obtained.

7.2. Color determination

The chromatic values of each samples were measured 6-8 times from different points of the inner surface of the cheeses cut in two halves, using a portable Hunterlab spectrocolorimeter (Miniscan™ XE, Hunter Associates Laboratory, Reston, VA, USA), with a cool white fluorescent (Fcw) illuminant, observer at 10º and CIElab scale. L* value describes lightness-darkness (ranges between 100-0), a* value indicates greenness (negative values) to redness (positive values) and b* value reflects blueness (negative values) to yellowness (positive values). Total color differences (ΔE) of HHP treated samples referred to control cheese during the ripening were calculated using the following equation.

$$\text{Total color difference: } \Delta E = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)}$$

7.3. Sensory properties

Sensory textural and flavor characteristics of 60 old cheese samples were evaluated by a panel consisting of 10 volunteers from the Centre Especial de Recerca Planta de Tecnologia dels Aliments (CeRPTA), related with dairy products and familiarized with caprine and ovine milk cheeses produced in Spain.

Representative slices of each batch of HHP treated and control caprine and ovine raw milk cheeses were presented to panelist to distinguish the differences perceived between a pair of samples (HHP1 and control; HHP15 and control and HHP1 and HHP15, depending the case) and to point out the samples which showed the highest intensity on each of the following tributes evaluated: texture (firmness, elasticity and crumbliness) and taste (intensity, saltiness, acidic and bitterness). Likewise, a preference test was also made, where the panelist had to choose between each pair of samples.

8. DETECTION OF BIOGENIC AMINE FORMING BACTERIA

8.1. Strain isolation

A total of 688 isolates were randomly picked out from the different selective media used for the microbiological analysis of cheese (MRS, M17, KF, VRBG and BP-RPF media). The purification of each isolated was made by streaking single colonies on Petri plates with Tryptone Soy Agar (Oxoid) and incubating at 30 ºC for 24-48 h; this procedure was made 2-3 times. Three pure cultures (two TY producing strains of *Lactobacillus brevis* and *Lactobacillus casei* and a HI producing strain of *Staphylococcus epidermidis*) isolated from previous studies (Hernández-Herrero et al., 1999b; Roig-Sagués et al., 2002) and belonging to the culture collection of the Animal and Food Science Department of the Univ. Autònoma de Barcelona, were used as positive controls. These cultures were revived in 10 mL of Tryptone Soy Broth (Oxoid) and incubated at 30 ºC for 24 h. The purity of each culture was verified by subculturing the *Lactobacillus brevis* and *Lactobacillus casei* strains onto MRS agar (Oxoid), incubated at 30 ºC for 24 h, and the *Staphylococcus epidermidis* strain on BP-RPF agar (Biomerieux) incubated at 37º C for 24 h.

Before performing the decarboxylase assay, each strain was suspended in a tube with physiological solution of NaCl 0.85% (Panreac, Barcelona, Spain) until reaching a turbidity of about 0.5 in the McFarland scale.

8.2. Decarboxylase media

According to the previously mentioned studies to evaluate amino acid decarboxylase activity, two synthetic media were formulated to determine the ability to form the most toxic BA (HI and TY) and their enhancers (PU and CA): the low nitrogen medium (LND) was elaborated with a reduced nitrogen source (0.25%) while the low glucose medium (LGD) contained a small amount of glucose with the objective to decrease the false positive and negative responses in bacteria with a strong peptidase (or deaminase) and fermentative activity (Table 3). Both kinds of media were subdivided in base broth (without amino acids); total amino acid broth, supplemented with all four amino acids: L-Lysine monohydrate (Merck, Darmstadt, Germany), L-Ornithine monohydrate (Farmitalia Carlo Erba, Milan, Italy), L-Histidine monohydrochloride (Merck) and L-Tyrosine disodium salt (Farmitalia Carlo Erba); and individual amino acid broths, supplemented individually with each amino acid. All media were adjusted to the pH values indicated in Table 3 and autoclaved at 120 ºC, 1 atm, during 5 min.

Table 3. Composition of decarboxylase broth media (%)

Reagent	Low nitrogen decarboxylase broth (LND)	Low glucose decarboxylase broth (LGD)	pH
Tryptone	0.125	0.25	
Yeast extract	0.125	0.25	
NaCl	0.25	0.25	
$CaCO_3$	0.01	0.01	
Pyridoxal 5'-phosphate	0.03	0.03	
Glucose	0.05	0.001	
Bromocresol purple	0.01	0.01	
Amino acids (Total broth)	1.0	1.0	5.5
L-Lysine (L-L broth)	1.0	1.0	5.0
L-Ornithine (L-O broth)	1.0	1.0	5.5
L-Histidine (L-H broth)	1.0	1.0	5.7
L-Tyrosine (L-T broth)	0.25	0.25	5.5

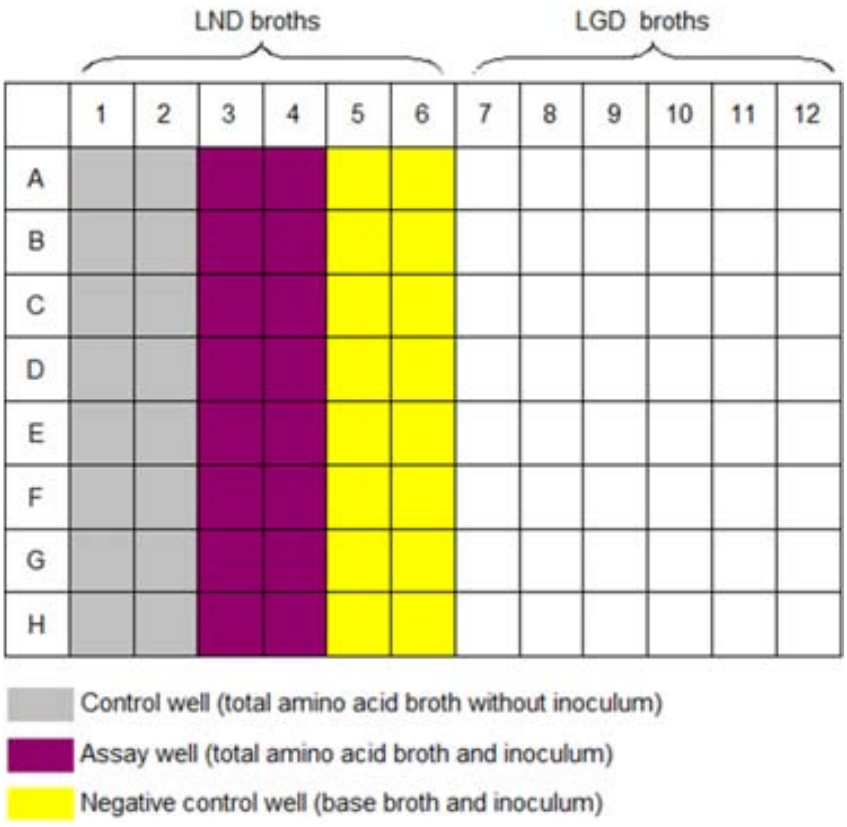
8.3. Qualitative microplate assay of amino acid decarboxylase activity

8.3.1. Assessment of total amino acid decarboxylase

In order to detect the capacity of the isolates to form any BA and to determine which one of both media tested showed the best response to each strain activity, a screening test was performed, using all the isolates, on sterile 96 bottom flat well microtiter plates (12 x 8) with sterile top.

As can be observed in Figure 6, the microplate was divided in two parts whose cells were filled with LND and LGD media, respectively. The first two columns of each part were used as control wells (with total amino acid broth), the next two were the assay wells (also with total amino acid broth) and the cells of the last two columns, containing base broth without amino acids, were used as negative control. Two-hundred µL of the corresponding broth media were place with 20 µL of the culture suspension into the assay and negative control wells. Microplates were covered with a sterile top and incubated at 30 ºC for 24 h. This distribution allowed to test up to eight different strains in each microplate.

Figure 6. Microplate diagram in the total amino acid decarboxylase assay

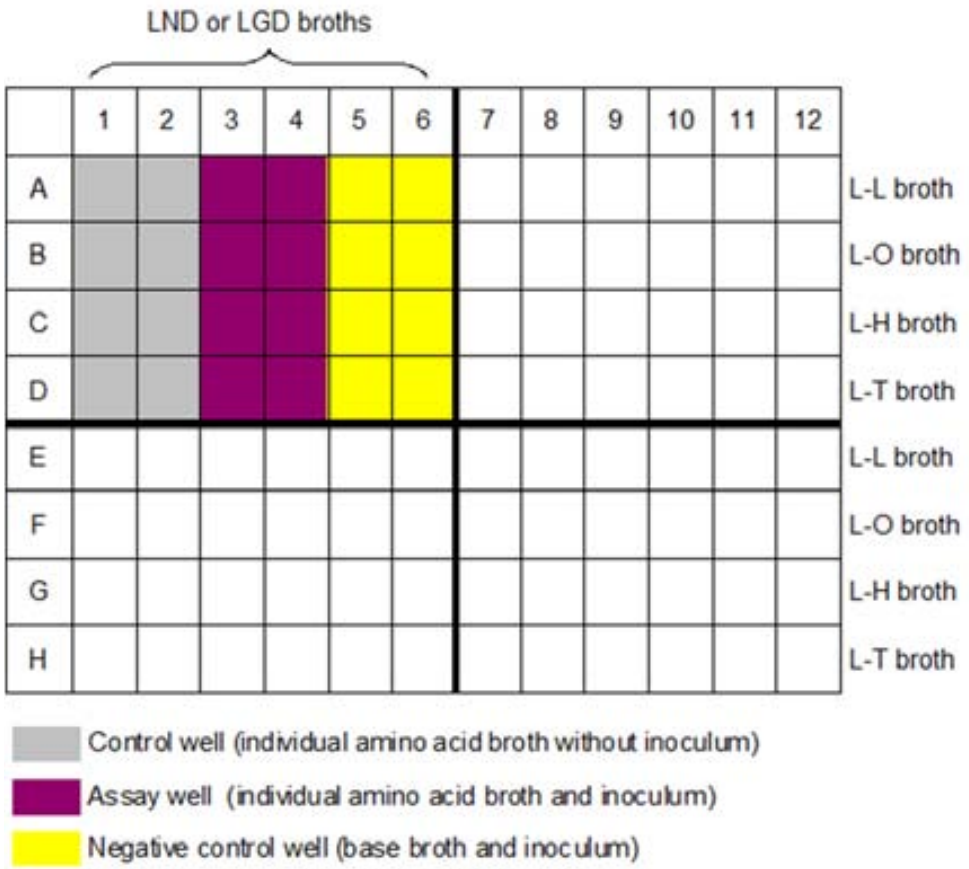


8.3.2. Assessment of specific amino acid decarboxylase

The analysis was followed out with the positive BA producer strains with the purpose of knowing the response to specific lysine, ornithine, histidine and/or tyrosine decarboxylase activity.

The microplate was prepared as shown in Figure 7. In this evaluation, 20 µL of broth cultured with each positive BA producing strain were placed with 200 µL of the corresponding broth media (LND or LGD), according with the best response obtained during the previous assay. In general, LND was commonly used to test strains obtained from the VRBG, BP and KF media and LGD was used to assay strains picked out from the M17, MRS and KF media. With this system 4 different strains could be individually assayed, for the 4 amino acids, in the same microplate.

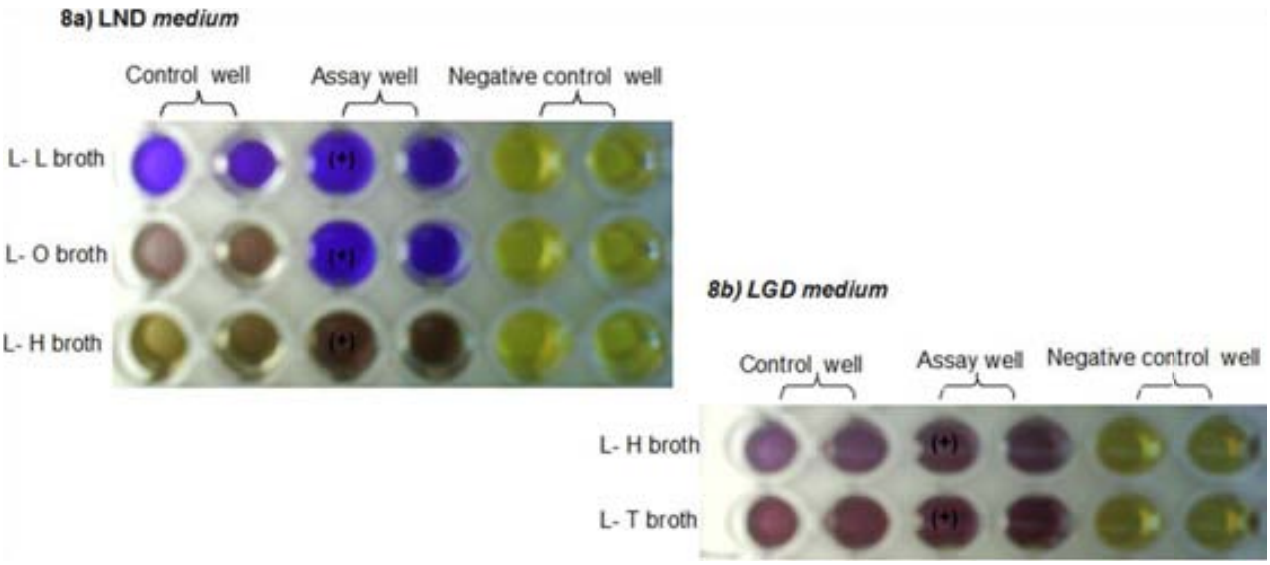
Figure 7. Microplate diagram in the specific amino acid decarboxylase assay



8.3.3. Response interpretation

A) Positive decarboxylase activity. Figures 8a and 8b display an example of affirmative responses in LND and LGD media, respectively. The assay-plate wells containing broth added with amino acids and inoculated with the strain indicated a positive response by an increase of alkalinity in the media expressed by purple color, in response of bromocresol purple, which has got a pKa of 6.3 and whose color changes from yellow-to-purple when the pH shift 5.8 to 6.1 (Brooker et al., 1973). The yellow color in negative control wells indicated acidification by the growth of the strain. In the case of LGD medium, the absence of color change in the assay well was considered also as positive result, provided the negative control wells reflected strain growth (yellow color).

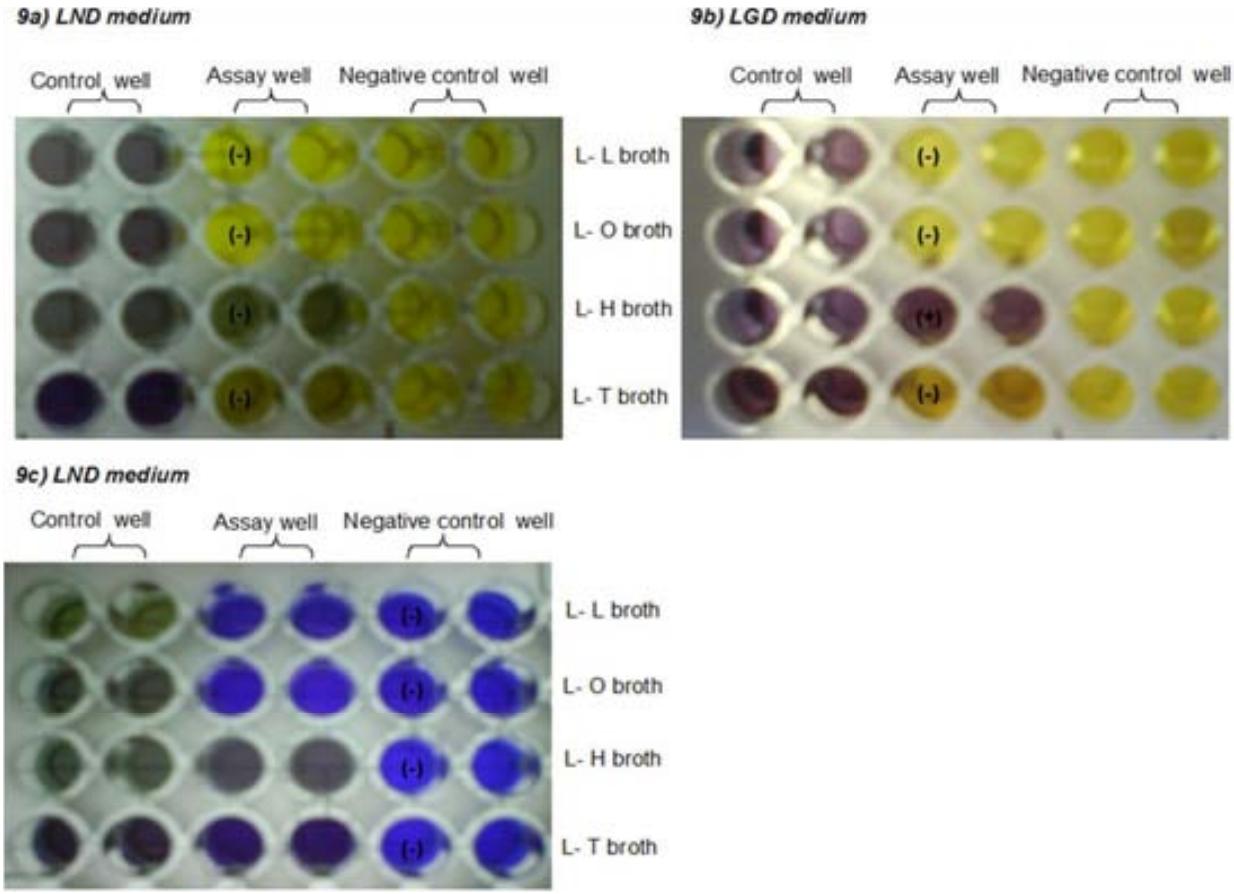
Figure 8. Example of positive responses to amino-acid decarboxylase activity using LND and LGD media



(+): Positive response.

B) Negative decarboxylase response was indicated by a yellow color in the assay wells compared to control wells (Figures 9a and 9b). A purple color in negative control wells indicated that bacterial metabolism produced different alkaline compounds than BA (Figure 9c). In this case the response was considered as a negative result.

Figure 9. Example of negative responses to amino-acid decarboxylase activity using LND and LGD media



(-): Negative response (+): Positive response.

8.4. Quantitative analysis to confirm amino acid decarboxylase activity

The positive decarboxylase strains were confirmed by the quantitative analysis of BA (PU, CA, HI and TY) produced in the decarboxylase broth by means of reverse-phase High Performance Liquid Chromatography (HPLC), using an automated HPLC system (HPLC P680, Dionex, Sunnyvale, California, USA) equipped with a UV detector (UVD170U, Dionex). Briefly: one mL of each bacterial suspension (0.5 McFarland) was inoculated into a tube containing 4 mL of LND or LGD total amino acid broth (according with the total amino acid decarboxylase assay), supplemented with 0.02% of $MgSO_4$ (Panreac); 0.005% of $MnSO_4$ (Panreac); and 0.004% of $FeSO_4$ (Panreac). After 4 days of incubation at 30 ºC, the media was centrifuged (88905 g, 10 min, 20ºC) and 3 mL of the supernatant were extracted with 2 mL of 0.4 M $HClO_4$ (Panreac). Aliquots of 1 mL were kept at -20ºC until BA analysis.

The determination of BA in the broth extracts was carried out according to Eerola et al., (1993). One mL of extracted supernatant was derivatized with dansyl chloride reagent (Sigma-Aldrich Chemical). The separation was performed on a Waters Spherisorb S5 ODS 2 45x150mm column (Waters Corporation, Milford, Massachusetts, USA). All reagents were of analytical grade and all solvents involved in derivatization and in the separation processes were of HPLC grade. The BA standards: putrescine (PU), cadaverine (CA), histamine (HI), tyrosine (TY), and the internal standard 1,7-diaminoheptane, were purchased from Sigma-Aldrich Chemical.

8.5. Biochemical identification of strains

Confirmed isolates were selected according their group and BA producing capacity. All were tested for their Gram reaction and shape. Gram positive strains were tested for catalase and Gram-negative strains were tested for catalase and oxidase. Further identification to the species level was carried out by a variety of biochemical tests using API 20-E, API 20-Strep, API-Staph, API 50-CH (Biomerieux), BBL Crystal Gram-positive ID system (Becton, Dickinson and Company, Sparks, Maryland, USA) and Bacillus-ID 24 test (Microgen Bioproducts, Surrey, UK).

9. DETERMINATION OF BIOGENIC AMINES IN CHEESE

The extraction, separation and quantification of BA were carried out according to Eerola et al., (1993). Briefly: two g of cheese sample were extracted with a 0.4 M $HCLO_4$ solution (Panreac). One mL of extracted supernatant was derivatizated with dansyl chloride reagent (Sigma-Aldrich Chemical) and analyzed by reverse-phase HPLC as described in point 8.4. The separation was performed on a Waters Spherisorb S5 ODS 2 45x150mm column at constant temperature of 40ºC and detected at wavelength of 254 nm. All reagents used were of analytical grade and all solvents involved in the derivatization and the separation were of HPLC grade. The BA standards β- phenylethylamine (PHE), tryptamine (TR), putrescine (PU), cadaverine (CA), histamine (HI), tyrosine (TY), spermidine (SD) and spermine (SM) and internal standard 1,7-diaminoheptane were purchased from Sigma-Aldrich Chemical. All analyses were done in duplicate.

10. STATISTICAL ANALYSIS

10.1. Analysis of data from physicochemical, microbiological, proteolysis, color, texture and biogenic amines determination

To test the main effects of the HHP treatments and the time of application on physicochemical, microbiological, proteolysis indexes and BA profile in cheeses, an analysis of variance (ANOVA) was performed on all data from each batch and treatment of caprine and ovine milk cheeses at different ripening stages. Comparisons of mean values of physicochemical, microbiological and BA were followed by Duncan test with significance level set on $P<0.05$. Comparisons of mean values of proteolysis indexes, color and uniaxial compression test were followed by Student-Newman-Keuls test with significance level set for $P<0.05$. All tests were performed with the SPSS for windows (v.15.01) program (Chicago, IL, USA).

10.2. Analysis of sensory data

The results of perceived sensory differences and preferences were carried out by the non-parametric Chi-Square test. All test were performed with the SPSS for windows (v.15.01) program (Chicago, IL, USA) setting the significance level on $P<0.05$.

10.3. Analytical validation of the qualitative microplate method of amino acid decarboxylase activity

The sensitivity, specificity, and the positive and negative predictive values were obtained to determine the diagnostic properties of the qualitative method (Altman and Bland, 1994a; Altman and Bland, 1994c) and were calculated by the following equiations.

$$\text{Sensitivity} = \frac{TP}{TP+FN}$$

Where TP is the truly positive amino acid decarboxylating isolates that are correctly identified by the screening test and FN is the false negative responses obtained.

$$\text{Specificity} = \frac{TN}{TN+FP}$$

Where TN is the truly negative (TN) amino acid decarboxylating isolates that are correctly identified by the screening test and FP is the false positive responses obtained.

$$PPV = \frac{(\text{sensitivity} \times \text{prevalence})}{(\text{Sensitivity} + \text{prevalence} + (1 - \text{specificity}) + (1 - \text{prevalence})}$$

Where PPV is the positive predictive value that reflects the proportion of isolates with positive screening test results which are correctly diagnosed; and the prevalence is the number of the positive results obtained among the total number of strains tested.

$$NPV = \frac{(specificity \times (1 - prevalence)}{(1 - sensitivity) + prevalence + specificity + (1 - prevalence)}$$

Where NPV is the negative predictive value that reflects the proportion of isolates with negative screening test results which are correctly diagnose.

By comparing the amino acid decarboxylase microplate screening results with the values of the HPLC confirmation method, Receiver Operating Characteristic (ROC) curves were assessed using the MedCalc statistical software, version 11.2.1 (Mariakerke, Belgium), to know the discriminative power of the qualitative method referred to the HPLC method. The ROC curve is obtained by the calculation of the sensitivity and specificity of every observed data value and plotting sensitivity against 1-specificity or false positve rate. A global evaluation of the test results is given by the area under the ROC curve (AUC). This area is equal to the probability that a random isolate with amino acid decarboxylase capacity has a higher value of the measurement than a random isolate without this ability. Thus, the better overall diagnostic performance of the test is when the AUC value is closer to 1, and a test with an AUC value of 1 is one that is perfectly accurate. The practical lower limit for the AUC of a diagnostic test is 0.5 (equivalent to tossing a coin) (Altman and Bland, 1994b; Park et al., 2004). MedCalc statistical software provides the ROC curve and AUC with its 95% confidence interval based on the method developed by Hanley and McNeil, (1982) in which a classification of diagnostic accuracy for the qualitative method is given according to AUC value: AUC 0.90–1.0 excellent, 0.80–0.90 good, 0.70–0.80 fair, 0.60–0.70 poor, 0.50–0.60 deficient and 0.50 null

After knowing the discriminative power of the qualitative test the optimal cut-off has been set. The value lying nearest to the point of intersection of the ROC curve and the 100% of sensibility to 100% of 1-specificity diagonal was chosen as the best discriminator value, being the optimal cut-off the value corresponding with the highest accuracy, the lowest false negative (FN) and the highest false positive (FP) results.

SECTION III

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

11. EVALUATION OF HYGIENIC MANUFACTURING PRACTICES IN TWO ARTISAN SPANISH CHEESE FACTORIES

11.1. General data of cheese factories and production

The first cheese factory (GCF) is situated in a rural region of the Autonomous Community of Catalonia, Spain. This region is characterized as semiarid-arid zone, with continental Mediterranean weather and an average annual temperature of 13-15 ºC with great differences between summer (40 ºC) and winter (-10 ºC). In this factory three workers are in charge of transforming the milk in cheese. The annual volume of milk transformed was approximately 100 000 L. The raw milk is acquired in two milk farms with goats of Murciano-Granadina breed located in the same region (around 10 km).

Two types of cheese were produced in this facility: *a)* a cheese elaborated from raw milk, with starter culture addition, enzymatic curd, pressing paste and wash rind. This cheese has a ripening period of approx. 60 days in a semi-nature chamber and a cylindrical format of weight of approx. 500 g; and *b)* a soft paste cheese made from raw milk and acid curd, with approx 60 days of ripening in a semi-natural chamber. This cheese has a cylindrical format and a weight of approx. 1300-1500 g .This cheese is elaborated only during the fall and winter seasons.

The second cheese factory (ECF) is conformed as a family factory, with three people working every day. The facilities are located in a small village with transhumance tradition in the Autonomous Community of Castilla-León, Spain. This factory transforms approximately 150.000 L of Lacaune ewe milk per year. They acquired the milk from a farm sited in the same region (at about 10 km of the facilities).

The cheeses produced in this factory are made of raw milk, with starter culture addition, enzymatic curd and pressed paste. The cheese has a cylindrical shape with a weight of approx. 1500-3000 g. The ripening period is around 3-10 months depending on the size of the cheese.

11.2. Hazard analysis and critical control point system (HACCP)

The European regulation EC 852/2004 (Anonymous, 2004a) states 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 HACCP principles.

HACCP plan has been performed in both cheese farms by external consultants, but the system has not been completely implemented, reflecting a failure in the system. The HACCP plan in many aspects was observed as complicate and incomprehensible and sometimes was perceived just as an administrative requirement. On the other hand, many consultants offer their services to implement the HACCP system, but without involving the cheese industry operators and did not take care about the work routines in the process, and consequently the procedures are established in the manual but not implemented in the cheese factory. These observations are in agreement with a report from the Commission to the Council and the European parliament about the experience gained from the application of the hygiene regulations. In this report is noted that most industrial food companies have HACCP-based procedures in place but delays in implementation were noted in former small capacity. Moreover, difficulties with such procedures were identified in small food businesses and more particularly, in micro retail establishments. Provision of generic systems from external consultants did not reflect, in some cases, not the reality of the establishment (Anonymous, 2009d). However, cheese makers knew very well that in order to have and keep a good cheese quality they must do several tasks and controls along the manufacturing process. On this line, the quality system of the artisan cheese factories has been performed according to their necessities and consists in the application, in different forms, of the prerequisites programs, although in most cases these controls are not written or registered on paper.

According to the elaboration process, both cheese factories usually register, in a manufacture sheet and also in other record forms, the data corresponding to the measured point. Table 4 shows the monitored points which the cheese workers follow during the process.

Table 4. Measure points during the cheese manufacture

	Ovine milk cheese Factory (ECF)	Caprine milk cheese Factory (GCF)
Raw milk	Temperature, pH and acidity Periodic milk analysis in qualified laboratory	Periodic milk analysis in qualified laboratory
Starter culture addition	Milk temperature, time and type of culture starter	Milk temperature
Curdling	Temperature, pH and time of curdling	Temperature, pH and time of curdling
Coagulum cutting	Total time of cutting	-
Reheating	Temperature (initial and final) and total time.	Not apply
Pressing	pH, pressure, temperature of workroom and time	-
Salting	Temperature, pH, and density of the brine. Time of salting	-
Airing	Temperature and % relative humidity of the room	Temperature of the room
Ripening	Temperature and % relative humidity of the ripening room. Ripening time (90-120 days.)	Temperature and % relative humidity of ripening room, time (60 days)
End product	Temperature. Periodic analysis in qualified laboratory	Temperature of cold room. Periodic analysis in qualified laboratory

Artisan cheese-makers understand that a closer monitoring of temperatures, times and acidity levels throughout cheese making process is needed to obtain a good quality and safer product. It is important to perform control parameters based on objective measurements, evaluating whether the procedures are being done correctly, diminishing thus the appearance of any undesirable risk and/or characteristic in the product. As example, ECF factory adjusted periodically the brine solution to maintain it within the specifications; likewise they renewed it every six months. In GCF cheese factory, the brine solution was changed depending on the season and the quantity of cheese produced, generally from 2 to 4 weeks. In addition, no monitoring of any kind was done in the brine during the period it was used.

The European Commission noted that record-keeping is sometimes perceived as an

administrative burden by small food industries, and that different member states have taken initiatives to simplify and minimize the necessary steps and documentation (Anonymous, 2009d). Romero del Castillo et al., (2005) in a study about the Spanish and French Pyrenees cheese making system it is reported that 66% of the interviewed Spanish artisan cheese makers applied HACCP procedures and the 24% carry out a self-control. On the contrary, 57% of the French cheese makers manifested that they did not perform any HACCP or self-control procedure.

11.3. Pre-requisites program

The quality control system of artisan cheese factories and its grade of conformity were evaluated according to the requirements from European regulations EC 852/2004 and EC 853/2004 and the pre-requisites program guide elaborated by the US Food and Drug Administration (FDA) (Anonymous, 2004a; Anonymous, 2004b; Anonymous, 2009c).

11.3.1. Facilities

The establishments should be located, constructed and maintained according to sanitary design principles. There should be linear product flow and traffic control to minimize cross contamination from raw to processed materials. There should be a suitable temperature-control and enough capacity to maintain foodstuffs (Anonymous, 2004a; Anonymous, 2009c).

In general, both cheese manufacturers had the suitable dimensions according with their activities and size of production. The installation of the cheese factory ECF had a reception room, workroom, aeration and maturation chamber. As far as maintenance is concerned, it was observed that some tile walls of the workroom showed cracks in some points and temporary arrangements with packing tape (Figure 10a). Likewise, the presence of molds in some upper parts (the joints of the ceiling and wall) and some spots of condensation and stains of moisture on the ceiling were evidences that the ventilation system was not adequate. On the other side, illumination in these facilities was observed as adequate, although one of the workroom lamps lacked of the corresponding cover (Figure 10b). The ECF were provided of suitable temperature-control handling and storage conditions in their aeration and maturation chamber. However, the maturation chamber was observed as rather small. The washing hands station was located in a specific area in the workroom and it was

supplied with tap water and provided with an adequate supply of soap, single service towels, and a covered waste receptacle.

Figure 10. ECF work room

The GFC cheese facilities counted with a reception area with a tank for cold milk, workroom, maturation room and cold chamber. During the visit it was observed that the floor, walls and ceiling from the reception and workroom were made from adequate materials, and these areas had adequate illumination and ventilation. While the floor, the wall and the ceiling of the maturation room were made of concrete. This surface was not smooth and was inappropriately finished. Besides, the painted surface was cracked by the humidity and showed dirtiness and mould, which reflected the poor ventilation of the area. In this case, the illumination was scarce and some equipment and other objects no longer used were stored into this room (Figure 11). The cold chamber was provided with a temperature control device with an automatic display. However, during the visit this chamber was at full capacity. On the other hand, the maturation chamber did not count with this control system as it is supposed in a semi-natural room, although there was a wet-bulb psychrometer used eventually to measure the temperature and humidity of the chamber. The washing hands station was a common washing sink, and it was provided with the necessary elements to do adequately this practice.

Dioguardi et al., (2005) reported that the major weaknesses observed in small cheese factories were due to non functional layouts, finishing materials and building design that contribute to get dirt accumulation. Microbial proliferation is very frequent if work surfaces that are not continuous and this makes the surfaces difficult to clean. The use of a tiled surface can be unhygienic if it is not properly constructed and regularly cleaned (Dioguardi and Franzetti, 2010).

Figure 11. GCF ripening room

11.3.2. Production equipment

All equipment items, fittings and equipment which come in contact with food should be designed, fabricated, constructed, and installed according to sanitary design principles and they should be kept in such good order, repair and condition as to minimize any risk of contamination (Anonymous, 2004a; Schmidt and Erickson, 2005).

The materials of the equipments, tools and items used in both cheese factories were made of stainless steel (cold tank, curd tub, work table press, milk churn and some others); plastic coated steel (ripening shelves) and polypropylene (molds, ripening trays, milk churns and other items). In general, they were maintained in good conditions. However, some equipment and items, such as lyres, were in bad conditions, and corrosion was found in some external points of a brine tank together with some broken parts and leakages (Figure 12).

Poor plant design and construction and no preventive maintenance have been identified among the ten principal food safety problems across all food processing industry (Sertkaya et al., 2006; Anonymous, 2009d). Likewise, these problems could provoke others, like difficult to clean the equipment and poor equipment sanitation that could increase the risk of contamination.

Figure 12. Equipment and items maintenance

11.3.3. Water supply

There is to be an adequate supply of potable water, which is to be used whenever necessary to ensure that foodstuffs are not contaminated. Recycled water used in processing or as an ingredient is not to present a risk of contamination. It is to be of the same standard as potable water, unless the competent authority is satisfied that the quality of the water cannot affect the wholesomeness of the foodstuff in its finished form (Anonymous, 2004a).

The ECF used water from the public net supplier. Likewise, the water was verified in the factory facilities by checking its chlorine levels and in a qualified external laboratory to determine its drinkability. On the other side, GCF cheese factory counted with a water well which supplied this element. The water was treated with chlorine before being used in this cheese house and the workers checked from time to time its chlorine levels. However, a drinkability analysis was not performed.

Romero del Castillo et al., (2005) found that in Spain the 85% of the artisan cheesemakers interviewed used water from the public water supplying system. On the contrary, the 63% of the cheese houses in France were supplied by water springs. Likewise, 16% of French cheesemakers used chlorinated water, 7% treated the water by ultraviolet radiation and 12% did not make any treatment to this element.

11.3.4. Cleaning and Sanitation

All the facilities, articles, fittings and equipment should be effectively cleaned and, where necessary, disinfected. Cleaning and disinfection have to take place at a sufficient frequency

to avoid any risk of contamination and the cleaning agents and disinfectants are not to be stored in areas where food is handled. All procedures for cleaning and sanitation of the equipment and the facility should be written and revised. A master sanitation schedule should be put in place (Anonymous, 2004a).

Both cheese houses counted with basic procedures for cleaning and disinfection. Different specific cleaning agents, such as alkaline and acid detergents and disinfectant were used. These factories counted with a specific storage area for chemical agents. However these chemical agents were applied in different ways. In ECF, usually cleaning and disinfecting agents were used according to their technical data sheet. This operation was periodically verified through microbiological analysis. During the visit it was observed that the cleaning and disinfection plan was followed mainly for the equipments, items and parts which have direct contact with the milk, whey, curd and cheese. However, other areas such as the plastic stripes curtain, some walls areas and chemical storage zones, presented dirtiness and/or mould growing (Figure 13).

The cleaning and disinfection in GCF facilities were applied according with their daily necessities, without implementing a standard operating procedure. It has to be pointed out that some the chemical cleaning agents were use by intuition, due to the lack of technical data sheets, and in some cases they were substituted by common soap and bleach. During the visit was observed that the facilities were clean, although the correct disinfection was not verified.

According with Sertkaya et al., (2006), poor plant and equipment sanitation and the difficulty to clean the equipment occupied the 3rd and 6th place in the ranking of food safety problems recorded in the food industry. Romero del Castillo et al.,(2005) observed that the majority of Spanish artisan cheese houses interviewed counted with written cleaning and disinfection plans, while in France the cheese makers had displayed these activities according with their specific needs. They emphasized that in Spain the authorities give more pressure in the formal aspects.

Figure 13. Cleaning and disinfection

11.3.5. Pest control

To prevent infestation, the processor must create a proactive program for stopping these pests from threatening the safety and quality of the product. The pest control program is a stand-alone program and is also a part of the plant's food safety system. Most small food plants must decide whether to maintain a pest control program themselves or contract the program to a pest control company (Keener, 2007).

Both cheese factories had installed elements to avoid and control insects, such as curtains, mosquito and fly nets and electronic insect exterminator devices, among others, although in some cases these elements were not used (Figure 14). The pest control for rats in the GCF cheese factory was done by the use of rat string traps; While ECF counted with a periodical service (quarterly) of an outsider pest controller (rats and insects). It has to be pointed out that in any of the cheese factories showed signs of presence of rodents or insects.

Figure 14. Electric pest control device

11.3.6. Supplier control

Each facility should be assured that its suppliers have effective good manufacture practices (GMP) and safety programs. Raw milk must come from animals that are in a good general state of health with no symptoms of infectious diseases, in particular, as regards to brucellosis and/or tuberculosis (Anonymous, 2004b; Anonymous, 2009c)

Both GCF and ECF did not count with written specifications for all ingredients, products, and packaging material. However they knew the farm facilities and the herds where the milk came from, likewise requested the consistency of sanitary qualification or green card according the respective herds were free of brucellosis.

Current European regulations permits manufacturing cheeses from raw milk with less than 60 days of ripening provided certain requirements such as the milk must come from animals officially free or free of brucellosis and/or tuberculosis, the low counts of somatic cells and germs in milk, among others. If the milk does not satisfy the required levels of germs and somatic cells it could be used to manufacture cheese provided that the cheese is aged 60 days as a minimum. In this case, the regulation demands a ripening control, with their corresponding records, to ensure this minimum period (Anonymous, 2004b; Anonymous, 2006).

The cheese factories did periodical verifications by means of microbiological and physicochemical controls of the milk through a qualified external laboratory. In the case of GCF, the microbiological analysis were made monthly and included the determination of indicator microorganisms (coliforms, *Staphylococcus aureus,* total mesophilic aerobic bacteria and *Escherichia coli*) and pathogens (*Salmonella* spp. and *Listeria* spp.), whereas the physicochemical analysis is done every year, including fat, protein and dry matter. ECF made the controls monthly, including indicator microorganisms (total mesophilic aerobic

bacteria and coliforms), protein, fat, inhibitors and somatic cells. In both cases, the manufactured cheeses have a minimum of 60 days of ripening.

It is known that for raw milk cheeses, milk is an important point to be considered in cheese making HACCP plans. Likewise, artisan cheese makers know that good quality raw milk coming from healthy, well-fed animals containing fewer pathogens gives good quality raw milk cheeses.

11.3.7. Reception, storage and shipping

All raw materials, ingredients and products should be stored under optimal sanitary and environmental conditions, such as adequate temperature and humidity to assure their safety and wholesomeness (Anonymous, 2004a; Anonymous, 2009c).

In the ECF, milk was transported in an isothermal tank, and usually the temperature of reception was around 5°C, according with written protocols. Usually after the arrival, the milk was poured into the curd tub for its transformation or into the cold tank for storage. The maximum storage of milk time was 48 hours after milking.

The milk was transported to GCF in stainless steel and plastic churns without any temperature regulation system. Later, depending on the day and time of reception, the milk was placed in the curd tub, cold tank or cold chamber. The maximum storage time of milk, before being transformed, was about 48 h from the milking. It has to be pointed out that in this cheese factory the reception temperature of the milk was usually estimated by touching the churn and asking the producer about the temperature in the cold tank when the container was filled. The tempera temperature was supposed to be around 6ºC. Taking into consideration the distance between the milking farm and the cheese factory (less than 10 km) the risk of temperature raise and consequent multiplication of microorganisms was considered as minimal. However it is convenient to verify temperature mainly in summer time. Nevertheless, the cold chamber was used for storing both raw material and finished product, which could increase the risk of crossed contamination.

11.3.8. Traceability

The traceability of food and food ingredients at all stages of production, processing and distribution is an essential element in ensuring food safety. To this end, such operators shall have in place systems and procedures which allow identifying the other industries to which their products have been supplied. Food that is released to the market shall be adequately labeled or identified to facilitate its traceability, through relevant documentation or

information in accordance with the relevant requirements of more specific provisions (Anonymous, 2004a; Anonymous, 2004b)

Both GCF and ECF cheese factories carried out control tasks from the reception of the raw material up to the packing of the final product. These included diverse records such as manufacture sheets in which the general manufacture data (date, type of cheese, production lot number), the quantity of raw material and conditions (milk, starter culture and rennet), and the details of manufacturing processes and cheese yielding (number of cheeses obtained, weight, cheese yielding) were registered; aeration and ripening identification label (with lot number, manufacturing date, number and type of cheeses); end packed product label, and the order form. It was observed that the traceability was frequently followed until the first customer considering the different ways of marketing: direct sale, fairs or markets, stores and/or restaurants and wholesale distributors.

11.3.9. Personal hygiene and training

Every person working in a food-handling area has to maintain a high degree of personal cleanliness and has to wear suitable, clean and, where necessary, protective clothing. All employees should receive documented training about the aspects of cheese making, personal hygiene, GMP, cleaning and sanitation procedures, personal safety, and their role in the HACCP program (Anonymous, 2004a).

The personal training on cheese manufacturing tasks in both cheese factories was given to new workers when they arrived and this labor was carried out by an experimented worker. However, this training was not frequently done because the scarce rotation of the personnel.

All employees from both cheese factories had the proper accreditation for food handling and also some of the workers have attended to specialized courses of cheese elaboration. However, there was not a continuous formation program for the personnel that worked at the factory, some of which have been there for a long time (more than 10 years) and received such courses just when they entered to the company.

In both cheese houses the use of working clothes was established, being caps, boots and aprons the usual uniform in the GCF, while pants, working coats, aprons and boots were used in the ECF house. During the visit to the GCF facilities it was observed that the workers went out the cheese building and returned back without changing boots and caps. In ECF it was noted that the working coat of one employee was in bad conditions.

The microbiological quality of cheese is influenced by milk quality, equipment and environmental hygiene during manufacture, packaging and handling (Robinson, 2010). For example, incorrect cleaning operations and poor hygiene habits by workers can facilitate microbial transfer from environment to food, where microorganisms find suitable conditions to grow. Sertkaya et al., (2006) ranked deficient employee training as the first problem facing food processors today and poor employee hygiene as the 9th. Additionally, the study showed that dairy products had the second highest overall and factor risk score within food sectors where these two problems were mainly associated. The analysis of a panel of experts revealed that ongoing and targeted training on GMP, cleanliness and sanitation as well as quality aspects are recommendable.

11.3.10. Waste and by-products management

Food waste, non-edible by-products and other disposals are to be deposited in appropriated, good condition and clean containers and removed from rooms where food is present as quickly as possible, so as to avoid their accumulation. Likewise, all waste is to be eliminated in a hygienic and environmentally friendly way so it will not be a direct or indirect source of contamination (Anonymous, 2004a).

The main food waste generated in cheese factories was the whey derived from the production of cheese. In this case, ECF factory was provided with a conduct that directed this residue from the workroom to a tank where it was stored to be used later for animal feeding in some farms in the vicinity. On the other hand, GCF did not count with a strategy for handling this residue. Both cheese manufacturers expressed their concern about the disposal of this residue because the quantity was too high for them, approximately 88 000 liters per year, and the farm animals around could not consume it completely. Likewise the whey transforming industry that could use this product charged a considerable amount of money for picking the whey up. Besides, in some cases there was no interest in picking these relative low quantities, or simply the cheese factory was located in a place with difficult access.

As far as solid residues are concerned, both factories counted with containers (with pedal activated cover and changeable plastic bags) placed at the workroom as well as bigger containers outside the factory. However, it was observed that, in one case, the trash containers were over their capacity and/or were uncovered (Figure 15). Likewise, in the surrounding areas of the cheese factory, there were found several things stored, such as ripening trays, empty gas cylinders and equipment parts, among others.

Figure 15. Solid residues

Romero del Castillo et al., (2005) reported that whey disposal was the major problem when discharging waste in small cheese houses. In this study the main use for whey were animal feed, followed by the production of cottage cheese, fertilizers for agricultural land, sewers and black well. It has to be noted that whey waste is an old problem solved with older methods of disposal and the newest solution become less available and too expensive and without the support of the authority.

Frequently, a strict food safety and hygiene regulation is difficult to apply for small cheese factories. Even though it is true that particular attention has to be devoted to improve their safety quality in production, the requirements and measures have to be adequate and functional so these artisan cheese houses can preserve the singular and diverse characteristics of their products. The pressure exerted by the authorities could provoke that artisan cheese makers have more formal aspects that are not frequently implemented and correlated with their reality. By the other side, they know the importance to maintain the quality and safety of their products and to implement autonomous control systems adjusted to their characteristics and needs. During the visits of these cheese houses there was found that the main weaknesses in these systems were preventive maintenance of facilities and equipments; cleaning and sanitation and whey waste disposal. In addition the lack of water supply control has been observed in one cheese house. Improvement in these points is indispensable to assure the quality and hygienic safety of traditional cheeses, but it is also indispensable to get cheese-makers also involved.

12. EFFECT OF HIGH HYDROSTATIC PRESSURE ON PHYSICOCHEMICAL AND MICROBIOLOGICAL PROPERTIES OF CAPRINE AND OVINE MILK CHEESES THOUGHOUT THE RIPENING

12.1. Physicochemical analysis

The results obtained in physicochemical analysis during the ripening of both caprine and ovine milk cheeses, respectively are shown in Tables 5 and 6. The mean fat and total nitrogen (TN) content at 60 day of ripening in caprine and ovine milk cheeses for control, farm and treated cheeses samples did not show significant differences. In general, fat contents in caprine and ovine milk cheeses, expressed in dry matter basis, were about 34.60% and 38.50%, respectively, while the means on TN content in both types of cheeses were 6.28% and 5.50%, respectively.

In general, the pH values of all cheeses decreased during ripening. Control and farm cheeses presented no significant differences in this value. Nevertheless it is possible to emphasize that in caprine and ovine cheeses the pH diminished mainly over the first 30 days, thereafter values remained stable. On the other hand, the samples with HHP1 treatment showed higher pH values compared with control samples, especially EHHP1, but at the end of ripening no differences were found in ovine milk pressurized cheeses, while GHHP1 samples presented significantly higher values. Likewise, during the ripening period no significant differences were observed between EC and EHHP15 samples over 30 days of ripening, even so at the end of this period pH values were higher in pressurized cheeses. An increase of pH post-HHP treatment has also been described in caprine milk and ovine milk cheeses (Messens et al., 1999; Saldo et al., 2002a; Juan et al., 2004) being this associated to a reduction in acid-lactic bacteria (LAB) counts after the HHP application and later recovery during the ripening period, which causes a slow lactose fermentation and the production of lactic acid. Moreover, Casal and Gomez, (1999) observed that pressure-treated LAB cells in bovine skim milk exhibited lower acidification rates, even with treatments that did not affect cell viability. Messens et al., (1998) also related the decrease of acidification with the dissolution of colloidal calcium phosphate due to HHP-treatment which causes a disintegration of casein micelles.

Moisture content decreased during the ripening in all cheeses and no significant differences were observed in control and HHP-treated caprine and ovine milk cheeses during the time. Nonetheless moisture content was slightly higher in HHP-treated cheeses. Pressure application affects protein matrix, forming more continued structures with water retention in

cheese (O'Reilly et al., 2003; Juan et al., 2004). Farm cheeses in general had the highest moisture content in both type of cheeses with significant differences in ovine milk ones. This could be explained by the fact that the format (diameter, weight) and rind in this cheeses differ from control and HHP-treated cheeses losing therefore less water.

There were no differences in salt-in-moisture content between treated and untreated ovine milk cheeses (Table 6). In caprine milk cheeses the differences were observed at the end of the ripening when control cheeses had higher salt content than farm and pressurized cheeses (Table 5). Some authors related HHP-treatments with a faster salt diffusion from the rind to the core of Gouda, Garrotxa and ewe ripened cheeses (Messens et al., 1998; Saldo et al., 2002a; Juan et al., 2008). In our study, control and HHP treated cheeses were cut to adjust them to the diameter of HHP machine cylinder, thus the initial format and rind was reduced, so the high amount of salt in control cheeses may be due to the speed of water losing and recovering of the rind.

Table 5. Changes in the physicochemical properties of the caprine milk cheeses during the ripening

	Day of ripening	Control	Farm	HHP1
pH	6	5.11 ± 0.05^{a}		5.13 ± 0.06^{a}
	15	5.08 ± 0.05^{a}	5.09 ± 0.01^{a}	5.11 ± 0.05^{a}
	30	5.05 ± 0.04^{ab}	5.05 ± 0.01^{ab}	5.09 ± 0.02^{ab}
	45	4.99 ± 0.04^{b}	4.98 ± 0.01^{bc}	5.04 ± 0.03^{ab}
	60	4.90 ± 0.08^{c}	4.95 ± 0.01^{c}	5.01 ± 0.03^{b}
Moisture	6	41.77 ± 0.60^{a}		42.16 ± 0.73^{a}
(%)	15	39.76 ± 1.53^{a}	38.37 ± 2.27^{a}	40.10 ± 1.08^{ab}
	30	35.55 ± 0.72^{b}	35.35 ± 0.16^{b}	36.23 ± 0.34^{c}
	45	31.99 ± 0.82^{c}	32.65 ± 1.48^{c}	31.97 ± 2.03^{d}
	60	28.89 ± 1.73^{d}	30.20 ± 1.23^{c}	29.51 ± 1.29^{d}
NaCl in	6	2.69 ± 0.11^{a}		2.75 ± 0.23^{a}
moisture	15	3.25 ± 0.47^{a}	3.21 ± 0.49^{a}	3.26 ± 0.23^{ab}
(%)	30	3.92 ± 0.49^{b}	3.89 ± 0.15^{b}	3.82 ± 0.14^{bc}
	45	4.69 ± 0.44^{c}	4.14 ± 0.43^{b}	4.41 ± 0.33^{c}
	60	5.68 ± 0.47$^{d\ B}$	4.82 ± 0.09$^{c\ A}$	5.09 ± 0.39$^{d\ A}$
Fat	6	35.65 ± 1.91		34.59 ± 1.29
(%)	15	34.83 ± 2.74	35.02 ± 0.23	34.49 ± 1.67
	30	35.67 ± 2.14	33.90 ± 1.27	34.76 ± 1.47
	45	35.77 ± 2.04	34.40 ± 1.23	34.40 ± 1.02
	60	35.84 ± 2.05	32.74 ± 2.33	35.21 ± 1.73
TN	5	6.15 ± 0.28		6.08 ± 0.27
(%)	15	6.02 ± 0.20	6.01 ± 0.17	6.13 ± 0.25
	30	6.29 ± 0.30	6.13 ± 0.40	6.21 ± 0.29
	45	6.40 ± 0.25	5.95 ± 0.17	6.17 ± 0.42
	60	6.43 ± 0.37	6.08 ± 0.47	6.32 ± 0.39

Fat and total nitrogen are expressed in dry basis.
(±) Values are means standard deviations from 3 different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significantly (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significantly (P<0.05)

Table 6. Changes in the physicochemical properties of the ovine milk cheeses during the ripening

	Day of ripening	Control	Farm	HHP1	HHP15
pH	5	5.05 ± 0.06$^{a\,A}$		5.20 ± 0.04$^{a\,B}$	
	15	5.02 ± 0.07$^{ab\,A}$	5.00 ± 0.09^{A}	5.20 ± 0.07$^{a\,B}$	5.09 ± 0.07^{AB}
	30	4.94 ± 0.09$^{ab\,A}$	4.95 ± 0.09^{A}	5.09 ± 0.09$^{ab\,B}$	5.03 ± 0.02^{AB}
	45	4.89 ± 0.07$^{b\,A}$	4.91 ± 0.06^{AB}	5.01 ± 0.06$^{bc\,B}$	5.01 ± 0.05^{B}
	60	4.93 ± 0.08$^{b\,A}$	4.91 ± 0.08^{A}	4.93 ± 0.03$^{c\,A}$	5.07 ± 0.04^{B}
Moisture	5	42.91 ± 1.89^{a}		42.06 ± 2.71^{a}	
(%)	15	37.77 ± 0.83$^{b\,A}$	40.45 ± 1.18$^{a\,B}$	38.71 ± 1.38$^{b\,AB}$	38.18 ± 1.92$^{a\,A}$
	30	32.92 ± 1.06$^{b\,A}$	37.11 ± 1.74$^{b\,B}$	34.93 ± 1.03$^{c\,A}$	33.96 ± 0.26$^{b\,A}$
	45	29.75 ± 1.73$^{d\,A}$	35.74 ± 1.49$^{b\,B}$	30.17 ± 0.43$^{d\,A}$	29.80 ± 0.99$^{c\,A}$
	60	26.35 ± 0.47$^{e\,A}$	32.65 ± 0.88$^{c\,B}$	27.40 ± 0.37$^{e\,A}$	26.78 ± 1.76$^{d\,A}$
NaCl in	5	2.08 ± 0.18^{a}		2.34 ± 0.49^{a}	
moisture	15	2.75 ± 0.18^{b}	3.08 ± 0.37^{a}	2.90 ± 0.43^{ab}	2.90 ± 0.23^{a}
(%)	30	3.60 ± 0.30^{c}	3.67 ± 0.31^{b}	3.44 ± 0.37^{b}	3.49 ± 0.11^{b}
	45	4.09 ± 0.72^{c}	4.23 ± 0.25^{b}	4.18 ± 0.40^{c}	4.14 ± 0.24^{b}
	60	4.93 ± 0.51^{d}	4.80 ± 0.14^{c}	4.92 ± 0.30^{d}	5.27 ± 0.45^{c}
Fat	5	42.29 ± 1.26		41.76 ± 1.97^{a}	
(%)	15	40.07 ± 0.80	39.37 ± 1.30	39.78 ± 0.56^{a}	40.04 ± 0.64
	30	39.70 ± 1.25^{A}	39.05 ± 0.80^{A}	49.51 ± 18.00$^{b\,B}$	39.24 ± 0.72^{A}
	45	38.89 ± 0.50	38.42 ± 0.78	38.39 ± 1.54^{a}	38.22 ± 0.28
	60	38.74 ± 0.85	38.13 ± 1.16	38.60 ± 1.69^{a}	38.48 ± 0.76
TN	6	5.50 ± 0.10		5.48 ± 0.26	
(%)	15	5.51 ± 0.19	5.31 ± 0.17	5.40 ± 0.28	5.50 ± 0.15
	30	5.52 ± 0.17	5.31 ± 0.17	6.86 ± 2.76	5.65 ± 0.13
	45	5.62 ± 0.16	5.31 ± 0.14	5.52 ± 0.06	5.73 ± 0.03
	60	5.52 ± 0.17	5.21 ± 0.13	5.54 ± 0.11	5.75 ± 0.13

Fat and total nitrogen are expressed in dry basis.
(±) Values are means standard deviations from 4 different batches
a-e Means with different superscript in the same column for the same parameter and sample differ significantly (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significantly (P<0.05)

12.2. Microbiological analysis

Microbial population of caprine and ovine milk cheeses was generally influenced by the type of treatment, with a significant reduction on bacterial counts when HHP was applied (Table 7 and 8).

LAB was the major microbial group in both kinds of cheese, with around 8 log cfu g^{-1}. Lactoccocci and lactobacilli counts were stable in farm and control samples and no differences were observed between them during all ripening stages. These results agreed with those reported by other authors in ripened caprine and ovine raw milk cheeses (Novella-Rodréguez et al., 2004a; Arqués et al., 2006; Cabezas et al., 2007; Juan et al., 2007a). However in CG samples, at the end of ripening, lactoccocci counts showed a significant reduction (Table 7). Similar results were reported by (Buffa et al., (2001a) who found that lactoccocci counts decreased 1-2 log cycles in 45 days in cheese made from raw and HHP-treated caprine milk.

The application of HHP affects significantly in the reduction of LAB counts. The decrease for lactoccocci counts one day after the HHP1 treatment in caprine and ovine cheeses, were 2.2 log cfu g^{-1} and 1.5 log cfu g^{-1} and for lactobacilli 3.41 log cfu g^{-1} and 1.03 log cfu g^{-1}, respectively. However, a subsequent recovery of these counts was observed and no statistical differences were found with control samples at the end of the ripening. In ovine cheeses with HHP-treatment at day 15 (EHHP15) a reduction about 1.91 log cfu g^{-1} and 1.33 log cfu g^{-1} for lactoccocci and lactobacilli was observed, respectively, although the subsequent recovery of the cells was not enough to reach similar loads as control (Table 8). Similar results were obtained by Novella-Rodríguez et al., (2002b) who observed that starter counts reduced about 2 log cycles in pressurized goat cheeses, although a subsequent increase was found during ripening. Rynne et al., (2008) reported significant reductions, about 1.5 log cycles, in starter and non starter lactic acid bacteria (NSLAB) in cheddar cheese treated at 400 MPa on the first day and also, in ovine milk ripened cheeses treated at 300 MPa and 400 MPa on days 1 and 15 proved to produce similar reductions although recovery of LAB counts only was observed in samples treated on day 1 (Juan et al., 2004; Juan et al., 2007a) (Juan et al., 2004; Juan et al., 2007a).

In general enteroccocci counts in untreated ovine and caprine milk cheeses were similar between control and farm samples. Enterococci counts in caprine milk cheeses remained practically constant throughout ripening, whereas a decrease of around 2 log cfu g^{-1} was observed on day 45 in ovine milk cheeses, remaining constant until the 60^{th} day (Tables 7

and 8). The enteroccocci counts were similar to those reported in other types of caprine and ovine raw milk cheeses, where this group are usually present, and remained stable with slight variations at the end of the ripening, reflecting the resistance to a variety of adverse conditions, such as high salt concentration, high or low pH values, low water activity and high or low temperatures (Mas et al., 2002; Novella-Rodríguez et al., 2004b; Ortigosa et al., 2006; Cabezas et al., 2007). Presence of high amounts of enteroccoci is associated with unhygienic production and processing of milk, but is also considered as important for in the development of the typical aroma and flavor of traditional Mediterranean cheeses made from raw and pasteurized ovine and caprine milk, were their counts can range from 10^4 to 10^6 cfu g^{-1} in curds and 10^5 to 10^7 cfu g^{-1} in ripened cheeses (Foulquié Moreno et al., 2006).

When the HHP-treatment was applied it caused a significant reduction in enteroccocci counts of 1.79 log cfu g^{-1}, 2.43 log cfu g^{-1} and 2.28 log cfu g^{-1} in GHHP1, EHHP1 and EHHP15 samples, respectively (Table 7 and 8), remaining steady up to the 60^{th} day of ripening. Arqués et al., (2006) reported a reduction of 2.68 log cycles in enteroccocci counts when a treatment of 400 MPa for 10 min at 10 ºC was applied on 2 days old "La Serena" cheese, and subsequently these counts remained constant throughout ripening. Nevertheless, Buffa et al., (2001a) found, in cheese made from HHP-treated caprine milk, an initial reduction around 3 log units of enteroccocci, and thereafter these counts maintained stable with a slight decrease during ripening. However, these authors did not found any statistical difference between the HHP-samples and the control cheeses at the end of ripening Bover-Cid et al., (2009) studied the behavior of 8 strains of enterococci isolated from meat enviroment after the application of HHP at 200, 400 and 600 MPa and observed that the inactivation extent depended on the treatment and strain.

The maximum counts of *Enterobactericeae* were observed at the initial stages of ripening (6.33 log cfu g^{-1} and 4.65 log cfu g^{-1} in GC and EC samples, respectively), decreasing gradually throughout the time until reaching counts of around 2.5 log cfu g^{-1} at day 60. Similar reductions on the enterobacteria counts were reported by other authors in cheeses made from raw ovine and caprine milk (Novella-Rodriguez et al., 2004b; Ortigosa et al., 2006). Similarly, *Escherichia coli* counts decreased gradually throughout ripening (Table 7 and 8). Levels below the detection limit at the 60^{th} day of ripening were found in untreated carpine cheeses and only a slight number of cells remained viable in ovine milk control and farm cheeses. These counts complies with the limits established by the European Regulation EC 2073/2005, for the heat-treated milk cheeses (*Escherichia coli* n =5, c = 2, m= 10^2cfu g^{-1}, M = 10^3cfu g^{-1}) (Anonymous, 2005). High counts of *Enterobactericeae* at the beginning of

the ripening and the remaining loads of this family at the end of this period reflected that the conditions of the cheese matrix during the 60 days of ripening were not tough enough to eliminate them. Quality control for raw material and Good Manufacturing Practices are important to follow.

EHHP1 and GHHP1 cheeses showed enterobacteria counts reduction of about 4.5 log cfu g^{-1} after the treatment, reaching not detected and 1.92 log cfu g^{-1} levels, respectively. Afterwards, a slight growth was noted on the 15th day, although these counts were not detected at the 30th and 45th days of ripening in ovine and caprine milk cheeses, respectively (Tables 7 and 8). Likewise, EHHP15 samples showed no detected levels of enterobacteria after the treatment, with a slight increase on the 30th day and it is subsequently reduction. This behavior could be due to a possible recovery of the sublethal injured cells after the HHP treatment, although cheese ripening conditions, such as the low pH, the salt concentration and the presence of LAB make difficult this recovery. Juan et al., (2007a) detected in an ovine milk cheese a reduction of about 3 and 3.92 log cycle after a 400 MPa treatment applied the 1st and the 15th days, respectively, being not detected during the ripening.

Undetectable levels of *Escherichia coli* in all HHP-treated cheeses were observed one day after the application with a little increase detected on day 30 in GHHP1-samples and on day 45 in EHHP15 samples. However no detectable levels were discovered at the end of ripening (Tables 7 and 8). Our results denoted that *Enterobactericeae* and *Escherichia coli* were more pressure sensitive than other bacteria and are in accordance to different studies. Capellas et al., (1996) observed a reduction range 6.7 to 8.7 log cycles of *E. coli* inoculated in fresh caprine milk Mató cheese treated with pressures 400 to 500 MPa for 5-15 min at 2-25 ºC. Likewise, De Lamo-Castellvi et al., (2006) inoculated in bovine milk model cheeses produced with starter culture with strains of *Escherichia coli* and observed that a maximum lethality was achieved when cheeses were treated at 400 and 500 MPa for 10 min at 20 ºC. They also observed that the presence of starter and the subsequent low pH of the samples can control the ability of cells to repair and grow during the 15 days of storage at 8 ºC. O'Reilly et al., (2000) observed that *E. coli* exhibited a greatest sensitivity to HHP in Cheddar cheeses than in the cheese slurries system. They suggested that the acid injury during the fermentation and the phase of growth may explain this behavior.

Staphylococcus aureus counts in untreated cheeses decreased progressively during ripening to undetectable levels at the end of ripening, except in control ovine milk cheeses, where a slight number remained (Tables 7 and 8). These counts were achieved within the limits established by the European Regulation EC 2073/2005 for raw milk cheeses

(*Staphylococcus aureus* n =5, c = 2, m= 10^4 cfu g^{-1}, M = 10^5cfu g^{-1}) (Anonymous, 2005). In caprine milk pressurized cheeses a significant diminution of 2.21 log units was found, reaching undetectable levels at the 60th day. EHHP1 and EHHP15 samples showed a count reduction of about 1.28 log cfu g^{-1} and 1.41 log cfu g^{-1}, respectively, with a progressive decrease to undetectable levels the 45th day. Reduction on counts of coagulase-positive staphylococci were also observed by (Arqués et al., (2006), who reported an initial reduction of 1.45 log units until being undetectable the 30th day in *La Serena* cheeses treated at 400 MPa on the second day. Moreover, a reduction of *S. aureus* of around 3 and 2 log cycles was reported by O'Reilly et al., (2000) in inoculated Cheddar cheeses and cheese slurries treated at 400 MPa for 20 min at 20 ºC. In bovine milk model cheeses inoculated with two strains of *S. aureus*, López-Pedemonte et al., (2007b) found 2 and 3 log cycles of inactivation the 1st day after applying 400 and 500 MPa for 10 min at 20 ºC HHP-treatments, respectively. The maximum mean reductions were reached for 500 MPa treatments after the 30th day ripening period at 8 ºC (around 6 log units). However, *S. aureus* enterotoxin was detected in all samples before and after treatments. *S. aureus* is one of the agents mostly related with foodborne intoxication caused by milk and dairy products consumption in many countries and has also been described as one of the most HHP resistant non-sporulated Gram positive bacteria (López-Pedemonte, 2006).

Lysteria monocytogenes or *Salmonella spp.* were not detected in any caprine or ovine milk cheeses studied. Therefore, no conclusion about the efficiency of HHP-treatment on these pathogenic microorganisms can be obtained, although some studies on the destruction of foodborne pathogens in cheeses have been carried out by different researchers. Wuytack et al., (2002) reported around 5 log cycles of inactivation in a cell suspension of stationary phase *Salmonella tiphymurium* by the application of 300 MPa at 25 ºC for 15 min. In the same line, HHP-treatments of 400 MPa at 25ºC for 15 min caused the inactivation of around 6 log units of *L. inocua* in a cell suspension. López-Pedemonte et al., (2007a) evaluated the inactivation of two strains of *L. monocytogenes* (Scott A and NCTC 1194) inoculated at levels of 7.5 log cfu g^{-1} in model wash-curd cheeses by HHP treatments. They found ranges of inactivation of 1.5–2.9 log and around 5 log cycles when 400 MPa and 500 MPa during 10 min at 20 ºC treatments were applied, respectively. After 30 days of storage at 8 ºC, the *L. monocytogenes* counts reached levels between 2.5 and below the quantification limit. The study of De lamo-Castellvi et al., (2006) observed around 5 log cycles of lethality for *Salmonella enteritidis* and *S. tiphymurium* in a model cheese treated at 400 MPa for 10 min. Both strains were not able to repair after 15 days of storage at 12 ºC and keep their counts

bellow the detection level.

In general, our results are in accordance to many authors, whom related the degree of microbial inactivation with many factors such as the type of cheese, the stage of ripening, HHP processing conditions applied, the microbial species, type of strain and their phase of growth (Smenlt, 1998; O'Reilly et al., 2001; Trujillo et al., 2002b; López-Pedemonte et al., 2007; Juan et al., 2007a).

Table 7. Changes in microbial population (log cfu g^{-1}) of the caprine milk cheeses during the ripening

	Day of ripening	Control	Farm	HHP1
Lactococci	6	9.55 ± 0.27$^{a\,A}$		7.30 ± 0.22^{B}
	15	8.75 ± 0.34$^{b\,A}$	8.63 ± 0.54^{A}	6.76 ± 0.49^{B}
	30	8.47 ± 0.26$^{b\,A}$	8.67 ± 0.17^{A}	7.03 ± 0.64^{B}
	45	8.26 ± 0.20$^{b\,A}$	8.50 ± 0.26^{A}	7.19 ± 0.25^{B}
	60	7.54 ± 0.51$^{c\,A}$	8.20 ± 0.54^{B}	7.05 ± 0.17^{A}
Lactobacilli	6	6.54 ± 0.31$^{a\,A}$		3.13 ± 0.26$^{a\,B}$
	15	8.36 ± 0.42$^{b\,A}$	8.45 ± 0.49^{A}	5.23 ± 1.54$^{b\,B}$
	30	8.28 ± 0.15$^{b\,A}$	8.64 ± 0.39^{A}	6.31 ± 0.33$^{c\,B}$
	45	8.18 ± 0.19$^{b\,A}$	8.08 ± 0.08^{A}	6.80 ± 0.50$^{c\,B}$
	60	7.89 ± 0.45$^{b\,AB}$	8.35 ± 0.35^{A}	7.03 ± 0.22$^{c\,B}$
Enterococci	6	6.23 ± 0.24^{A}		4.44 ± 0.44$^{ab\,B}$
	15	6.10 ± 0.70^{A}	5.54 ± 0.75^{A}	3.99 ± 0.24$^{ab\,B}$
	30	6.17 ± 0.47^{A}	6.24 ± 0.34^{A}	4.60 ± 0.96$^{b\,B}$
	45	6.17 ± 0.48^{A}	5.81 ± 0.69^{A}	3.45 ± 0.34$^{a\,B}$
	60	5.32 ± 0.64^{A}	5.43 ± 0.73^{A}	3.85 ± 0.59$^{ab\,B}$
Enterobacteria	6	6.33 ± 0.26$^{a\,A}$		1.92 ± 0.54$^{a\,B}$
	15	3.15 ± 2.75$^{b\,AB}$	4.34 ± 0.34$^{a\,A}$	2.49 ± 0.36$^{a\,B}$
	30	4.54 ± 0.15$^{b\,A}$	4.09 ± 0.68$^{a\,A}$	0.86 ± 1.48$^{a\,B}$
	45	3.58 ± 0.40$^{b\,A}$	3.27 ± 0.60$^{ab\,A}$	ND ± ND$^{b\,A}$
	60	2.94 ± 0.54$^{b\,A}$	2.21 ± 0.49$^{b\,A}$	ND ± ND$^{b\,A}$
E. coli	6	2.56 ± 0.62$^{b\,A}$		ND ± ND^{B}
	15	0.90 ± 0.78^{ab}	1.47 ± 0.48^{ab}	ND ± ND
	30	1.60 ± 1.96^{ab}	2.31 ± 2.15^{b}	0.83 ± 1.44
	45	0.77 ± 0.68^{ab}	0.33 ± 0.58^{a}	ND ± ND
	60	ND ± ND^{a}	ND ± ND^{ab}	ND ± ND
S. aureus	6	2.64 ± 0.15$^{a\,A}$		0.43 ± 0.75^{B}
	15	1.06 ± 0.96$^{b\,A}$	0.33 ± 0.58^{A}	ND ± ND^{A}
	30	1.18 ± 0.31$^{b\,A}$	ND ± ND^{B}	ND ± ND^{B}
	45	0.77 ± 1.33$^{b\,A}$	0.57 ± 0.98^{B}	0.33 ± 0.58^{B}
	60	ND ± ND	ND ± ND	ND ± ND

ND. Not detected (<10 cfu g^{-1})
(±) Values are means standard deviations of from the different batches
a-c Means with different superscript in the same column for the same parameter and sample differ significantly (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significantly (P<0.05)

Table 8. Changes in microbial population (log cfu g^{-1}) of the ovine milk cheeses during the ripening

	Day of ripening	Control	Farm	HHP1	HHP15
Lactococci	5	$9.34 \pm 0.33^{a\,A}$		$7.80 \pm 0.31^{a\,B}$	
	15	$9.16 \pm 0.20^{a\,A}$	$9.36 \pm 0.10^{a\,A}$	$6.87 \pm 0.39^{b\,B}$	$7.25 \pm 0.22^{a\,B}$
	30	$9.21 \pm 0.18^{a\,A}$	$9.15 \pm 0.33^{a\,A}$	$8.04 \pm 0.74^{b\,B}$	$7.67 \pm 0.14^{ab\,B}$
	45	$8.74 \pm 0.20^{ab\,A}$	$8.88 \pm 0.38^{ab\,A}$	$8.07 \pm 0.61^{b\,B}$	$7.92 \pm 0.39^{ab\,B}$
	60	$8.38 \pm 0.16^{b\,A}$	$8.37 \pm 0.40^{b\,A}$	$8.32 \pm 0.76^{b\,A}$	$7.57 \pm 0.32^{b\,B}$
Lactobacilli	5	$5.57 \pm 0.35^{a\,A}$		$4.54 \pm 0.64^{a\,B}$	
	15	$7.52 \pm 1.07^{b\,A}$	7.74 ± 0.23^{A}	$5.60 \pm 0.37^{b\,B}$	$6.19 \pm 0.50^{a\,B}$
	30	$8.64 \pm 0.41^{c\,A}$	8.62 ± 0.56^{A}	$6.61 \pm 0.82^{c\,B}$	$6.78 \pm 1.00^{ab\,B}$
	45	$8.68 \pm 0.12^{c\,A}$	8.62 ± 0.41^{A}	$8.09 \pm 0.55^{d\,A}$	$7.82 \pm 0.27^{bc\,A}$
	60	$8.15 \pm 0.39^{c\,A}$	7.89 ± 0.05^{A}	$8.12 \pm 0.51^{d\,A}$	7.53 ± 0.15^{cB}
Enterococci	5	5.59 ± 0.74^{A}		3.16 ± 1.05^{A}	
	15	6.18 ± 2.14^{AB}	$6.91 \pm 2.46^{a\,A}$	3.50 ± 1.13^{C}	3.90 ± 1.11^{B}
	30	6.16 ± 1.18^{A}	$622 \pm 1.44^{a\,A}$	1.76 ± 1.53^{B}	3.96 ± 1.32^{A}
	45	4.63 ± 1.04^{A}	$4.19 \pm 0.70^{b\,A}$	2.76 ± 2.26^{B}	3.27 ± 0.48^{A}
	60	4.23 ± 1.40^{A}	$4.02 \pm 1.25^{b\,A}$	2.58 ± 1.93^{B}	3.88 ± 0.93^{A}
Enterobacteria	5	$4.65 \pm 0.23^{a\,A}$		$ND \pm ND^{B}$	
	15	$4.46 \pm 0.30^{a\,A}$	$4.46 \pm 0.42^{a\,A}$	0.29 ± 0.58^{B}	$ND \pm ND^{B}$
	30	$4.42 \pm 1.92^{a\,A}$	$4.52 \pm 0.31^{a\,A}$	$ND \pm ND^{B}$	1.22 ± 1.11^{B}
	45	$2.87 \pm 1.21^{b\,A}$	$3.71 \pm 0.54^{ab\,A}$	$ND \pm ND^{B}$	0.57 ± 0.98^{B}
	60	$2.35 \pm 0.96^{b\,A}$	$2.64 \pm 0.43^{b\,A}$	$ND \pm ND^{B}$	$ND \pm ND^{B}$
E. coli	5	1.16 ± 1.01		$ND \pm ND$	
	15	1.15 ± 1.37	0.33 ± 0.58	$ND \pm ND$	$ND \pm ND$
	30	0.65 ± 0.79	0.50 ± 0.58	$ND \pm ND$	$ND \pm ND$
	45	0.64 ± 0.73	0.93 ± 0.64	$ND \pm ND$	0.78 ± 1.35
	60	0.73 ± 0.91	0.25 ± 0.50	$ND \pm ND$	$ND \pm ND$
S. aureus	5	$2.35 \pm 0.58^{a\,A}$		1.07 ± 1.35^{A}	
	15	$1.74 \pm 1.19^{ab\,AB}$	$2.23 \pm 1.33^{a\,A}$	0.50 ± 0.58^{B}	0.33 ± 0.58^{B}
	30	$1.17 \pm 0.35^{ab\,A}$	$1.08 \pm 1.57^{ab\,A}$	0.29 ± 0.58^{A}	0.33 ± 0.58^{A}
	45	$0.61 \pm 0.71^{bc\,A}$	$0.99 \pm 1.33^{ab\,A}$	$ND \pm ND^{A}$	$ND \pm ND^{A}$
	60	$0.25 \pm 0.50^{c\,A}$	$ND \pm ND^{b\,A}$	$ND \pm ND^{A}$	$ND \pm ND^{A}$

ND: Not Detected (<10 cfu g^{-1})
(±) Values are means standard deviations from the different batches
a-c Means with different superscript in the same column for the same parameter and sample differ significantly (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significantly (P<0.05)

13. ASSESMENT OF PROTEOLYSIS DURING THE RIPENING OF CAPRINE AND OVINE MILK CHEESES TREATED BY HIGH HYDROSTATIC PRESSURE

13.1. Proteolysis in caprine milk cheeses

The evolution of the proteolysis measured by the ratio of pH 4.6 water soluble nitrogen (WSN/TN), trinitrobencensulphonic acid (TNBS) and free amino acids (FAA) during the ripening of caprine milk cheese is presented in Table 9. Farm cheeses showed lower TNBS and FAA values than those observed in control samples, although on FAA content significant differences were only observed at day 45. In general TNBS and FAA values increased during the ripening of the cheeses. Significant differences between control and GHHP1 samples in TNBS were obseved from the 45th to the 60th day of ripening, while the differences in FAA content were noted from day 30 to 60. In both parameters control samples showed higher values

The application of HHP resulted in a decrease of TNBS and FAA values in proximately 30% in relation to control samples, at the end of the ripening, on both parameters. The ratio of pH 4.6 WSN/TN displayed a different trend. Proteolysis was more intense during the first 15 days period, where an increment of around two times was observed in control and HHP-treated samples, without differences between them, after this point the proteolysis rate became slower. Cheeses with HHP treatment showed slightly higher values than control samples from the 30th to the 60th day, although no significant differences were found. The evolution of this parameter in farm cheeses were similar than control samples.

13.2. Proteolysis in ovine milk cheeses

An increment on the three proteolysis index evaluated was observed during the maturity period (Table 10). EHHP15 samples presented slightly higher values than EC samples in all these parameters, especially in TNBS, although no significant differences were observed at the end of the ripening. The samples pressurized on the first stages of maturity (EHHP1) showed an intense proteolysis on the first 30 days. After this time, a decrease on the rate was noticed, obtaining a considerable reduction of TNBS, WNS/TN, and FAA values on the 60th day, reflecting, in this case, that pressure treatment provoked a decelerated rate of proteolysis, mainly at the last stage of ripening. On the other hand, the proteolysis in farm samples was significantly lower than those obtained in control samples, showing around 36% lower TNBS

and WNS/TN values, and 50% FAA values at day 60.

Table 9. Evolution of proteolysis index during the ripening of caprine milk cheese with and without HHP treatment

	Day of ripening	Control	Farm	HHP1
TNBS	5	10.40 ± 2.24^{a}		9.41 ± 1.80^{a}
	15	14.90 ± 1.21^{ab}	13.93 ± 3.61^{a}	15.33 ± 2.15^{ab}
	30	22.54 ± 2.50^{b}	24.21 ± 2.32^{ab}	21.14 ± 4.59^{b}
	45	44.89 ± 1.19$^{c\,B}$	32.34 ± 4.89$^{bc\,A}$	32.65 ± 5.81$^{c\,A}$
	60	53.66 ± 2.14$^{d\,B}$	38.30 ± 6.15$^{c\,A}$	39.96 ± 4.52$^{c\,A}$
% WNS/TN	5	10.99 ± 3.05^{a}		11.09 ± 2.60^{a}
	15	22.64 ± 0.56^{b}	19.42 ± 0.88^{a}	22.97 ± 1.23^{b}
	30	21.88 ± 0.60$^{b\,AB}$	19.38 ± 2.22$^{a\,A}$	24.39 ± 3.15$^{bc\,B}$
	45	22.14 ± 1.78^{b}	20.74 ± 0.90^{ab}	25.71 ± 2.15^{bc}
	60	25.07 ± 0.66^{b}	24.52 ± 2.06^{b}	28.03 ± 0.35^{c}
FAA	5	2.08 ± 0.57^{a}		1.48 ± 0.72^{a}
	15	4.85 ± 1.42^{a}	3.71 ± 1.17^{a}	3.71 ± 1.06^{ab}
	30	10.05 ± 2.06$^{b\,B}$	8.98 ± 0.76$^{b\,AB}$	5.90 ± 1.59$^{bc\,A}$
	45	15.07 ± 3.52$^{c\,B}$	10.34 ± 1.43$^{b\,A}$	8.40 ± 1.43$^{cd\,A}$
	60	15.71 ± 3.06$^{c\,B}$	14.45 ± 1.00$^{c\,B}$	10.40 ± 1.29$^{d\,A}$

TNBS and FAA expressend in mg L-Leu g^{-1}
(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

Table 10. Evolution of proteolysis index during the ripening of ovine milk cheese with and without HHP treatment

	Day of ripening	Control	Farm	HHP1	HHP15
TNBS	6	7.66 ± 1.65^{a}		7.92 ± 0.44^{a}	
	15	13.87 ± 1.37^{ab}	9.84 ± 2.18^{a}	13.36 ± 0.82^{a}	15.03 ± 0.40^{a}
	30	22.13 ± 8.22$^{b\ AB}$	12.40 ± 1.16$^{a\ A}$	26.33 ± 17.48$^{b\ BC}$	34.31 ± 6.11$^{b\ C}$
	45	34.93 ± 2.60$^{c\ BC}$	19.08 ± 4.05$^{a\ A}$	28.00 ± 2.84$^{b\ AB}$	44.79 ± 4.93$^{bc\ C}$
	60	47.40 ± 2.74$^{d\ BC}$	31.48 ± 2.79$^{b\ A}$	37.59 ± 6.86$^{b\ A}$	52.50 ± 2.21$^{c\ C}$
%WNS/TN	6	9.70 ± 3.55^{a}		10.44 ± 4.39^{a}	
	15	19.38 ± 4.24^{b}	11.81 ± 2.61	18.51 ± 4.23^{b}	19.15 ± 2.57^{a}
	30	24.96 ± 3.64$^{bc\ B}$	14.93 ± 4.33^{A}	23.35 ± 2.38$^{b\ B}$	25.39 ± 3.57$^{ab\ B}$
	45	29.59 ± 2.97$^{c\ B}$	18.56 ± 1.35^{A}	25.09 ± 2.95$^{b\ A}$	30.54 ± 2.46$^{b\ B}$
	60	31.92 ± 6.63$^{c\ B}$	19.52 ± 3.88^{A}	26.38 ± 3.77$^{b\ A}$	31.62 ± 2.21$^{b\ B}$
FAA	6	2.36 ± 0.04^{a}		2.18 ± 0.22^{a}	
	15	5.01 ± 1.27^{a}	2.65 ± 1.11^{a}	3.66 ± 1.79^{ab}	5.59 ± 2.94^{a}
	30	11.66 ± 2.76$^{b\ B}$	4.31 ± 0.29$^{ab\ A}$	6.85 ± 3.07$^{ab\ AB}$	11.22 ± 1.11$^{b\ B}$
	45	11.39 ± 2.69$^{b\ B}$	5.46 ± 0.74$^{ab\ A}$	6.55 ± 1.10$^{ab\ AB}$	14.86 ± 4.89$^{bc\ C}$
	60	16.56 ± 3.51$^{c\ B}$	8.26 ± 1.07$^{b\ A}$	8.63 ± 1.71$^{b\ A}$	16.03 ± 5.14$^{c\ B}$

TNBS and FAA expressend in mg L-Leu g^{-1}
(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-C Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

In ovine and caprine milk cheeses, TNBS, WSN/TN and FAA values indicated an increase of proteolysis during the ripening. In ovine and caprine milk farm cheeses, the degree of proteolysis was similar to those reported by other authors in ripened bovine, ovine and caprine raw milk cheeses (Izco et al., 2000; Mas et al., 2002; Trujillo et al., 2002a; González et al., 2003; Novella-Rodriguez et al., 2004b; Cabezas et al., 2007; Marino et al., 2008). In our results ovine milk farm cheese presented lower values than control samples in all three parameters, suggesting that the proteolysis, in general, was slower. This phenomenon could be explained by the fact that the characteristics of the farm cheeses, such as diameter (25 cm), weight (1300 g) and kind of rind differ from the control cheese (8 cm and 300 g). The different size of these cheeses probably affected at three important factors in the proteolysis rate as the degree of anaerobiosis, salt and a_w values (Bover-Cid et al., 1999; Suzzi and Gardini, 2003). On the other hand, the differences in the format (diameter and weight) of caprine milk farm and control cheeses were not too big and this could permit that both type of samples had a similar trend for WSN/TN and FAA indexes, although in TNBS parameter the control cheeses had significantly higher values.

In caprine and ovine milk cheeses the proteolysis in the first 15 days of ripening was similar in control and HHP treated samples, and values measured by WNS/TN indicated that this process was more intense in this period. HHP treatment provoked a decrease on lactic acid bacteria counts (Seccion 2.2), thus in the first 15 days of ripening the main proteolytic activity was probably caused by rennet and milk proteinases. On fact, WSN is produced mainly by rennet and to a lesser extent, by plasmin or cellular proteinases, whereas those starter proteinases/peptidases are primarily responsible for the formation of small peptides and free amino acids (Fox, 1989). Messens et al., (1999) observe that chymosin and plasmin activity in Gouda cheese were not influenced by pressure (from 50 to 400 MPa for 20-100 min). Similary, Malone et al., (2003) in a study of HHP effects (100-800 MPa, 5 min, 25 ºC) on the activity of proteolytic and glycolytic enzymes, observed that plasmin was insensible to pressure treatment and chymosin activity was unaffected by treatments up to 400 MPa, decreasing by 50% after a 800 MPa treatment. On the other hand, Juan et al., (2007a) found in ovine milk cheese that the chymosin activity decrease depending on the age of the cheese and the pressure applied (≥400 MPa, 1-day old cheese), whereas plasmin activity was not significantly affected by HHP treatments (200-500 MPa, for 10 min) applied on the 1st and 15th day of ripening.

On the three proteolysis index evaluated, EHHP15 cheeses showed slightly higher values than control samples during the ripening period, although no significant differences were observed

at the end of the ripening (Table10), reflecting that this kind of treatment did not significantly affect the proteolysis process. However, HHP application during the initial stages of the ripening in ovine and caprine milk cheeses led to a decrease of the proteolysis rate.

Similar results about the decrease of proteolysis rate caused by HHP application were obtained by other authors (Messens et al., 1999; Juan et al., 2004; Rynne et al., 2008). Juan et al., (2004), in pressurized ovine milk cheeses (200-500 MPa, 10 min 12ºC), reported that HHP-treatment applied on the 15th day of ripening had similar WSN/TN values than control samples, at the end of the ripening, but higher than those obtained in samples with HHP-treatment on rhe 1st day. However the FAA levels obtained in treatments at 400 MPa applied at day 1 and 15 were lower than those registered by untreated samples. In contrats, Messens et al., (1999) observed that HHP treatments (50-500 MPa, with holding times from 20 min to 3 days) in Gouda cheese did not influenced the proteolysis indexes and the FAA values during ripening, although these treatments led to an increase in pH shift. Likewise, in full-fat HHP-treated Cheddar cheeses (400 MPa for 10 min) it was observed little or no effect on levels of pH 4.6 WSN, which was the consistent with measured plasmin and chymosin activities, that were unaffected by HHP treatment. In addition this treatment had no significant effect on levels of total FAA in cheese over ripening (Rynne et al., 2008).

On the contrary, some surveys reported the increase of proteolysis related with HHP-treatments. Saldo et al., (2000) noticed that caprine milk cheeses HHP-treated at 400 MPa for 5 min exhibited a faster proteolysis and higher pH values than the untreated cheeses. They suggested that HHP accelerates ripening by increasing water retention and pH by releasing bacterial enzymes and by increasing enzyme activity under pressure. Messens et al., (2000) observed that the application of 50 MPa HHP treatments for 8 h on "Père Joseph" cheese, a semi-hard smear-ripened cheese, resulted in an increased pH value which intensified the proteolitic activity of *Brevibacterium linens* and the peptidases of the starter bacteria. Also, Trujillo et al., (2002a) observed that caprine milk cheeses made from HHP-treated milk (500 MPa, 15 min, 20 ºC) showed higher contents of WSN and free amino acids than the raw milk control cheeses.

It is generally assumed that proteolytic enzymes of starter cells are released in the cheese matrix when the cells lyses after death (Fox, 1989). Starter bacteria are one of the primary sources of ripening enzymes, many of which required for proteolysis (proteinases and peptidases) are intracellular and the lysis of bacteria is required for their liberation (Thomas and Pritchard, 1987). Meyer et al., (1989) reported three types of peptidase activity, aminopeptidase, dipeptidylpeptidase and dipeptidase, exclusively in the cytoplasm of *S.*

thermophilus and *Lactobacillus lactis,* which became active during cheese ripening only after cell lysis. O'Donovan et al., (1996) found a positive relationship between proteolysis in Cheddar cheese and lysis of starters (*Lactococcus lactis* ssp. c*remoris* AM2, 303 and HP), suggesting that inter-strain differences in the level of cell lysis/leakage is a major factor influencing the rate of secondary proteolysis in cheese. HHP-induced cell lysis is pressure and strain-dependent (Malone et al., 2002) and causes membrane damage and increases cellular permeability (Cheftel, 1992). Malone et al., (2003), evaluated the effect of HHP-treatments (400 MPa, 5 min, 25 ºC) on proteolytic and glycolytic enzymes extracted from from *Lactococcus lactis*, found that this treatments could cause activation of aminopeptidase C, inactivation of: acid production, of cell envelope protease, aminopeptidase N, X-prolyl-dipeptidyl aminopeptidase, Lys-Ala-ρ-nitroanilide hydrolysis; and unchanged activity of plasmin, chymosin and aminopeptidase A. Saldo et al., (2002a) suggested that in caprine milk cheeses the release of starter enzymes probably causes an increase in proteolysis and FAA amounts found on cheese two weeks after applying a pressure treatment at 400 MPa for 5 min. However, Juan et al., (2004) noticed that are necessary HHP-treatments above 400 MPa to delay the proteolysis and the FAA formation in ovine milk cheeses. Messens et al., (1999) indicated that in Gouda cheese the possible lysis of the starter bacterial cells resulting from the damage suffered at 400 MPa did not lead to an increase of FAA content, and suggested that the enzymatic system of endocellular enzymes was inactivated also by pressure and/or the absence of substrate for these enzymes. Also O'Reilly et al., (2003) pointed out that ripening enzymes in Cheddar cheese would probably begin to denature after the application of pressure treatments between 350-400 MPa.

14. EFFECT OF HIGH HYDROSTATIC PRESSURE ON TEXTURAL AND SENSORY CHARACTERISTICS OF CAPRINE AND OVINE MILK CHEESES

14.1. Uniaxial compression test

Tables 11 and 12 show the values of fracture stress and fracture strain obtained during the ripening of ovine and caprine milk samples, respectively. Fracture stress (σ_f) is the stress required for fracture and collapse of cheese mass beyond the point of recovery. This parameter is related with the strength of the cheese matrix, and low values indicate greater fracturability (O'Callaghan and Guinne, 2004). In general all ovine and caprine milk cheeses showed a significant increase of the fracture stress values throughout maturity, indicating that cheeses were less fracturable on day 60. It has been previously reported the progressive increase of breaking force during the ripening of artisan Manchego (Gaya et al., 1990; González Viñas et al., 2007). This increase appears to be caused specially by the decrease of moisture during the ripening period so that its protein matrix is strengthened and the softening produced by the protein degradation is eliminated (Núñez et al., 1986)

Cheeses with HHP1 treatment displayed a slightly decrease on fracture stress values one day after the application of the treatment, being significant in EHHP1 samples, with the following increase of these values over the time, showing significant higher levels than control samples from day 30 through the end of the ripening. Likewise, EHHP15 samples presented significant higher fracture stress values than EC samples in the last stages of the ripening (day 45 to 60), but significantly lower than EHHP1 samples from day 30 to 60. The increase of fracture stress values has been described by Rynne et al., (2008) on one day old Cheddar cheeses treated at 400 MPa, for 10 min and by Juan et al., (2007b) in ovine milk cheese treated on the first or the 15th day of ripening, at 400 MPa, for 10 min. Likewise, similar differences depending on the day of HHP-application were also observed (Juan et al., 2007b). This behavior could be due to the structural changes in cheese when the treatment was applied. HHP induced more continuous and closer protein matrix with more uniform and more homogeneously distribution of fat globules (Capellas et al., 2001; Buffa et al., 2001b). In addition, Creamer and Olson, (1982) related that the diminution of the fracturability of cheddar cheese during ripening was related to the content of intact casein in cheese. In our case, the HHP-treatment applied on the first stages of ripening decreased the proteolysis rate in ovine and caprine milk cheese. This fact combined with the structural changes caused by the HHP could contribute to reduce the fracturability of cheeses. However, the HHP treatments applied on ovine milk cheeses on the 15th day of ripening presented similar

proteolysis than control cheese. Thus the increase of the fracture stress values could be only due to of the formation of a more homogeneous microstructure.

Fracture strain (ε_f) is the deformation required to induce fracture. This parameter is related with brittleness and shortness or longness of cheese (O'Callaghan and Guinne, 2004). In this case higher numerical values express greater deformability (Zoon, 1991). In general, it was observed that this parameter significantly decreased throughout the ripening in all type of cheeses. The decrease of fracture strain with the aging has been previously described in Manchego (González Viñas et al., 2007) and cheddar cheese (Hort and Le Grys, 2001).

HHP1 caprine and ovine milk cheese samples showed about 2 and 1.6 times higher fracture strain values than control cheeses, respectively, one day after the pressure treatment. However, no differences were observed from day 15 toward the end of the ripening between control and HHP1 samples. EHHP15 cheeses showed similar fracture strain levels than EC samples, even so significant higher values were only observed at day 30. Besides, no differences in this parameter were found between EHHP1 and EHHP15 samples. All this indicated that cheese deformability was not significantly influenced by HHP. Some surveys found that the application of HHP in cheese could provoke higher fracture strain values. These studies observed that this coincides with the increase of pH values in HHP cheeses, which would contribute to a better solvation of casein (Juan et al., 2007b; Rynne et al., 2008). In our case, higher strain values were only observed in HHP-samples one day after the treatment and this could be attributed to the changes in the physical conformation of the protein matrix.

Table 11. Fracture stress (x10 kPa) and fracture strain values throughout the ripening of caprine milk cheeses with and without HHP treatment.

	Day of ripening	Control	HHP1
Fracture Stress	6	3.95 ± 0.66^{a}	3.31 ± 0.75^{a}
	15	6.53 ± 0.95^{b}	6.59 ± 0.66^{b}
	30	9.65 ± 0.81^{c}	10.12 ± 1.04
	45	14.70 ± 2.32^{d}	15.54 ± 1.68^{d}
	60	19.63 ± 3.26^{eA}	21.26 ± 3.15^{eB}
Fracture Strain	6	0.50 ± 0.05^{aA}	1.03 ± 0.45^{aB}
	15	0.35 ± 0.05^{b}	0.42 ± 0.06^{b}
	30	0.26 ± 0.03^{bc}	0.30 ± 0.02^{c}
	45	0.23 ± 0.03^{c}	0.24 ± 0.02^{c}
	60	0.20 ± 0.03^{c}	0.21 ± 0.02^{c}

(±) Values are means standard deviations of data from the different batches
a-e Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

Table 12. Fracture stress (x10 kPa) and fracture strain throughout the ripening of ovine milk cheeses with and without HHP treatment

	Day of ripening	Control	HHP1	HHP15
Fracture Stress	5	4.32 ± 0.93$^{a\,B}$	3.38 ± 0.57$^{a\,A}$	
	15	4.77 ± 0.89^{b}	5.09 ± 0.76^{b}	4.80 ± 1.05^{a}
	30	5.40 ± 1.17$^{c\,A}$	7.59 ± 0.84$^{c\,B}$	6.03 ± 0.96$^{a\,A}$
	45	7.93 ± 2.12$^{d\,A}$	10.98 ± 1.94$^{d\,C}$	9.85 ± 2.34$^{b\,B}$
	60	10.52 ± 2.63$^{e\,A}$	14.19 ± 2.97$^{e\,C}$	12.66 ± 1.84$^{c\,B}$
Fracture Strain	5	0.64 ± 0.26$^{a\,A}$	1.03 ± 0.38$^{a\,B}$	
	15	0.44 ± 0.08^{b}	0.48 ± 0.07^{b}	0.52 ± 0.09^{a}
	30	0.32 ± 0.03$^{c\,A}$	0.35 ± 0.06$^{bc\,AB}$	0.42 ± 0.06$^{b\,B}$
	45	0.27 ± 0.03^{cd}	0.27 ± 0.05^{c}	0.28 ± 0.04^{c}
	60	0.23 ± 0.02^{d}	0.24 ± 0.02^{c}	0.23 ± 0.04^{c}

(±) Values are means standard deviations of data from the different batches
a-e Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

14.2. Color determination

The mean of L* (lightness- darkness); a* (greenness-redness); b* (blueness-yelowness) and ΔE (total color difference) values of ovine and caprine milk cheeses are shown in Tables 13 and 14. In general, a significant effect of the HHP treatments in the color parameters was observed, especially on the first period of ripening, and the effect of time on these values. HHP-treatment applied at the beginning of the ripening, in both type of cheeses, resulted in a significant decrease of lightness values with respect to control samples, however these differences became imperceptible toward the end of the ripening. On the other hand, no differences in L* values were observed when the HHP was applied at the 15^{th} day of ripening.

The a* values of HHP-samples in both type of cheeses reflected an increase in greenness (negative a* values) one day after the treatment, being significant in GHHP1 and EHHP15 samples. However the subsequent decrease of greenness (increase of a* values) was observed during the aging, and the significant differences relative to control only persisted until day 60 in GHHP1 samples. Likewise, one day after the application of pressure, b* values markedly increased the yellowness in both types of cheeses, with a subsequent decrease of these values during the ripening. However, at the end of ripening, significant higher values were observed in GHHP1 samples, while EHHP1 and EHHP15 samples showed lower b* values than control samples.

The decrease of L* values after the application of HHP have been reported by Sheehan and Guinee, (2004) in low-fat mozzarella cheese treated on the first day at 400 MPa during 5 min at 21 ºC. They related the decrease of lightness to the increase of protein hydration induced by the application of HHP, evidenced by the increase of non-expressible serum, which is an indicator of water holding capacity of cheese. They also reported the increase in greenness after the pressurization of cheeses, but this effect was reversed during the storage (75 days at 4 ºC). Okpala et al., (2010) in fresh cheese treated at up to 291 MPa and 29 min, observed that increased pressure led to increase b* values with a pronounced effect of the yellowing in cheese. Likewise, Capellas et al., (2001) in fresh milk cheeses treated at 500 MPa for 5, 15 or 30 min at 10 ºC; Saldo et al., (2002b) in hard caprine cheeses HHP-treated at 400 MPa for 5 min and Juan et al., (2008) in ovine milk cheeses treated at 300 MPa for 10 min at day 1 or 15 of ripening, reported the decrease of lightness and the augment of yellowness. These authors related the differences of color to the structural changes induced by HHP treatments and to a more continuous and closer protein network with smaller and more uniform fat globules.

Total color differences (*ΔE*) values relative to control cheeses were higher during the first stages of the ripening, decreasing as the sample aged.

Table 13. Changes in color parameters during the ripening of control and HHP-treated caprine milk cheeses

	Day of ripening	Control	HHP1
L*	6	91.79 ± 0.73^{aA}	90.34 ± 1.16^{aB}
	15	90.33 ± 0.55^{bA}	88.99 ± 1.24^{bB}
	30	88.92 ± 0.95^{cA}	88.16 ± 0.51^{cB}
	45	86.44 ± 0.65^{dA}	86.53 ± 0.72^{dA}
	60	85.18 ± 0.96^{eA}	84.60 ± 0.88^{eA}
a*	6	0.53 ± 0.05^{aA}	-1.21 ± 0.16^{aB}
	15	0.41 ± 0.38^{bA}	-1.14 ± 0.12^{aB}
	30	0.37 ± 0.25^{bcA}	-0.97 ± 0.11^{bB}
	45	0.33 ± 0.04^{bcA}	-0.94 ± 0.15^{bcB}
	60	0.27 ± 0.10^{cA}	-0.86 ± 0.15^{cB}
b*	6	14.15 ± 0.84^{aA}	17.95 ± 1.08^{aB}
	15	10.06 ± 0.15^{bA}	12.43 ± 0.37^{bB}
	30	10.83 ± 0.40^{cA}	12.56 ± 0.27^{bB}
	45	11.86 ± 0.36^{dA}	13.23 ± 0.34^{cB}
	60	12.58 ± 0.35^{eA}	13.59 ± 0.35^{dB}
ΔE	6		4.45 ± 0.51^{a}
	15		3.31 ± 1.01^{b}
	30		2.61 ± 0.66^{bc}
	45		2.04 ± 0.38^{c}
	60		1.83 ± 0.48^{c}

(±) Values are means standard deviations of data from the different batches
a-e Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

Table 14. Changes in color parameters during the ripening of control and HHP treated ovine milk cheeses

	Day of ripening	Control		HP1		HP15	
L*	5	88.40±	1.97$^{a\,A}$	86.48±	1.81$^{a\,B}$		
	15	85.87±	2.51^{b}	84.00±	1.54^{b}	86.19±	1.92^{a}
	30	81.91±	1.77^{c}	82.82±	2.15^{b}	81.89±	2.62^{b}
	45	78.47±	3.47$^{d\,B}$	82.74±	2.44$^{b\,A}$	80.08±	2.33$^{c\,B}$
	60	79.11±	2.48$^{d\,AB}$	80.62±	1.57$^{c\,A}$	78.59±	2.68$^{c\,B}$
a*	5	0.30±	0.52	-0.53±	1.05		
	15	0.51±	0.65^{A}	-0.35±	1.18$^{b\,AB}$	-0.76±	1.01$^{a\,B}$
	30	0.26±	0.73	-0.07±	1.24	-0.34±	1.02^{ab}
	45	0.41±	0.78	0.14±	1.28	0.13±	0.99^{ab}
	60	0.49±	0.99	0.16±	1.36	0.48±	1.04^{b}
b*	5	12.46±	2.79$^{a\,A}$	15.66±	1.20$^{a\,B}$		
	15	13.12±	1.31$^{a\,A}$	15.73±	1.38$^{a\,B}$	15.75±	0.49$^{a\,B}$
	30	14.69±	1.24^{bc}	14.44±	1.47^{b}	14.07±	1.21^{b}
	45	14.91±	1.23$^{c\,B}$	12.67±	1.34$^{c\,A}$	12.58±	0.45$^{c\,A}$
	60	13.64±	1.65$^{ab\,B}$	12.27±	1.38$^{c\,A}$	12.31±	0.75$^{c\,A}$
ΔE	5			4.42±	0.89^{a}		
	15			3.09±	1.30^{b}	3.38±	1.27^{a}
	30			2.04±	0.86^{c}	1.85±	0.95^{c}
	45			5.19±	1.30$^{a\,A}$	3.29±	1.15$^{b\,B}$
	60			2.90±	2.01^{bc}	2.07±	0.97^{c}

(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-B Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

14.3. Sensory properties

The perceptual differences in the color, texture (firmness, elasticity, crumbliness) and taste (intensity, saltiness, acidty and bitterness) attributes between HHP-treated and control untreated 60-old aged cheeses showed that color and firmness were the only characteristics in which the panel detected significant differences. Whereas samples with HHP treatments applied at the beginning and on the 15^{th} day of ripening presented no sensory differences between them (Table 15).

A 96.67% of the panel members perceived differences in color between HHP-treated and control caprine milk cheeses. This fact coincided with the significant differences observed in a* and b* values obtained at day 60, although the ΔE-value in these type of cheeses was just of 1.83. Likewise, sensory differences on the color perceived between EHHP1 and EC samples were detected by 90.48% of the panel, while 57.14% of them found differences between EHHP15 and EC samples. In this case the total color differences instrumentally measured showed slightly higher ΔE-values in EHHP1 samples than EHHP15 at the end of ripening.

On the other hand, 90.48% and 95.23% of the panel members detected that EHHP1 and EHHP15 samples, respectively, differed from control cheese in firmness and indicated that HHP-cheeses had higher intensity in this attribute. This perception was in concordance with the results obtained in the uniaxial compression test, concretely on fracture stress parameter, where samples with HHP-treatments applied on the 1^{st} and 15^{th} day presented 35% and 20% higher values than control cheeses at the end of ripening, respectively. On the contrary, the panel did not found differences between pressurized and control caprine milk cheeses. Although significant higher fracture stress values were obtained in GHHP1 samples, (around 8%). In addition, the preference test between samples did not show significant results, despite 65% of the panel members preferred ovine and caprine milk cheeses with HHP treatment at the first stages of ripening than their respective control samples (data not shown).

Rynne et al., (2008) noted that HHP-treated 1 day old-cheddar cheese had altered color values and was discriminated from the control cheese by the descriptive sensory analysis. In other surveys, sensory assessment of textural properties showed that HHP cheeses were perceived as less crumbly and more elastic than untreated cheeses by the tasting panel members (Saldo et al., 2000; Juan et al., 2007b). In our results, these attributes were not significantly differentiated between samples

Table 15. Differences (%) in the sensory characteristics of control and HHP-treated caprine and ovine milk cheeses

		Caprine milk cheese	Ovine milk cheese		
		HHP1- Control	HHP1-Control	HHP15-Control	HHP1-HHP15
	Colour	96.67 *	90.48 *	57.14 ns	23.81 ns
Texture	Firmness	53.33 ns	90.48 *	95.23 *	14.29 ns
	Elasticity	36.67 ns	61.90 ns	66.67 ns	52.38 ns
	Crumbliness	60.00 ns	61.90 ns	61.1 ns	52.38 ns
Taste	Flavour intensity	63.33 ns	63.16 ns	52.38 ns	33.33 ns
	Saltiness	33.33 ns	42.86 ns	57.14 ns	–
	Acidic	60.00 ns	38.10 ns	28.57 ns	42.86 ns
	Bitterness	33.33 ns	33.33 ns	14.29 ns	9.52 ns

Values are percentage of means obtained from the panel responses in different batches
* Statistical significance P<0.05
ns. Not significant

15. IMPROVED SCREENING PROCEDURE TO DETECT AMINO ACID DESCARBOXYLASE ACTIVITY OF BACTERIA

15.1. Decarboxylase media

Several qualitative methods and their modification to determine decarboxylase activity in microorganism have been described in order to adapt the method for different applications and to reduce the number of false positive and negative responses (Table 2). These qualitative methods have in consideration different factors such as initial pH, glucose concentration, presence of pyridoxal-5-phosphate, and others. Niven et al., (1981) set the pH of differential plating medium in 5.3 to detect the histidine decarboxylase activity of Enterobacteria. A lower pH may inhibit the bacterial growth. Joosten and Northolt, (1989) improved the decarboxylase response of lactobacilli isolated from cheese by lowering the initial pH of the medium to 5.0 and adding some metal sulphates to promote the growth of the bacteria. Pircher et al., (2007) test pH 6.7 and 5.2 on amino acid decarboxylase broth and found that TY formation by LAB was higher in broth with initial pH 5.2, while Enterobacteria formed less CA at the same pH. It seems practical to use a pH which mimics the original food system or product from which the organism has been isolated (Bover-Cid and Holzapfel, 1999). In our study, the pH of the media was set to 5.5 in order to enhance the growth of bacteria and the decarboxylase activity. Besides, metal sulphates were only added when the medium was used to quantify the amine-forming capacity by a HPLC method.

The presence of glucose in the decarboxylase media could enhance the growth of bacteria, provoking the lowering of pH media to favor their amino acid decarboxylase activity (Halázs et al., 1994; Marcobal et al., 2006a). Glucose concentrations in the range of 0.5-2.0% have been reported to be optimal, while levels in excess of 3% inhibited enzyme formation (Halázs et al., 1994). However, the fermentative activity of certain bacteria that produce acid along with BA could constitute a problem, especially in the screening of decarboxylase activity of LAB. Joosten and Northolt, (1989) established the optimal glucose concentration in 0.1%, based in the acid production of homofermentative strains such as *Lactobacillus*. However, Bover-Cid and Holzapfel, (1999) used 0.05 % of glucose in the media and increased the buffer effect with the addition of di-potassium phosphate and calcium carbonate and Maijala, (1993) omitted glucose from the modified decarboxylating agar to prevent false negative results arising when it is converted to acid by LAB. In our decarboxylase broths, the glucose concentration was established in 0.05% in low nitrogen decarboxylase broth (LND), and 0.001% in low glucose decarboxylase broth (LGD) with the aim to reduce the false negative

responses originated by the acid production of fermentative strains. The glucose amount in the LND medium was similar to this suggested by Bover-Cid and Holzapfel, (1999). However, 0.05% of glucose in LGD medium caused false negatives responses by a high acid production (results did not show).

The majority of the authors suggested a content of 1-1.5% of nitrogen substances in the decarboxylase media (Table 2); however the amount of nitrogen source in LND media was decreased to 0.25% in order to reduce the false positive responses due to microorganisms with peptidase and deaminase activity. Some reports described the occurrence of false positive reactions in decarboxylase media due to other alkaline bacterial products and not only by BA produced in the media (Rodríguez- Jerez et al., 1994; Roig-Sagués et al., 1997a; Hernández-Herrero et al., 1999b). Özogul and Özogul, (2007) detected in their study that all culture strains tested and especially *H. alvei* produced ammonia.

The positive control strains showed a decarboxylase activity in both microplate screening and HPLC methods as expected. *Lactobacillus brevis* and *Lactobacillus casei* expressed tyrosine decarboxylase activity in LGD broth, and *Staphylococcus epidermidis* presented histidine decarboxylase capacity in LND broth. All were also able to produce low amounts of PU (around 1 mg L^{-1}), detected only by the confirming method (Table 16).

Table 16. Biogenic amine production by positive control bacteria strains in the amino acid decarboxylase microplate assay and HPLC analysis

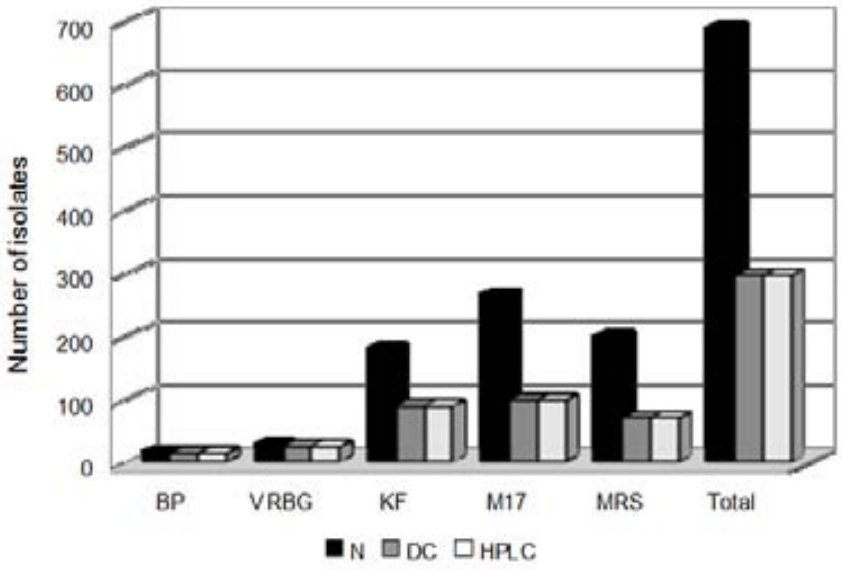
Strain	Total positive	PU		CA		HI			TY		
		DC	HPLC	DC	HPLC	DC	HPLC		DC	HPLC	
L. brevis	1/1	ND	1	ND	ND	ND	ND		1	1	(109.8)
L. casei	1/1	ND	1	ND	ND	ND	ND		1	1	(77.14)
S. epidermidis	1/1	ND	1	ND	ND	1	1	(46.48)	ND	ND	

Total positive. Number of decarboxylase positive strains with respect to the total number assayed
DC. Amino acid decarboxylase microplate assay
HPLC. Number of positive strains detected by RP- HPLC and BA concentration in parenthesis (mg L^{-1})
ND. Not detected

15.2. Assessment of total amino acid decarboxylase

A total of 688 isolates were obtained from different culture media. Only a 43.02% of them gave a positive response in the microplate assay with total amino acid broth, being subsequently confirmed by HPLC. As can be observed in Figure 16, the amino acid decarboxylase activity was more frequent on isolates obtained from VRBG and BP media (87.5 and 92% respectively), than in those obtained from KF, M17 and MRS media (49.7, 36.8, and 35% respectively). Several studies found that the decaboxylase activity was more evident in most of Enterobacteria strains (80-95%) and in less extension in LAB strains (9.5-65 (Roig-Sagués et al., 1997a; Roig-Sagués et al., 2002; Moreno-Arribas et al., 2003; Pircher et al., 2007; Marino et al., 2008). Staphylococci strains capable to produce BA have been reported in fewer incidences (2-14.6%) in raw and slightly fermented sausages (Roig-Sagués et al., 1996; Martín et al., 2006). However, Hernández-Herrero et al., (1999b) found that the main positive histidine decarboxylase confirmed isolates from salted anchovies belonged to *Staphyloccoccus* genus. Generally the presence of enterobacteria and staphylococci bacteria in ripened cheeses made under hygienic conditions never reach high counts and were no longer detectable after few days of ripening, thus LAB with decarboxylase activity could be the main BA formers in this product.

Figure 16. Number of positive amino acid decarboxylase isolates detected by total amino acid decarboxylase screening assay and HPLC analysis



N. Number of isolates assayed
DC. Number of positive isolates detected by amino acid decarboxylase screening assay
HPLC. Number of positive strains detected by RP-HPLC

15.3. Assessment of specific amino acid decarboxylase

The assessment of specific amino acid decarboxylase activity was followed out with isolates which gave positive responses in the total amino acid decarboxylase screening assay. Up to 150 of this isolates were tested in LND and LGD media, respectively. In general the capability to decarboxylate tyrosine was the most frequent activity detected in 91.6% of the total isolates tested in the specific amino acid decarboxylase screening assay, followed out by the ability to decarboxylate lysine and ornithine, in around 33.5%. In these cases, the 95, 96 and 94% of them were confirmed by the HPLC analysis, respectively. On the other hand, histidine decarboxylase activity was detected by the specific screening assay in 24% of the isolates tested and only the 76% of them were confirmed.

The receiver operating characteristic (ROC) curves and their analytical validation data were used to know the discriminative power and the cut-off of the amino acid decarboxylase screening method with both media (LND and LGD) to diagnose the specific amino acid decarboxylase activity (Figures 17 and 18).

The tyrosine decarboxylase test showed an area under the ROC curve (AUC) around 0.98, with an optimal cut-off value at 25 and 20 mg tyramine L^{-1} on LND and LGD media, respectively. This means that the microplate screening method could discriminate the isolates with tyrosine decarboxylase activity in 98% of times at the optimal cut-off. The sensitivity and specificity values obtained with both type of media were similar (92.5 and 100% respectively), reflecting that the qualitative method gave in general a reduced number of false negative (FN) and false positive (FP) responses. However the negative predictive value (NPV) was considered low (below of 66%), this could be due to the fact that the number of negative tyrosine decarboxylase isolates at the optimal cut-off values (25 isolates in LND and 13 in LGD) represented a small proportion, and the number of isolates that gave a positive reaction in the qualitative method with a TY production lower than the cut-off values were also considered as FN (6 and 8 cases, in LND and LGD broths respectively).

The assay to detect ornithine decarboxylase with the LND broth showed the highest diagnostic values (over 98%) with the lowest cut-off concentration (10 mg L^{-1}) (Figure 17) and an AUC exceeding 0.995. Besides, this screening test showed that only 3.7% of the total negative responses were FN and 1.5% of the total of positive responses was FP. On the other hand, for the same test using LGD medium, an 84.4% of sensitivity and a 97.46% of specificity were reached at a cut-off value of 15 mg L^{-1} with an AUC of 0.907. Likewise, FN and FP results were detected in a propotion of 2.5% and 15%, respectively.

The corresponding ROC curves for the lysine decarboxylase test displayed an area AUC of 0.992 and 0.935, with an optimal cut-off concentration of 15 and 10 mg L^{-1} for LND and LGD media, respectively. In this case, the sensitivity and specificity values using LND broth were 98.7 and 93% respectively, while the same diagnose values using LGD medium were 88 and 97.6%, respectively. FN and FP were detected in six out of 73 negative and one out of 76 positive responses in LND broth, respectively, while LGD broth showed FN and FP responses in five out 125 and three out 25, respectively.

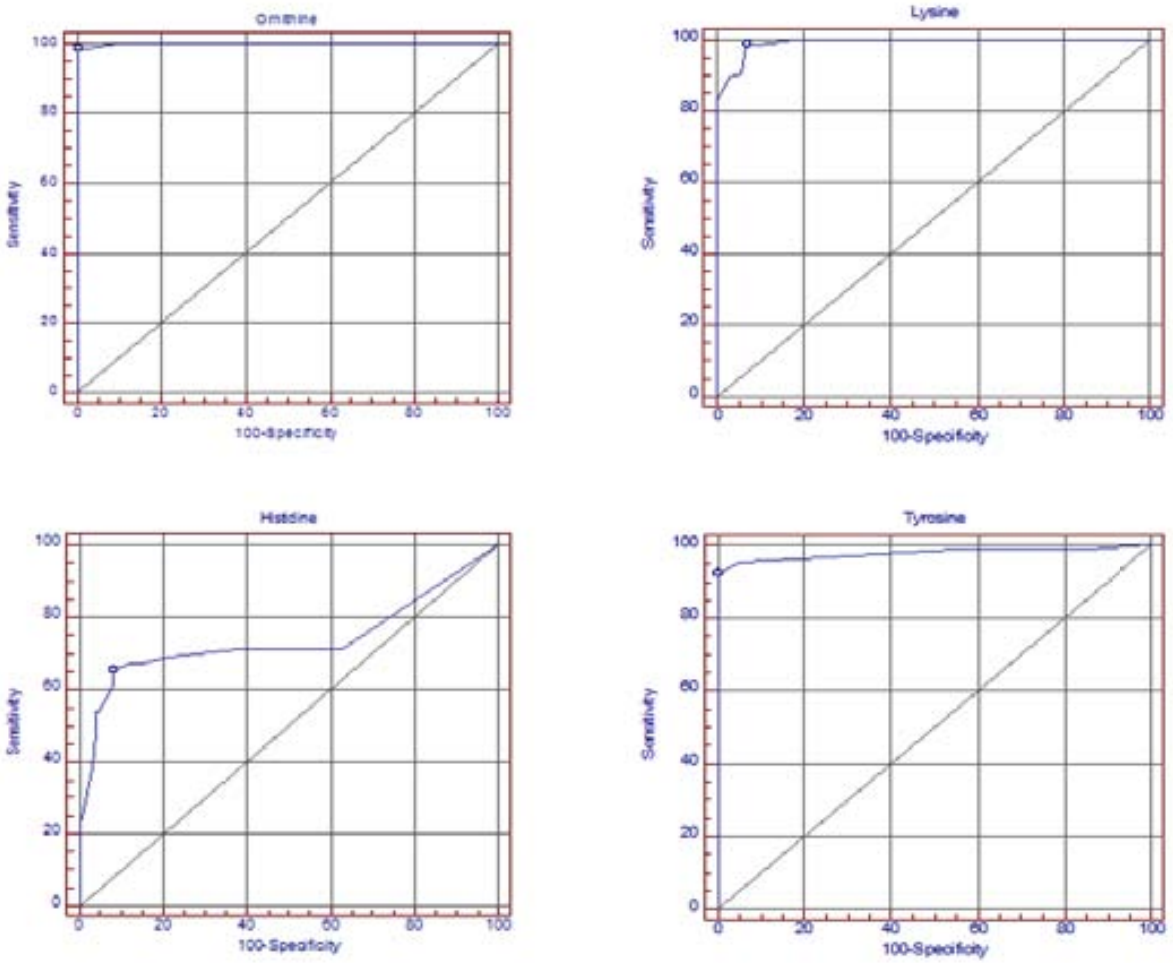
The diagnostic performance of the histidine decarboxylase test with both types of media gave the lowest sensitivity values (below 0 %), with the highest optimal cut-off concentration (50 mg L^{-1}) and specificity values up to 90%. Likewise, the AUC were the lowest registered (0.737 and 0.592 to LND and LGD broth, respectively). As a consequence, from the total total positive responses obtained in the test using LND and LGD broths, at the optimal cut off, around of 70% were FP reactions, whereas 11% and 1.5% of the total negative responses, using the same respectively broths, were FN results.

In many occasions it has been reported that qualitative screening decarboxylase methods have some limitations in terms of sensitivity in detecting BA production. The presence of false positive and false negative reactions reported has not been insignificant (Hernández-Herrero et al., 1999b; Suzzi and Gardini, 2003). Hernández-Herrero et al., (1999b) observed that 96.5% of the suspected histamine formers detected by Niven decarboxylase media were finally considered as false positives. Likewise Roig-Sagués et al., (1996) found that only 15.8% of the total presumptively histamine-formers isolates obtained in Joosten and Northolt media were confirmed. Similar results were observed when tyramine decarboxylase capacity was tested in the same media, where only 8.4% of the suspected isolates with tyrosine decarboxylase activity where confirmed. The false positive results were attributed to the production of other substances able to alkalinize the media (Roig-Sagués et al., 1997b). On the same line, Moreno-Arribas et al., (2003) used the Maijala modified decarboxylase media and noticed a high number of false positive reactions related to PU and agmathine production, but lower false reactions were found in tyrosine decarboxilase activity test. On the contrary, de las Rivas et al., (2008) did not find any correlation between the positive responses in the decarboxylase activity media (31 out of 200 strains) and the BA detected by HPLC (45 out of 200 strains). They suggested that the screening Maijala modified decarboxylase media underestimates the number of BA-producing strains, giving false negative results that could be produced by an insufficient growth of the strain. Bover-Cid and Holzapfel, (1999) reported 102 strains with decarboxylase activity out of 194 LAB strains tested in their improved

screening media. They did not observe FP reactions from all strains tested and confirmed by HPLC analysis and only 3 strains gave a FN response with this screening procedure. They justified these false negative results by the meaning of low amine amount production which could not cause the pH shift of the media, especially when considering the acid production of LAB. Although these authors proposed their improved decarboxylase medium as a rapid preliminary method to select strains with low decarboxylase activity which detection limit estimated was around 350 mg L^{-1}.

Some studies have proposed diverse qualitative screening procedures to detect amino acid decarboxylase activity in bacteria and some others had applied these methods. Few surveys have been reported some evaluation data of the qualitative method. These surveys frequently tested a reduce number of strains with known decarboxylase amino acid, but none of these surveys displayed the main validation parameters. In our case, a diagnostic test was assessed to evaluate the capability of prediction of the qualitative method. The data indicates that, in general, the specific amino acid decarboxylase assay with LND and LGD broths had satisfactory diagnostic parameters to discriminate bacterial isolates with ornithine, lysine and tyrosine decarboxylases with AUC above 0.90. Moreover the sensitivity and specificity values for ornithine, lysine and tyrosine decarboxylase test with both types of media were acceptable with low number of FP and FN responses. Generally FN responses were due to weak BA producers. On the other hand, the identification of histidine decarboxylase activity in bacterial isolates using LND and LGD broths have obtained high specificity value, but low sensitivity and the corresponding area under the ROC curve has been considered as acceptable (0.74) for LND and poor (0.60) for LGD (Hanley and McNeil, 1982), reflecting less capacity to detect HI-producers. The microplate screening method allows a rapid preliminary selection of strains with decarboxylase activity, with a detection limit estimated around 50 mg L^{-1}. Moreover, the use of microtiter wells allows large numbers of samples to be assayed, reducing the volume of material and culture media needed. However, more experiences need to be assayed to adjust the characteristics of diagnostic amine formers, especially for histamine producers.

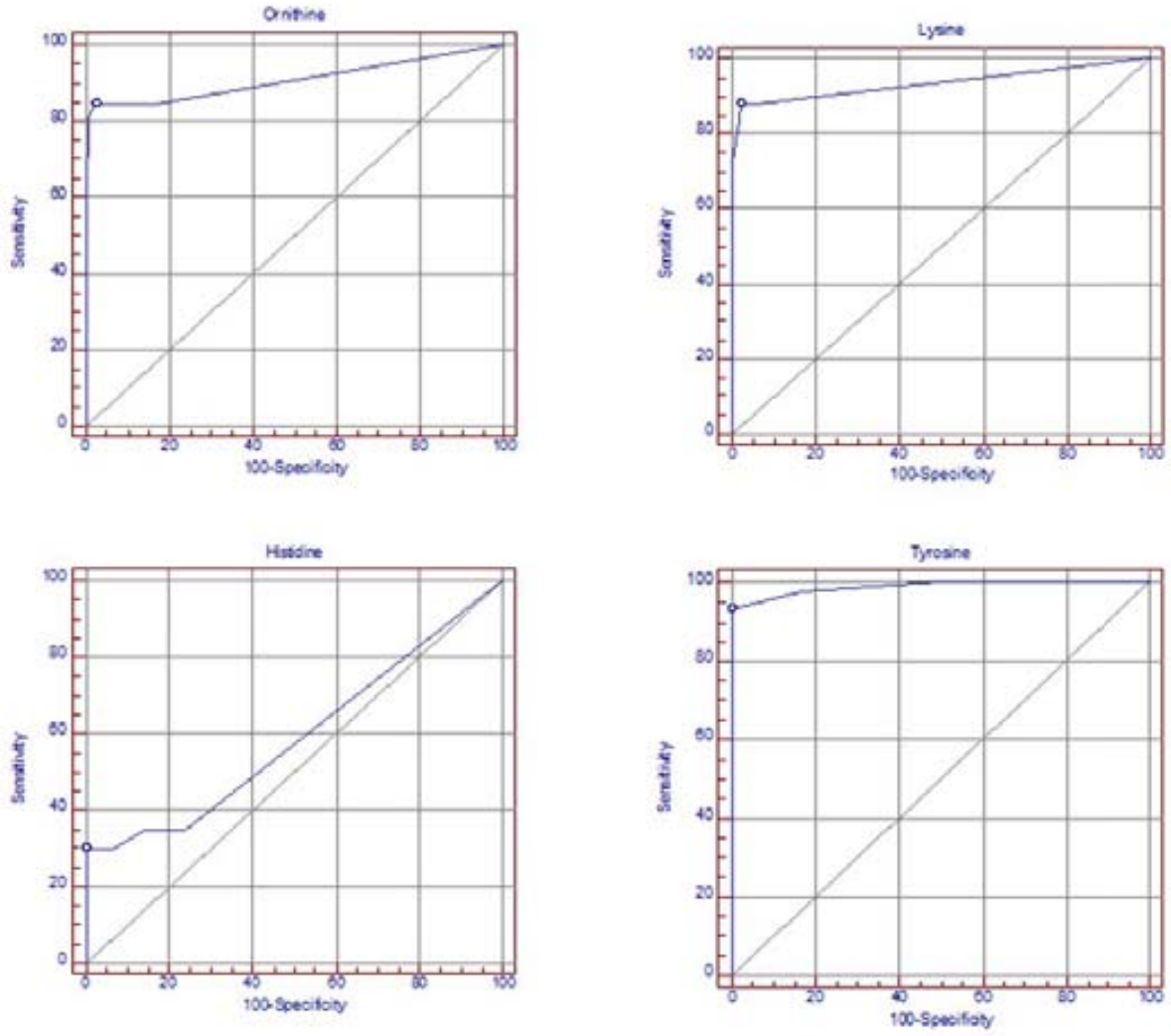
Figure 17. ROC curves and analytical validation data of the qualitative method to detect specific amino acid decarboxylase activity in isolates using LND broth



	Ornithine	Lysine	Histidine	Tyrosine
AUC	0.999 (0.97-1)	0.992 (0.961-1)	0.737 (0.659-0.806)	0.98 (0.943-0.996)
Optimal cut-off (mg L^{-1})	10	15	50	25
Sensitivity at optimal cut-off (%)	98.5	98.7	65.4	92.3
Specificity at optimal cut-off (%)	100	93.2	91.8	100
PPV (%)	100	93.7	81	100
NPV (%)	98.8	98.6	83.2	66.7
FN at cut-off	3	6	11	1
FP at cut-off	1	1	17	6

In ROC curve the point with a circle mark correspond to the optimal cut-off value.
AUC. Area under ROC curve and its 95% confidence interval
PPV. Positive predictive value NPV. Negative predictive value
FN. Number of false negative FP. Number of false positive

Figure 18. ROC curves and analytical validation data of qualitative method to detect specific amino acid decarboxylase activity in isolates using LGD broth



	Ornithine	Lysine	Histidine	Tyrosine
AUC	0.907 (0.849-0.948)	0.935 (0.883-0.969)	0.592 (0.509-0.672)	0.989 (0.956-0.999)
Optimal cut-off (mg L^{-1})	15	10	50	20
Sensitivity at optimal cut-off (%)	84.37	88	30	93.06
Specificity at optimal cut-off (%)	97.46	97.6	100	100
PPV (%)	90	88	100	100
NPV (%)	95.8	97.6	90.3	37.5
FN at cut-off	3	5	2	0
FP at cut-off	5	3	14	8

In ROC curve the point with a circle mark correspond to the optimal cut-off value.
AUC. Area under ROC curve and its 95% confidence interval
PPV. Positive predictive value NPV. Negative predictive value
FN. Number of false negative FP. Number of false positive

16. BIOGENIC AMINE FORMING CAPACITY OF BACTERIA ISOLATED DURING THE RIPENING OF CAPRINE AND OVINE MILK CHEESES

Considering the conditions into which the isolates were subjected (inoculum level, nutrient media, temperature and time of incubation) on the LND and LGD media and the subsequent HPLC analysis, and also in order to simplify the discussion, the following four categories of amine production by bacterial strains were defined according to Aymerich et al., (2006): medium amine former (25-50 mg L^{-1}), good amine former (50-100 mg L^{-1}), strong amine former (100-1000 mg L^{-1}) and prolific amine former (>1000 mg L^{-1}).

16.1. Biogenic amine production by isolates from caprine and ovine milk cheeses

The ability to produce BA by the main microorganism groups in the cheese samples was studied. The 37.7% of the bacteria isolated from caprine milk cheeses could produce at least one BA. Among these, 28.57%, 29.59% and 41.84% were obtained from control, farm and HHP1 samples, respectively. In the same way, 47% of the strains picked up from ovine milk cheeses were BA-formers, being 27.36%, 19.0%, 38.8% and 14.93% isolated from control, farm, HHP1 and HHP15 samples, respectively.

As it can be observed in figure 19, the incidence of strains with decarboxylase activity isolated from M17, in both type of cheeses, was about 2.7 times higher in HHP1 samples than in control cheeses. In the same way, positive decarboxylase isolates obtained from MRS, in ovine milk samples with HHP-treatments, were 1.45 times more frequent. This could be related to the fact that in these type samples, in which the count of LAB was reduced by the HHP-treatment, it was possible to detect other indigenous bacteria species such as *Pediococci, Leuconosto*c and *Bacilli*.

Figure 19. Positive amino acid decarboxylase isolates (%) obtained from caprine (19a) and ovine (19b) milk cheeses with and without HHP treatment

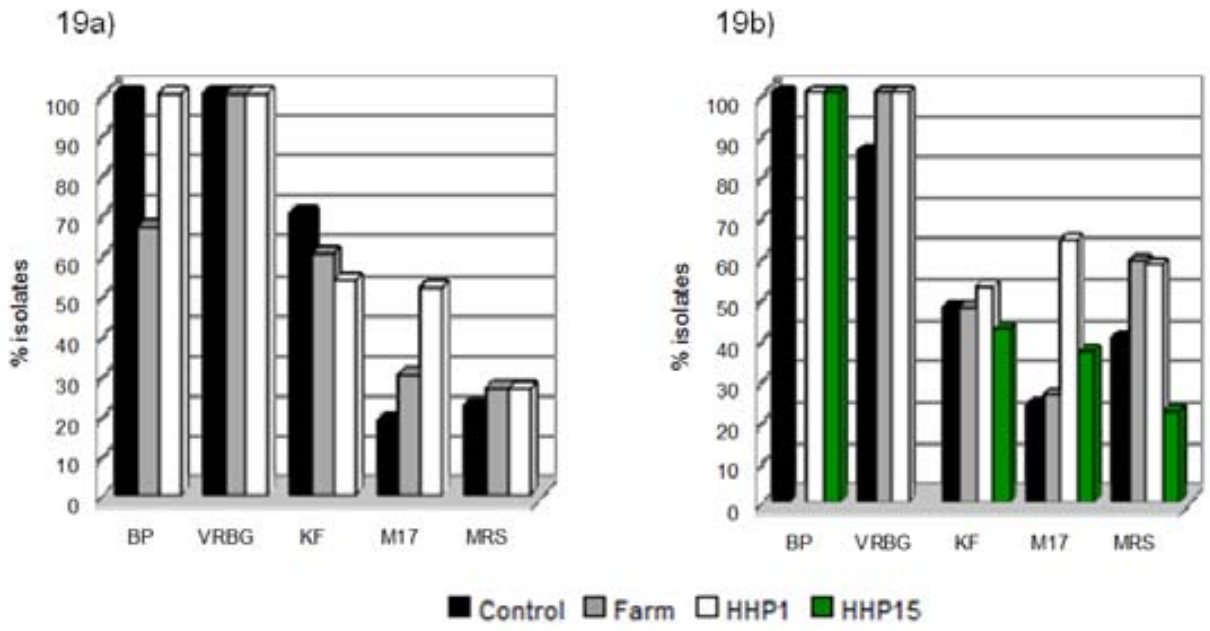


Table 17 displays the amine production capacity of bacteria isolated from caprine and ovine milk cheeses according to the culture media of origin, and Tables 18 and 19 show the identification of the BA-forming isolates obtained from both type of cheeses.

In general, amine producing capacity exceeding 100 mg L^{-1} was more frequent in isolates with TY-producing capacity, followed by those with CA and PU-forming ability. However, prolific amine production capacity (>1000 mg L^{-1}) was only observed in some diamine formers. The detection of HI in amounts above 100 mg L^{-1} produced by microorganisms isolated from both type of cheeses was a rare event.

The most important BA reported in cheeses have been TY, PU, CA and HI. However, great differences were observed in the type and amount of BA present depending on the type of cheese and manufacturing process (Roig-Sagués et al., 1998; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2003; Novella-Rodríguez et al., 2004b; Kalaç and Abreu Gloria, 2009). In food, BA are mainly generated by decarboxylation of the corresponding amino acid through substrate-specific enzymes of microorganisms present in the food (Komprda et al., 2004). The amount and type of BA formed depends on the nature of food and on the kind of amino acid decarboxylating microorganisms present (Bover-Cid et al., 2001).

The microorganisms isolated from VRBG culture medium with decarboxylase activity were able to mainly produce diamines in amounts above 100 mg L^{-1}. In caprine milk cheeses the 87.5% and 100% of the isolates picked up from this medium showed a positive ornithine and lysine decarboxylase activity, respectively. While in ovine milk cheeses the microorganisms isolated showed to possess PU and CA producing ability in a proportion of 85.35% and 94%, respectively. TY forming capacity was detected in bacteria isolated from VRBG medium, in this case only one isolate from caprine milk cheeses, and 8 from ovine milk samples possessed this ability. On the other hand, isolates with histidine decarboxylase activity were also observed, showing mainly a weak production capacity, although one and three isolates from caprine and ovine milk cheeses, respectively, were good HI-producers (50-100 mg L^{-1}) (Table 17). *Enterobactericeae* are known to decarboxylate several amino acids, specially lysine, ornithine (Joosten and Stadhouders, 1987; Joosten and Northolt, 1987; Marino et al., 2000; Bover-Cid et al., 2001; Suzzi and Gardini, 2003) and histidine (Halázs et al., 1994; Roig-Sagués et al., 1997a).

Among the Gram negative isolates from both type of cheeses *Hafnia alvei* was the most frequently identified (Tables 18 and 19) showing strong PU and CA production. Two of these strains, isolated from ovine milk cheeses, presented a strong HI-forming capacity as well. Likewise, strains of *Klebsiella oxytoca* and *Escherichia coli* with strong diamine production capacity were identified from ovine cheese samples. However only one strain of each specie and other identified as *Citrobacter freundii* were able to produce CA and TY in a considerable amount (above 100 mg L^{-1}). *H. alvei, K. oxytoca* and *E. coli* have been previously associated with the formation of PU, CA and/or HI in foods (Joosten and Northolt, 1987; Silla Santos, 1996; Roig-Sagués et al., 1996; Roig-Sagués et al., 1997a; Silla-Santos, 1998; Bover-Cid and Holzapfel, 1999; Hernández-Herrero et al., 1999b; Roig-Sagués et al., 2002; Özogul and Özogul, 2005; Pircher et al., 2007; Özogul and Özogul, 2007). Also some strains of these species have been reported to possess the ability to decarboxylate tyrosine (Roig-Sagués et al., 2002; Pircher et al., 2007). The formation of high amounts of PU and CA by strains of *C. freundii* has been previously reported (Marino et al., 2000; Pircher et al., 2007), indicating that strains of this specie mainly produced prolific amounts of PU but low of CA. The same surveys also reported some strains with TY-forming ability in a range 10-100 mL^{-1}.

In caprine milk cheeses the 33 and 44% of positive decarboxylase isolates obtained from BP culture medium displayed a strong PU and CA forming ability, respectively. Likewise, tyrosine decarboxylase activity was observed in 33% of isolates with the strong level of production, whereas histamine accumulation was detected in a range of 50-100 mg L^{-1} in the

33% of the cases. In ovine milk cheeses positive decarboxylase bacteria isolated from BP medium could decarboxylate ornithine and lysine in 50% of the cases, with prolific production of the respective amines, while the 66.6% of them showed a strong TY-forming capacity. On the other hand, HI-production by these isolates was lower than 50 mg L^{-1} (Table 17). Little information related with the production of BA by *Staphylococcus* genus in cheese is available. However, some species of this group have been related with a variable formation of TY, PU, CA and/or HI in meat and fish fermented products. Martín et al., (2006) found that 14.6% of the gram positive catalase-positive cocci obtained from slightly fermented sausages had BA producing capacity, being TY the most intense amine produced by 4.6% of the isolates, while PHE had more frequently detected amine (10.8%) but in low amounts. They observed that some of these strains also produced PU, CA and HI. Hernández-Herrero et al., (1999b) reported that the main HI-formers detected in salted anchovies belonged to *Staphylococcus* genus. Likewise, de las Rivas et al., (2008) reported some staphylococci strains isolated from "Chorizo" as TY-formers.

Different strains of staphylococci were identified among the positive decarboxylating bacteria isolated from the ovine milk cheeses (Table 19). *Staphylococcus chromogenes* showed the highest amine production, being prolific diamine and strong TY former. Likewise, strains of *Staphylococcus xylosus* with strong TY-forming capacity and *Staphylococcus aureus* with strong HI production were also found. On the other hand, the most frequent strains with high BA forming capacity from BP culture medium in caprine milk cheeses were identified as *Staphylococcus hominis,* with strong production of PU, CA and/or TY, and *Staphylococcus warneri,* which showed to be a strong diamine producer (Table 18).

S. chromogenes was previously reported as prolific PU, good CA, strong TY and HI-forming bacteria in Spanish salted anchovies (Pons-Sánchez-Cascado et al., 2005). de las Rivas et al.,(2008) reported *S. xylosus* as the most frequent species in "Chorizo", but any strain of this species showed decarboxylase activity. On the contrary, Masson et al., (1996) detected a weak TY-production capacity in strains of *S. xylosus* isolated from fermented sausages. Silla-Santos, (1998) observed HI-production in the 76% of *S. xylosus* strains isolated from Spanish sausages and Martín et al.,(2006) found some strains of *S. xylosus* in slightly fermented sausages capable to produce amounts above 100 mg $L^{-1}$of TY, PU and/or HI and concentrations between 50 and100 mg L^{-1} of CA. Strains of *S. warneri* have also been reported to possess tyrosine decarboxylase ability with variable amounts of production (Masson et al., 1996; Martín et al., 2006; de las Rivas et al., 2008) and strong diamine forming ability (Martín et al., 2006). Drosinos et al., (2007) in traditional fermented sausages isolated

one strain of *S. hominis novobiosepticus* with lysine and tyrosine decarboxylase activity.

Since enterobacteria and taphylococci are microbial groups related to the contamination of food during handling, it is reasonable to expect that they would be part of the microbiota of food products. Consequently, is important to follow always good manufacturing practices to avoid the proliferation of this kind of bacteria in the product.

Formation of 100-1000 mg L^{-1} of TY was observed in 76 and 81% of isolates obtained from KF culture medium in caprine and ovine milk cheese samples, respectively. Besides, around 30 and 34% of the isolates were able to produce PU and CA respectively, in amounts above 100 mg L^{-1} (Table 17). *Enterococcus faecalis, Enterococcus faecium* and *Enterococcus durans* were the most frequent amine producing bacteria identified from both types of cheese, with a varied production of TY, PU and CA. In addition, one strain of *E. facecium* and two of *E. durans* showed a strong histamine formation (Tables 18 and 19).

Several authors have described *E. faecalis, E. faecium* and *E. durans* as the most frequently TY formers in food (Joosten and Northolt, 1987; Roig-Sagués et al., 1997b; Leuschner et al., 1999; Bover-Cid and Holzapfel, 1999; Galgano et al., 2001; Roig-Sagués et al., 2002; Pircher et al., 2007; Burdychova and Komprda, 2007; Landete et al., 2007) and also some strains of *E. feacalis* and *E. faecium* have been registered as capable to produce amounts between less than 10 to 100 mg L^{-1} of PU (Martuscelli et al., 2005; Pircher et al., 2007); CA (Galgano et al., 2001; Pircher et al., 2007) and/or HI (Tham, 1988; Pircher et al., 2007). Nevertheless, Tham et al., (1990) reported a strain of *E. faecalis* which produced no histamine in synthetic decarboxylase broth, but it did so when it was inoculated in cheese. On the contrary, a strain of *E. faecium* that produced histamine *in vitro* produced only small amounts in cheese. No references concerning histidine decarboxylase activity of *E. durans* have been found in the literature.

The 79 and 65% of the bacteria isolated from M17 culture medium of caprine and ovine milk cheese samples, respectively, were capable to produce TY in amounts above 100 mg L^{-1}. Likewise, diamine formation was observed in less proportion: around 5% of the isolates obtained from both type of cheeses was capable to produce PU and only 4.7% of those obtained from ovine cheeses had the ability to form CA at the same level. Resembling results were found in the isolates obtained from MRS medium in both type of cheeses, where around 69% of them were considered strong TY-producers. Among the isolates obtained from caprine cheese samples no PU or CA formation was detected, whereas about 10% of those obtained from ovine cheese samples presented this ability, and forming up to >1000 mg L^{-1} (Table 17).

Several BA producing strains isolated from M17 and MRS media used in caprine milk cheeses were identified, being *Lactococcus lactis* subsp. *lactis,* the most frequent, followed by *Lactobacillus brevis, Lactobacillus plantarum,* and *Leuconostoc spp*. All of them showed strong TY forming ability and also a strong-prolific amount of PU was produced by *Leuconostoc spp.* In addition, two isolates belonging to the *Bacillus* genus were found as capable to decarboxylate tyrosine (Table 18). In the case of ovine milk cheeses, *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, Pediococcus pentosaceus, Lactobacillus paracasei* subsp. *paracasei, L. plantarum* and *Leuconostoc spp* were often associated with a strong TY-forming capability. Moreover, two strains identified as *L. lactis* subsp. *lactis* showed strong PU and CA forming ability, respectively, while two strains of *P. pentosaseus* species produced strong and prolific PU and CA amounts, respectively (Table 19).

Several studies have reported different species of LAB able to form BA, especially TY (Joosten and Northolt, 1987; Maijala, 1993; Straub et al., 1995; Roig-Sagués et al., 1997b; Bover-Cid and Holzapfel, 1999; Bover-Cid et al., 2001; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2002a; Novella-Rodríguez et al., 2002c; Pircher et al., 2007). Within the species of LAB that may occur in food some strains of *L. brevis and L. plantarum* were reported to posses the potential to form TY, PU and/or HI (Straub et al., 1995; Roig-Sagués et al., 1997b; Leuschner et al., 1998; Bover-Cid and Holzapfel, 1999; Roig-Sagués et al., 2002; Kung et al., 2005; Pircher et al., 2007; de las Rivas et al., 2008). Some trains of *P. pentosaceus* isolated from commercial starters (Maijala, 1993) and ripened sausages (Roig-Sagués et al., 1997b) were found capable to produce TY. On the other hand, *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *L. paracasei* subsp. *paracasei* are species usually used as starter cultures or probiotic strains, and were usually reported as non decarboxylating strains (Joosten and Stadhouders, 1987; Straub et al., 1995; Novella-Rodriguez et al., 2002a). On the contrary, some *L. lactis* and *L. paracasei* strains have been reported with the ability to produce TY, HI, PU and/or CA in amounts from less of 10 to 1000 mg L^{-1} (Maijala, 1993; Roig-Sagués et al., 1997b; Bover-Cid and Holzapfel, 1999; Roig-Sagués et al., 2002; Kung et al., 2005; Pircher et al., 2007; Burdychova and Komprda, 2007). However, *L. lactis* subsp. *cremoris* has not been described as BA-producer.

Among LAB, *Leuconostoc* have been described as a frequent member of the microbiota in several Spanish farm house cheeses (Fontecha et al., 1990; González de Llano et al., 1992; Cuesta et al., 1996), and has also been described to possess tyrosine, lysine and/or histidine decarboxylase activity (Marino et al., 2008). Likewise, González de Llano et al., (1998) reported the production of TY in a range of 100-1000 mg L^{-1} by strains of *Leuconostoc*.

Pircher et al.,(2007) found that strains belonging to this genus could form TY, PU, CA and /or HI in amounts up to100 mg L^{-1}.

Strains belonging to the *Bacillus* genus isolated from ripened salted anchovies, cheese and raw sausages have also been described as BA-formers (Rodríguez-Jerez et al., 1994; Roig-Sagués et al., 1996; Hernández-Herrero et al., 1999b). One of them was *Bacillus macerans* isolated from Italian cheese, which was capable to from prolific amounts of HI. Formation of CA and PU was also observed in this strain (Rodríguez-Jerez et al., 1994).

Several authors mentioned that the specificity of the amino acid decarboxylases is specially strain dependent (Straub et al., 1995; Bover-Cid et al., 2001; Suzzi and Gardini, 2003; de las Rivas et al., 2008). A great variability in BA production by different groups and species of bacteria, either in type or amount, was found in this survey. Cheese is considered as a living product with complex relations between different microorganisms, thus the type and concentration of BA produced *in vitro* do not implicate the same behavior in the product.

Table 17. BA forming capacity of bacteria isolated from caprine and ovine milk cheeses according to the culture media of origin

			PU [A]				CA				HI				TY			
	Sample	**Pos**	**±**	**+**	**++**	**+++**	**±**	**+**	**++**	**+++**	**±**	**+**	**++**	**+++**	**±**	**+**	**++**	**+++**
VRBG	GC	8	0	1	4	3	0	0	5	3	2	5	1	0	2	2	1	0
	EC	17	1	1	13	1	0	0	12	4	6	5	3	0	1	4	8	0
BP	GC	9	1	1	3	0	0	0	4	0	0	3	0	0	2	1	3	0
	EC	6	0	0	0	3	0	0	0	3	4	1	1	0	0	1	4	0
KF	GC	21	2	1	6	1	4	0	6	1	2	7	1	0	1	2	16	0
	EC	68	3	2	16	3	9	0	16	8	17	14	3	0	3	6	55	0
M17	GC	34	0	1	2	0	2	1	0	0	3	2	1	0	1	2	27	0
	EC	64	7	1	3	0	5	0	2	1	11	4	1	0	4	4	42	0
MRS	GC	26	0	0	0	0	0	0	0	0	0	0	0	0	1	4	18	0
	EC	46	2	2	2	3	0	0	2	3	7	0	0	0	4	4	32	0
Total		299	16	10	49	14	20	1	47	23	52	41	11	0	19	30	206	0

Pos. Number of amine producers GC. Caprine milk cheese EC. Ovine milk cheese
[A]. Number of BA-forming isolates detected depending their production:
(±) 25-50, medium; (+) 50-100, good; (++) 100-1000, strong; and (+++) >1000 mgL^{-1} , prolific

Table 18. Identification of some amine-forming bacteria isolated from caprine milk cheeses

	N		PU [C]		CA		HI		TY
Gram Negative									
Hafnia alvei	4	4	(537.7-889.54)	2	(641-1001.30)	4	(30.43-95.68)	4	(22.44-151.54)
Enterobacteriaceae	1	1	(1037.52)	1	(1173.24)	1	(111.43)	1	(73.41)
Gram Positive									
Staphylococcus cohni subsp. *cohni*	1	–		1	(1.50)	1	(1.57)	1	(25.78)
Staphylococcus warneri	2	2	(69.29-753.55)	2	(240.94-694.22)	1	(88.63)	1	(65.50)
Staphylococcus capitis	1	1	(310.30)	1	(246.37)	1	(95.13)	–	
Staphyilococcus lentus	2	1	(23.44)	2	(5.41- 19)	–		2	(39.58-191.69)
Staphyilococcus hominis	2	1	(890.96)	1	(998.20)	1	(91.63)	2	(102.42-245.22)
Enterococcus faecalis	8	8	(39.66-884.27)	8	(32.44-972)	6	(25.62-92.35)	8	(327.5-477.20)
Enterococcus durans	2	–		–		–		2	(337.40-357.44)
Enterococcus avium	1	–		1	(26.52)	1	(56.51)	1	(24.88)
Enterococcus faecium	6	3	(19.68-1113.80)	3	(1.32-1281.50)	3	(20.22-111.38)	6	(9.91-366.47)
Lactococcus lactis subsp. *lactis*	10	1	(7.25)	1	(7.50)	2	(21.24-33.38)	10	(198.77-450.77)
ediococcus pentosaceus	1	–		–		1	(33.44)	1	(55.71)
Lactobacillus brevis	5	–		–		1	(22.51)	5	(212.58-519.52)
Lactobacillus plantarum	3	1	(22.41)	–		1	(24.5)	3	(307.62-528.45)
Lactobacillus paracasei subps. *paracasei*	1	–		–		–		1	(307.03)
Leuconostoc spp.	3	2	(252.88-749.05)	2	(14.47-28.83)	2	(22.74-25.75)	3	(330.01-417)
Bacillus macerans	1	–		–		–		1	(418.10)
Bacillus licheniformis	1	–		–		–		1	(403.07)

N. Number of isolates [C]. Number of isolates and their amine production (mg L^{-1}) (–) Not detected

Table 19. Identification of some amine-forming bacteria isolated from ovine milk cheeses

	N		PU [C]		CA		HI		TY
Gram Negative									
Escherichia coli	2	2	(746- 857.48)	2	(762.35-983.93)	2	(41.37-74.12)	2	(21.7-281.48)
Hafnia alvei	5	5	(738.50-1049.51)	5	(787.20-1180.12)	4	(43.52-185.54)	5	(48.83-184.79)
Klebsiella oxytoca	2	2	(30.63-458.50)	2	(493.41-866.96)	2	(16.04-27.22)	2	(5.01-167.24)
Citrobacter freundii	1	1	(67.1)	1	(1095.3)	1	(45.62)	1	(372.38)
Enterobacteriaceae	1	1	(832.76)	1	(846.95)	1	(83.95)	1	(69.42)
Gram Positive									
Staphylococcus xylosus	2	—		—		2	(42.31-68.5)	2	(92.09-475.35)
Staphylococcus chromogenes	1	1	(1142.92)	1	(1760.06)	1	(31.12)	1	(441.40)
Staphylococcus aureus	1	—		—		1	(100.54)	—	
Enterococcus faecalis	5	3	(860.29-978.9)	4	(35.43-1394.87)	5	(20.7-92.98)	5	(338.01-461.50)
Enterococcus durans	9	9	(12.63-1160.44)	8	(13.32-1773.03)	9	(30.8-179.32)	9	(46.09-747.33)
Enterococcus faecium	2	2	(24.89-847.05)	2	(877.24-941.69)	2	(28.98-29.97)	2	(100.54-149.41)
Enterococcus hirae	2	2	(552.4-579.25)	2	(600.67-615.75)	2	(32.36-45.95)	2	(108.01-187.58)
Enterococcus avium	2	—		1	(15.17)	1	(2.18)	2	(19.93-434.25)
Streptococcus salivarius	1	—		—		—		1	(742.1)
Lacococcus lactis subsp. *lactis*	9	3	(34.39-795.26)	2	(14.95-844.13)	2	(16.38-30.72)	9	(211.93-566.2)
Lactococcus lactis subsp. *cremoris*	4	3	(4.78-37.63)	3	(5.23-22.94)	2	(11.15-19.03)	4	(229.47-406.87)
Pediococcus pentosaceus	4	3	(9.91-897.67)	3	(10.25-1018.4)	3	(27.06-88.78)	4	(34.58-411.66)
Lactobacillus paracasei subps. *paracasei*	6	2	(17.42-45.05)	1	(17.18)	1	(51.93)	6	(365.79-575.73)
Lactobacillus plantarum	4	1	(8.58)	1	(17.58)	1	(26.26)	4	(45.1-353.31)
Lactobacillus brevis	1	—		—		—		1	(240.08)
Lactobacillus pentosus	1	1	(50.79)	—		—		1	(422.75)
Leuconostoc spp.	5	2	(11.13-1162.76)	2	(22.52-1781.26)	3	(29.63-33.82)	5	(392.01-626.42)

N. Number of isolates [C]. Number of isolates and their amine production (mg L^{-1}) (—) Not detected

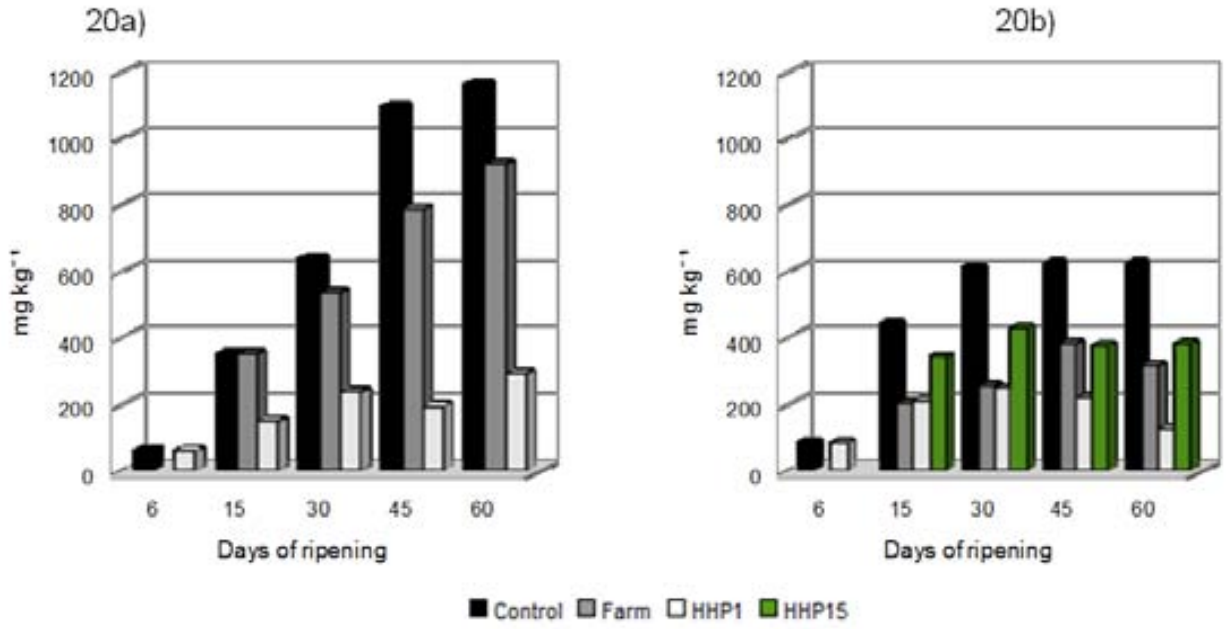
17. BIOGENIC AMINE CONTENT IN CAPRINE AND OVINE MILK CHEESES TREATED BY HIGH HYDROSTATIC PRESSURE

Figure 20 shows the values of the total BA content of the caprine and ovine milk cheeses. In general, BA content showed a constant and significant increase in control samples during ripening from the 5th (59.36 mg kg^{-1}) to the 60th (1156 mg kg^{-1}) day of ripening in the GC cheese batches and from the 6th (85.08 mg kg^{-1}) to the 60th day (622.39 mg kg^{-1}) in EC batches. This is in agreement with the results reported by other authors that observed accumulation of BA during the ripening of caprine and ovine milk traditional cheeses (Ordoñez et al., 1997; Gardini et al., 2001; Galgano et al., 2001; Martuscelli et al., 2005). However, in both types of cheeses, control samples presented higher BA content than farm samples with significant differences at the 60th day of about 20% and 50% in caprine and ovine milk cheese samples, respectively.

Farm ovine milk cheeses showed similar microbiological and physicochemical parameters than control samples except in water content, which was significantly higher on the 60th day. However, proteolysis values suggested that the maturity of the farm cheeses was slower than the control ones showing around 50% less FAA content than control samples. Thus, in this type of samples there was not a similar amount of precursor amino-acids to allow a similar BA formation.

In the case of caprine milk cheeses the difference in the format characteristics between farm and control samples was not too intense. However, it was observed that FAA content during the ripening was slightly higher in the control samples with significant differences at the 45th day. This factor could contribute to a higher formation of BA in control samples, showing a maximum amount the day 45. A deceleration of the BA accumulation was observed at the 60th day in control samples. At this time, this kind of cheeses showed a slightly lower content of water, higher salt in moisture values and lower lactoccocci and lactobacilli counts than farm samples. Joosten, (1988a) in experiments with Gouda cheeses showed that a high salt/water ratio contributes to decelerate the liberation of amino acids and results in a lower HY content after 6 months of ripening

Figure 20. Total biogenic amine profile in caprine (20a) and ovine (20b) milk cheeses



HHP treatment influenced significantly the BA content in both kinds of cheeses. The application of pressure during the first stages of maturity caused an initial reduction of BA formation from the 15th day, observing that at the end of the ripening these samples displayed concentrations around 75% lower than control samples. Ovine milk cheeses HHP treated at the 15th day also resulted in a decrease of the BA content with respect to control samples, although this reduction was less pronounced (38% lower than control ones). Espinosa et al., (2006) observed that the application of HHP treatments at 400 MPa during 10 min at the 3rd day of ripening in artisan Spanish goat milk cheeses resulted in a reduction of about 49% in the total BA amount. However, the application of HHP treatment at the 15th day of maturity hardly influenced the amount of BA formed.

The BA reduction in caprine and ovine HHP1-samples could be explained as a result of a significant decrease in the microbiological counts observed one day after the treatment (specially of lactoccocci, lactobacilli, enteroccocci, and enterobacteria) and the lower proteolysis presented in these samples with respect to control ones, showing a reduction of about 34 and 49% of FAA content, respectively, at the end of the maturity. On the other side, the application of pressure at the 15th day of ripening also caused a decrease in the microbial counts when compared with the untreated cheeses, but this treatment did not affect the proteolysis, and consequently the liberation of amino acids. Novella-Rodríguez et al., (2002b) found that the total BA amine content in caprine milk cheeses with a HHP treatment

of 400 MPa during 5 min was similar than the untreated cheeses, although TY content was significantly reduced in HHP samples. However, in this work the higher proteolysis induced by HHP-treatments was not correlated with a higher amine production. Ruiz-Capillas et al., (2007) observed that HHP treatment at 350 MPa for 15 min applied on chorizo slices caused a significant decrease of BA amines content (mainly TY, PU, CA and SM), being the reduction of these amines coincidental with the decrease in microbial counts, especially of LAB.

According with the monoamines and diamines content observed in caprine and ovine milk cheeses (Tables 20 and 21) TY and PU were the BA with the highest amounts in GC samples, showing concentrations of about 492 and 476 mg kg^{-1}, respectively at the end of the ripening. Whereas in EC samples the predominant BA were TY and CA with amounts of 277 and 106 mg kg^{-1}, respectively, at day 60. Several authors reported, in different ranges, TY (88.6-445 mg kg^{-1}), HI (not detecting -697 mg kg^{-1}), PU (74.15- 446.5 mg kg^{-1}) and CA (44-269.77 mg kg^{-1}) as the most abundant BA in caprine and ovine milk ripened cheeses (Ordoñez et al., 1997; Valsamaki et al., 2000; Pinho et al., 2001; Galgano et al., 2001; Novella-Rodríguez et al., 2004a; Martuscelli et al., 2005; Pintado et al., 2008). These variable levels depended on the type of cheese, length of the ripening period; manufacturing process and type of microorganisms present (starters and non starter bacteria with decarboxylase activity).

TY was the main BA detected in control and farm samples in both type of cheeses. This coincided with the high frequency of TY-forming bacteria detected in both type of cheeses; particularly those isolated from KF, M17 and MRS media. Many studies also reported the predominance of TY, especially in raw milk cheeses (Ordoñez et al., 1997; Valsamaki et al., 2000; Pinho et al., 2001; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2002b; Novella-Rodríguez et al., 2004a; Novella-Rodríguez et al., 2004b). Roig-Sagués et al. (2002) found that the TY-forming bacteria isolated from traditional Spanish cheeses containing the highest TY amounts were mostly enteroccocci and LAB. In our study, diverse isolates obtained from both type of cheeses and belonging to these families showed, in high frequency, the ability to form TY in amounts over 100 mg L^{-1}. Among these isolates, the most common strains identified were *Enteroccoccus faecalis, Enteroccoccus faecium, Enteroccoccus durans, Lactoccoccus lactis subsp. lactis, Lactobacillus paracasei subsp paracasei, Lactobacillus plantarum* and *Lactobacillus brevis*.

On the other hand, the TY levels found in pressurized samples were lower than those presented in control cheeses. This aromatic amine increased slowly on the first 15 days of

ripening in GHHP1 samples and remained constant throughout the rest of the ripening. At the end of maturity, the concentrations were about 93 and 91% lower than in GC and farm samples, respectively. Likewise, EHHP1 samples reached concentrations of 33 mg kg^{-1} at the 60^{th} day, being this value 88 and 78% lower than those obtained in EC and farm samples, respectively. Whereas, the application of HHP15-treatment resulted in a slight decrease of TY levels, even so, on the 60^{th} day this amine content was not significantly different from that presented in control samples (Table 21). The reduction of the TY levels in caprine and ovine milk pressurized cheeses coincided mainly with the decrease in LAB and enteroccocci counts and with the FAA values displayed in these samples. Novella-Rodriguez et al., (2002b) observed that goat cheeses with a HHP-treatment of 400 MPa for 5 min had a significant lower TY content than the control samples. They credited this behavior mainly to the occurrence of HHP which had reduce the microbiological counts, especially the non starter LAB, although tyrosine amounts in this kind of cheeses resulted in similar levels than untreated cheeses.

PU content in untreated caprine milk cheeses showed almost the same amount as TY, with no significant differences between farm and control cheeses at the end of the ripening (Table 20). On the contrary, GHHP1 samples registered a slightly PU increase on the first 15 days and remained almost stable throughout the ripening, reaching concentrations of about 83 and 68% lower than control and farm samples, respectively, at the 60^{th} day. The application of pressure in ovine milk cheeses also affected the PU amounts formed, showing that EHHP1 samples limited the production of this diamine around 93 and 50% when compared with control and farm cheeses, respectively, at the end of the maturity. In addition, this kind of treatment displayed a 28% more efficiency than HHP applied on the 15^{th} day of ripening (Table 21). Untreated and HHP-treated caprine milk cheeses displayed CA in amounts below 100 mg kg^{-1} without significant differences. On the other hand, in control ovine milk cheeses this diamine increased mainly during the first 15 days, while in farm, HHPI and HHP15 samples remained without significant changes throughout the ripening.

The highest incidence of PU and CA-forming ability in bacteria isolated from caprine and ovine milk cheeses was observed mainly in enterobacteria, being *Hafnia alvei* the most frequently identified strain showing a strong and prolific capacity of production (100-1000 and >1000 mg L^{-1}), although some strains of *Entercoccus, Lactococcus, Pediococcus* and *Leuconostoc* was also found capable to form considerable amounts of diamines (above 100 mg L^{-1}). In both type of cheeses, enterobacteria decreased slowly throughout the ripening, reaching levels, at the end of the maturity, around of 2.5 log cfu g^{-1} in control and farm

samples and no detected levels in HHP-treated samples. Consequently, the diamines found here could be formed by this kind of bacteria and, in a less extend, by LAB and enteroccocci, which remained active much longer.

Several authors related the production of PU and CA with *Enterobactericeae* (Joosten and Northolt, 1987; Ten et al., 1990; Novella-Rodriguez et al., 2004a; Martuscelli et al., 2005; Pircher et al., 2007), but also some strains of enterococccci (Leuschner et al., 1999; Galgano et al., 2001; Pircher et al., 2007), lactococci (Bover-Cid and Holzapfel, 1999; Pircher et al., 2007; Burdychova and Komprda, 2007) and lactobacilli (Joosten and Northolt, 1987; Leuschner et al., 1998; Bover-Cid and Holzapfel, 1999) were reported with this ability. Joosten, (1988a) detected PU formation in cheese made from milk contaminated with coliform bacteria, salt-tolerant lactobacilli and mixtures of non-salt tolerant lactobacilli. The author noted that it was unlikely that coliform could cause much greater putrescine formation because they die during the ripening period and suggested that the presence of high amounts of PU could be related to the ornithine decarboxylating lactobacilli.

Low levels of HI were detected in untreated caprine milk cheeses reaching amounts not greater than 15 mg kg^{-1} at the 60^{th} day. This aromatic amine increased during the ripening and reached the maximum content about the day 45. Thereafter, a small decrease was observed in its concentration, being only significant in farm samples and showing 49% lower content than control on the 60^{th} day. A similar behavior was observed in GHHP1 samples, but, in this case, the differences were found from the 45^{th} day, displaying levels of 68% lower than the control samples of day 60 (Table 20). The concentration of HI in ovine milk control cheeses at the end of the ripening was 91 mg kg^{-1}, while farm cheeses presented a 76.4% lower HI amount on the same day. Likewise, EHHP1 samples showed a reduction of 92 and 31% of this aromatic amine when compared with control and farm samples, respectively. The HHP15 treatment was less efficient and displayed 36.7% less HI amounts than control (Table 21). The HI-forming bacteria isolated from caprine and ovine milk cheeses showed a small frequency and intensity of the amounts produced (hardly above the 100 mg L^{-1}). Among these strains, *Hafnia alvei, Staphylococcus aureus, Enterococcus durans* and *Enterococcus faecium* were identified.

Diverse authors reported low HI amounts in cheese (below 100 mg kg^{-1}), and related the production of this aromatic amine with some LAB (Joosten and Northolt, 1989; Valsamaki et al., 2000; Roig-Sagués et al., 2002; Novella-Rodríguez et al., 2002c; Burdychova and Komprda, 2007; Pintado et al., 2008). Likewise, some surveys have described enterobacteria with histidine decarboxylase activity in diverse food (Roig-Sagués et al., 1996; Roig-Sagués

et al., 1997a; Silla-Santos, 1998; Hernández-Herrero et al., 1999b; Roig-Sagués et al., 2002; Pircher et al., 2007; Özogul and Özogul, 2007). On the contrary, enterococci have been scarcely related with histamine formation in cheeses (Tham et al., 1990; Leuschner et al., 1999; Galgano et al., 2001).

The amounts of TR and PHE increased during the ripening of caprine milk cheeses without significant differences between in control, farm and HHP samples. Wherreas, low amounts of these amines were detected in ovine milk cheeses and remained practically constant without showing significant differences between the treatments (Tables 20 and 21).

Polyamine content in caprine cheese batches was low. SD and SM concentrations remained stable during the ripening with slight variations. However, an increase in their amount was observed when HHP-treatment was applied (Table 22). High levels of SD were detected in control, farm and HHP1 ovine milk cheeses, showing maximum amounts the 30^{th} day (about 64, 86, 122 mg kg^{-1}, respectively) followed by a decrease, with no differences between these type of samples at the end of the ripening. On the other hand, SM amounts were constant throughout the ripening of control and treated samples (Table 22). Polyamines are described as natural amines with non microbial origin and generally with a concentration lower than those of the bacterial origin. In some surveys, SD has been found as the main polyamine in cheese (Ordoñez et al., 1997; Pinho et al., 2001; Novella-Rodríguez et al., 2003). Some others have also mentioned their importance for the intestine cell growth and proliferation in childhood (Bardócz, 1995; Mariné Font, 2005). Novella-Rodríguez et al., (2004a) found an increase of polyamines, specifically SD, in goat cheeses treated with 400 MPa for 5 min. However no data were reported to elucidate the cause of this phenomenon. Galgano et al., (2001) detected some proteolytic and non proteolytic enteroccocci strains able to produce small amounts of SD *in vitro*.

As it has been mentioned previously, HI and TY are the BA that have been most frequently related with food-borne outbreaks, being suggested threshold values in cheese below 400 mg kg^{-1} of HI (Rauscher-Gabernig et al., 2009) and 200 mg kg^{-1} of TY (Karovicova and Kohajdova, 2005). Besides, food with TY amounts over 6 mg kg^{-1} (McCabe, 1986), 4-5 mg of PHE (Mariné Font, 2005), or more than 20 mg of HI (Vind et al., 1991) would be toxic to sensitive individuals.

If 30g of cheese is considered as a usual serving, caprine and ovine control cheeses analyzed in our survey would provide around 15 and 8.3 mg of TY, respectively, which can be dangerous for the most sensible consumers. On the contrary, cheeses with HHP-treatment at the first stages of maturation would give less than 1 mg of TY with the same size of serving.

As a conclusion, the effectiveness of HHP treatment depends on different factors, such as the type of cheese, the stage of ripening, the HHP processing conditions applied, the microbial counts content and the type of microbial genus and species present. In this work, the use of High Pressure applied on initial phases of ripening affected significantly the microorganisms responsible of forming BA, and, in consequence, reduced its content, especially on those with the highest concentrations: TY and PU in caprine and TY and CA in ovine milk cheese samples.

Table 20. Monoamine and diamine content (expressed in dry basis, mg kg^{-1}) during the ripening of caprine milk cheeses with and without HHP treatment

	Day of ripening	Control	Farm	HHP1
TR	6	8.99 ± 7.94^{a}		23.12 ± 5.93^{a}
	15	27.76 ± 15.00^{ab}	16.35 ± 15.81	10.81 ± 9.37^{a}
	30	95.96 ± 34.38^{c}	57.63 ± 8.51	82.10 ± 26.33^{b}
	45	68.39 ± 20.42^{bc}	52.12 ± 11.30	70.11 ± 40.01^{b}
	60	63.69 ± 27.90^{bc}	50.99 ± 7.56	89.08 ± 49.98^{b}
PHE	6	1.40 ± 0.65^{a}		0.26 ± 0.45^{a}
	15	14.62 ± 1.42^{b}	1.97 ± 3.42^{a}	17.96 ± 4.07^{b}
	30	17.27 ± 5.94^{b}	13.31 ± 7.61^{ab}	17.16 ± 4.29^{b}
	45	20.75 ± 4.20^{ab}	27.90 ± 21.30^{bc}	14.80 ± 0.45^{ab}
	60	31.13 ± 14.19^{b}	35.58 ± 10.84^{c}	25.56 ± 7.47^{b}
PU	6	4.07 ± 0.51^{a}		3.29 ± 0.42
	15	136.56 ± 21.57^{ab}	96.14 ± 16.60^{a}	59.09 ± 7.34
	30	225.16 ± 22.31^{b}	176.72 ± 47.73^{a}	67.09 ± 8.50
	45	463.88 ± 60.73$^{c\,B}$	251.88 ± 134.72$^{a\,A}$	42.69 ± 33.89^{A}
	60	476.41 ± 126.21$^{c\,B}$	420.32 ± 310.16$^{b\,B}$	79.80 ± 19.51^{A}
CA	6	30.20 ± 16.19^{a}		24.07 ± 9.04
	15	29.63 ± 4.35^{a}	42.60 ± 13.13	26.20 ± 3.13
	30	50.14 ± 10.97^{ab}	39.03 ± 24.63	35.29 ± 12.70
	45	69.53 ± 16.34^{b}	69.40 ± 42.41	36.22 ± 17.00
	60	70.45 ± 27.21^{b}	52.03 ± 24.02	44.22 ± 15.61
HI	6	1.27 ± 0.56^{a}		1.00 ± 0.87
	15	3.02 ± 0.43^{a}	2.92 ± 0.49^{a}	2.44 ± 0.34
	30	6.38 ± 3.31^{a}	5.19 ± 0.97^{a}	6.27 ± 3.16
	45	18.04 ± 9.36$^{b\,B}$	17.86 ± 8.62$^{b\,B}$	6.51 ± 1.75^{A}
	60	15.41 ± 7.05$^{b\,B}$	7.72 ± 3.25$^{a\,A}$	4.85 ± 2.20^{A}
TY	6	10.04 ± 6.80^{a}		6.11 ± 6.95
	15	130.51 ± 42.98^{ab}	183.32 ± 72.84^{a}	18.96 ± 1.07
	30	234.74 ± 69.16$^{b\,B}$	237.79 ± 45.64$^{ab\,B}$	15.59 ± 3.56^{A}
	45	443.87 ± 105.10$^{c\,B}$	362.45 ± 258.95$^{b\,B}$	16.17 ± 0.68^{A}
	60	491.89 ± 67.45$^{c\,B}$	346.65 ± 160.29$^{ab\,B}$	28.93 ± 5.91^{A}

(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-C Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

Table 21. Monoamine and diamine content (expressed in dry basis mg kg^{-1}) during the ripening of ovine milk cheeses with and without HHP treatment

	Day of ripening	Control	Farm	HHP1	HHP15
TR	5	1.66 ± 3.31^{a}		4.92 ± 5.86	
	15	4.51 ± 3.32^{a}	3.97 ± 5.79	10.03 ± 7.29	8.76 ± 6.98
	30	9.87 ± 4.43^{ab}	11.51 ± 0.72	9.83 ± 4.66	6.25 ± 0.80
	45	9.51 ± 3.85^{ab}	11.01 ± 6.89	12.49 ± 6.76	12.32 ± 8.43
	60	15.73 ± 2.15^{b}	12.61 ± 8.99	11.06 ± 6.78	11.66 ± 6.50
PHE	5	1.02 ± 1.23		0.40 ± 0.48	
	15	2.78 ± 1.44	3.18 ± 3.86	2.39 ± 0.56	4.39 ± 1.84
	30	5.67 ± 5.25	2.71 ± 1.09	3.04 ± 1.01	3.71 ± 1.45
	45	12.69 ± 5.08	3.35 ± 1.70	4.37 ± 1.52	5.78 ± 1.53
	60	12.74 ± 2.62	9.86 ± 8.64	4.43 ± 2.37	13.31 ± 15.37
PU	5	3.62 ± 0.40^{a}		5.20 ± 2.41	
	15	42.42 ± 23.00^{ab}	6.33 ± 2.76	11.25 ± 9.80	15.14 ± 11.99
	30	65.89 ± 30.90$^{bc\ B}$	5.27 ± 1.97^{A}	5.45 ± 1.38^{A}	22.33 ± 14.49^{AB}
	45	87.89 ± 76.10$^{c\ B}$	11.20 ± 4.60^{A}	7.81 ± 3.03^{A}	22.92 ± 17.51^{A}
	60	74.89 ± 54.74$^{bc\ B}$	10.69 ± 11.87^{A}	5.24 ± 0.76^{A}	26.08 ± 6.26^{A}
CA	5	62.13 ± 33.00^{a}		55.55 ± 28.54	
	15	159.07 ± 37.23$^{b\ B}$	62.73 ± 61.50^{A}	48.75 ± 44.16^{A}	87.50 ± 70.43^{AB}
	30	141.63 ± 79.34$^{ab\ B}$	66.30 ± 37.02^{A}	48.40 ± 16.94^{A}	80.65 ± 76.20^{AB}
	45	129.82 ± 67.03$^{ab\ B}$	66.41 ± 39.16^{AB}	41.91 ± 22.89^{A}	72.47 ± 29.78^{AB}
	60	105.90 ± 21.87$^{ab\ B}$	45.43 ± 18.05^{AB}	28.51 ± 17.32^{A}	80.28 ± 71.97^{AB}
HI	5	4.81 ± 4.54^{a}		5.33 ± 3.79	
	15	39.98 ± 11.42^{b}	14.04 ± 16.88	12.71 ± 10.97	29.14 ± 22.84^{a}
	30	74.34 ± 29.42$^{c\ B}$	7.57 ± 2.43^{A}	6.38 ± 2.77^{A}	47.52 ± 31.34$^{ab\ B}$
	45	81.66 ± 46.30$^{c\ B}$	16.72 ± 10.68^{A}	5.94 ± 1.25^{A}	58.99 ± 3.14$^{b\ B}$
	60	91.02 ± 5.73$^{c\ C}$	21.45 ± 25.28^{A}	7.07 ± 4.30^{A}	57.65 ± 13.16$^{ab\ B}$
TY	5	4.37 ± 0.71^{a}		3.15 ± 1.94	
	15	123.72 ± 40.62^{ab}	62.99 ± 86.66	13.79 ± 5.28	115.79 ± 55.44
	30	222.32 ± 84.26$^{bc\ B}$	54.98 ± 29.25^{A}	22.16 ± 10.34^{A}	162.64 ± 36.21^{A}
	45	268.02 ± 130.91$^{c\ B}$	189.56 ± 174.94^{AB}	80.49 ± 126.16^{A}	147.73 ± 30.57^{AB}
	60	277.30 ± 114.08$^{c\ B}$	149.25 ± 109.89^{AB}	32.69 ± 16.54^{A}	147.62 ± 26.64^{AB}

(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-C Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

Table 22. Polyamine content (expressed in dry basis mg kg^{-1}) during the ripening of caprine and ovine milk cheeses with and without HHP treatment

		Day of ripening	Control	Farm	HHP1	HHP15
Caprine milk chese	SD	6	1.13 ± 0.51^{a}		0.32 ± 0.50^{a}	
		15	1.92 ± 0.18$^{a\,A}$	1.66 ± 0.04^{A}	4.34 ± 0.21$^{b\,B}$	
		30	2.09 ± 0.24$^{ab\,A}$	1.14 ± 0.49^{AB}	3.70 ± 0.89$^{b\,B}$	
		45	4.32 ± 1.75^{c}	2.39 ± 0.82	3.28 ± 1.30	
		60	3.92 ± 1.49$^{bc\,A}$	3.06 ± 1.40^{A}	6.53 ± 1.99$^{c\,B}$	
	SM	6	2.25 ± 2.07^{a}		1.47 ± 1.48^{a}	
		15	4.35 ± 1.25$^{ab\,A}$	2.85 ± 0.14^{A}	7.80 ± 0.60$^{bc\,B}$	
		30	3.03 ± 0.75$^{ab\,A}$	1.95 ± 2.11^{A}	6.83 ± 0.46$^{b\,B}$	
		45	5.57 ± 3.91$^{b\,B}$	2.10 ± 0.30^{A}	2.16 ± 0.30$^{a\,A}$	
		60	3.90 ± 0.75$^{ab\,A}$	3.44 ± 1.81^{A}	10.46 ± 2.18$^{c\,B}$	
Ovine milk chese	SD	5	0.73 ± 0.65^{a}		0.51 ± 0.90^{a}	
		15	55.94 ± 29.00^{ab}	43.08 ± 45.10	90.23 ± 67.99^{bc}	73.15 ± 38.57
		30	64.22 ± 8.22$^{b\,A}$	86.05 ± 12.87^{AB}	122.25 ± 48.16$^{c\,B}$	65.61 ± 10.97^{A}
		45	14.74 ± 14.76^{ab}	61.38 ± 41.46	52.04 ± 36.91^{ab}	34.49 ± 19.56
		60	30.14 ± 23.04^{ab}	44.20 ± 30.32	19.49 ± 33.57^{a}	17.36 ± 9.35
	SM	5	6.76 ± 5.17		5.94 ± 4.03	
		15	14.13 ± 12.84	3.47 ± 4.65	18.69 ± 14.57	8.00 ± 6.99^{a}
		30	25.18 ± 18.41	19.83 ± 19.09	28.39 ± 7.52	39.50 ± 32.16^{b}
		45	17.57 ± 9.32	20.54 ± 7.95	13.13 ± 7.26	20.08 ± 21.91^{ab}
		60	14.66 ± 17.02	22.14 ± 18.48	15.27 ± 17.76	26.92 ± 17.93^{ab}

(±) Values are means standard deviations of data from the different batches
a-d Means with different superscript in the same column for the same parameter and sample differ significant (P<0.05)
A-C Means in the same row for the same parameter and day of ripening with different superscript differ significant (P<0.05)

SECTION IV

CONCLUSIONS

CONCLUSIONS

- The evaluated artisan cheese factories followed hygienic control plans consisting in the application, in different forms, of the prerequisites programs, although in most cases these procedures and control results were not written or registered. The main weaknesses observed in both cheese factories were the maintenance, cleaning and sanitation of the facilities and equipments, as well as the whey waste disposal. In addition, the lack of control in the water supply was also detected in one of the cheese houses.

- The HHP treatments used in this work (400 MPa for10 min at 2 ºC) did not affect total nitrogen, fat and salt content when applied on ovine and caprine raw milk cheese either on the first days or on the 15^{th} day of ripening. HHP treated samples showed higher pH values although differences were only observed at the 60^{th} day in the GHHP1 and EHHP15 samples.

- The HHP treatment applied on the beginning of the ripening of the ovine and caprine milk cheese affected significantly the proteolysis, causing a deceleration mainly on the last stages of the ripening. However, the HHP treatment applied on the 15^{th} day did not significantly affect the proteolysis rate.

- The HHP treatments applied on both type of cheeses contributed to reduce the fracturability of samples. Whereas deformability showed to be affected only one day after the HHP1 treatment was applied.

- Total color differences *(ΔE*-values*)* in the HHP treated samples were higher during the first stages of the ripening, decreasing as the sample aged. The color and firmness were the main characteristics in which the panel detected significant differences between the HHP-treated and control cheeses. Besides, no differences were perceived between samples with treatments applied at the beginning and day 15^{th} of ripening.

- The application of HHP treatments on the beginning and on the 15th day of ripening reduced significantly the number of undesirable contaminating microorganisms such as *Enterobactericeae*, *Escherichia coli* and *Staphylococcus aureus* in the ovine and caprine raw milk cheeses. Likewise these HHP treatments caused a significant decrease of the lactic acid bacteria counts, although a subsequent recover was observed throughout the ripening.

- The improved method that was used to detect specific amino acid decarboxylase activity using the LND and LGD broths was considered as excellent in the diagnostic of ornithine, lysine and tyrosine decarboxylase according to the validation parameters evaluated. On the contrary, the method showed to be less reliable to detect the histidine decarboxylase activity.

- The most frequently found amino acid decarboxylase activity among bacteria isolated from cheeses was the tyrosine decarboxylase, followed by the lysine and the ornithine decarboxylase capacity, forming in most cases amounts above 100 mg L^{-1}.

- A great variability was found between the different microbial groups either in the type and amounts of BA production. The main TY-forming strains were identified as *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Leuconostoc*; while PU and/or CA-producers were more frequently identified as *Enterobactericeae* and *Staphylococcus*, although some *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* strains were also capable to produce diamines. On the other hand, the HI-production ability was frequently detected in *Enterobactericeae*, *Staphylococcus* and *Enterococcus* in amounts below of 100 mg L^{-1}. However five strains belonging to these groups showed to possess strong HI-forming ability.

- The HHP treatments applied at the beginning and on the 15th day of ripening resulted in a decrease of around 75% and 38% of the total BA amount, respectively. TY and PU were the main amines afected in caprine milk cheeses, while TY and CA were in ovine milk cheese samples.

SECTION V

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