UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE NUTRICIÓN, BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS

TESIS DOCTORAL

Prevención de la sobremaduración y de la formación de aminas biógenas en quesos mediante tratamientos de altas presiones

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Javier Calzada Gómez

Directores

Manuel Núñez Gutiérrez
Ana del Olmo Sánchez

Madrid, 2015

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos

PREVENCIÓN DE LA SOBREMADURACIÓN Y DE LA FORMACIÓN DE AMINAS BIÓGENAS EN QUESOS MEDIANTE TRATAMIENTOS DE ALTAS PRESIONES

Javier Calzada Gómez

Madrid, 2014

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

PREVENCIÓN DE LA SOBREMADURACIÓN Y DE LA FORMACIÓN DE AMINAS BIÓGENAS EN QUESOS MEDIANTE TRATAMIENTOS DE ALTAS PRESIONES

Memoria presentada por **Javier Calzada Gómez** para la obtención del grado de Doctor por la Universidad Complutense de Madrid

Director: Prof. Dr. Manuel Núñez Gutiérrez
Codirector: Dra. Ana del Olmo Sánchez
Departamento de Tecnología de Alimentos
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

EL DOCTORANDO VºBº DEL DIRECTOR VºBº DEL CODIRECTOR

Manuel Núñez Gutiérrez, Profesor de Investigación del Departamento de Tecnología de Alimentos del INIA, y la Dra. Ana del Olmo Sánchez

CERTIFICAN:

Que la Tesis doctoral titulada “Prevención de la sobremaduración y de la formación de aminas biógenas en quesos mediante tratamientos de altas presiones” de la que es autor Javier Calzada Gómez, ha sido realizada bajo su dirección en el Departamento de Tecnología de Alimentos del INIA, y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid.

Madrid, 28 de Octubre de 2014

Fdo. Manuel Núñez Gutiérrez

Fdo. Ana del Olmo Sánchez

Agradecimientos.

En primer lugar, me gustaría agradecer a mi director de Tesis, Manolo, el brindarme la oportunidad de realizar este trabajo y por toda su confianza durante estos años. Por supuesto a mi codirectora Anen, porque sin ella, sin su ayuda y su cariño, todo hubiese sido más difícil y nunca hubiese conseguido llegar hasta aquí, y por todos los buenos momentos que hemos pasado juntos superando los numerosos obstáculos y problemas que nos hemos encontrado, juntos seguiremos superándolo todo.

A Pilar Gaya por su trabajo con el HPLC y sobre todo y más importante por su cariño y alegría.

A Marta por todo lo que hemos pasado, en el departamento y fuera, y por su ayuda, cariño y su amistad. A Sonia por su simpatía, su ayuda y su disposición a echar siempre una mano. A Roci y Rakel por su amistad, que siempre me ha ayudado a tirar hacia adelante con una sonrisa (Roci vuelve!!!). A Izaskun, Natalia y África por su contagiosa alegría y porque sigáis siempre así. A Iria por su apoyo, su simpatía y esos momentos de cigarritos y desahogos (mucho ánimo, que ya queda menos). Y a todos mis compañeros del departamento por los buenos momentos y por hacer más llevadera esta etapa: Juan, Marga, Máximo, Tomi, Joaquín, Pilar, Sagrario, Olga, Nerea, Chiqui, Lucía, Eva, Chema, Ángela, Dani y Susana. Sin olvidarme de Ana GI por su ayuda siempre con todos los papeleos y demás trámites. También agradecer su apoyo a las "vecinas" de leguminosas: Isabel, Carmen C, Mercedes, Carmen B y especialmente a Blanca y Merche por todo su cariño. Gracias a todos por vuestro apoyo.

Son demasiadas las personas a las que me gustaría agradecer y dedicar esta Tesis. Desde que entré en el departamento de Tecnología de Alimentos del INIA, son muchas las personas con las que he compartido momentos que nunca olvidaré y de las que he aprendido tantas cosas en el trabajo y en la vida. A "Sita" por enseñarme esos primeros pasos en micro, y por su amistad y todos los buenos momentos también quiero agradecer a Pi, Victoria, Eugenia, Teresa, Nuri, Toñi, Ana Gómez… por todo lo compartido.

A mis amigos del INIA con los que tantos buenos momentos he compartido: Nines, Isabelita, Mila, Isabel, Jesús, José Luis y Enrique, sin olvidarme de Evaristo que debería de estar compartiendo este momento.

A Manuela, de Veterinaria, por su paciencia y su ayuda con todas las dudas y trámites necesarios para llegar hasta aquí. A Isabel por su ayuda y su apoyo y a todos los que me habéis dado ánimos para finalizar esta Tesis.

A Mary, John, Gabriel, Casi y Vlad, por todo vuestro apoyo y por que con esa pizca de mala leche, siempre sacáis una sonrisa en los momentos duros.

A mis amigos, que no sois pocos y que os tengo abandonados, pero que sé que siempre estáis para lo que sea. Por todo el apoyo y todos los buenos y malos momentos que hemos pasado durante todos estos años en los que nos han pasado tantas cosas. A Pedrito, Ramos, Manu, Jaime, Mati, Yeyo, Osky, Maca, Borjita, Juncal, Tranchete, Eric, Pablo, Alvarito, Sandrita, Luigy, Laura, Carmela y a Julián, el culpable de que fuese a parar en el INIA. Sé que faltáis mucha gente con la que he compartido grandes momentos y aquí os dejo este espacio …….. ……, para que lo rellenéis y tengáis vuestro hueco.

A mis padres, por su apoyo y su cariño, por siempre estar dispuestos a echar una mano, por comprender que les haya tenido un poco abandonados y por todo lo que me han dado. A mi abuela para que siga adelante con mucho ánimo y dando guerra.

A todos, gracias, se os quiere.

"Solo hay dos cosas infinitas, el universo y la estupidez humana, y de la primera no estoy seguro"

A. Einstein.

ÍNDICE GENERAL.

ÍNDICE DE TABLAS.

ÍNDICE DE FIGURAS.

- **Abreviaturas**

a*	tendencia al rojo
ADC	arginina descarboxilasa
AGL	ácidos grasos libres
a_w	actividad de agua
α_{S1}-CN	alfa-s1-caseína
α_{S2}-CN	alfa-s2-caseína
α-LA	alfa-lactoalbúmina
b*	tendencia al amarillo
BAL-NI	Bacterias ácido lácticas no iniciadoras
β-CN	beta-caseína
β-LG	beta-lactoglobulina
$C_{10:0}$	ácido cáprico
$C_{12:0}$	ácido laúrico
$C_{14:0}$	ácido mirístico
$C_{16:0}$	ácido palmítico
$C_{18:0}$	ácido esteárico
$C_{18:1}$	ácido oleico
$C_{18:2}$	ácido linoleico
$C_{18:3}$	ácido linolénico
$C_{4:0}$	ácido butírico
$C_{6:0}$	ácido caproico
$C_{8:0}$	ácido caprílico
CADC	cetoácido descarboxilasa
CADH	cetoácido deshidrogenasa
DAO	diamino oxidasa
DAT	diamino N-acetiltranferasa
DM	dry matter
DOP	denominación de origen protegida
ES	extracto seco
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FDC	fenilalanina descarboxilasa
GC	gas chromatography
GC-MS	gas chromatography-mass spectrography
GDL	glucono-δ-lactona
GRAS	Generally Recognized as Safe
γ-CN	gamma-caseína

HADH	hidroxiácido deshidrogenasa
HDC	histidina descarboxilasa
HPLC	high pressure liquid chromatography
HPP	high pressure processing
HTST	High Temperature Short Time
κ-CN	kappa-caseína
L*	luminosidad
LDC	lisina descarboxilasa
LDH	L-lactato deshidrogenasa
LPL	lipoproteína lipasa
λ-CN	lambda-caseína
MAO	monoamino oxidasa
MAT	metionina adenosiltransferasa
nd	no detectado
NS	no significativo
ODC	ornitina descarboxilasa
OPA	*orto*-ftalaldehído (técnica espectrofotométrica)
PAO	poliamino oxidasa
PCA	principal component analysis
PCP	pirrolidona carboxilil aminopeptidasa
Pep C	aminopeptidasa C
Pep D	dipeptidasa D
Pep DA	dipeptidasa DA
Pep G	aminopeptidasa G
Pep N	aminopeptidasa N
Pep T	tripeptidasa T
Pep V	dipeptidasa V
PepA	glutamil aminopeptidasa
PepF	oligoendopeptidasas intracelulares F
PepI	prolin aminopeptidasa
PepL	leucil aminopeptidasa
PepO	oligoendopeptidasas intracelulares O
PepP	aminopeptidasa P
PepQ	prolidasa Q
PepR	prolinasa R
PepX	prolildipeptidil aminopeptidasa
PrtP	proteinasa asociada a la pared celular
p-κ-CN	*para*-kappa-caseína
SAMDC	S-adenosil-L-metionina descarboxilasa
SPME	solid phase micro extraction
TDC	tirosina descarboxilasa

TrDC	triptófano descarboxilasa
UA	unidades de área
ufc	unidades formadoras de colonias
UHT	Ultra High Temperature

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

PREVENCIÓN DE LA SOBREMADURACIÓN Y DE LA FORMACIÓN DE AMINAS BIÓGENAS EN QUESOS MEDIANTE TRATAMIENTOS DE ALTAS PRESIONES

RESUMEN de TESIS

Javier Calzada Gómez

Departamento de Tecnología de Alimentos

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid

Director: Prof. Dr. Manuel Nuñez Gutiérrez, Departamento de Tecnología de Alimentos, INIA, Madrid.

Codirector: Dra. Ana del Olmo Sánchez, Departamento de Tecnología de Alimentos, INIA, Madrid.

El queso desarrolla sus características organolépticas durante la maduración, debido a la acción de diferentes microorganismos y enzimas sobre los componentes de la cuajada. Los microorganismos y enzimas continúan ejerciendo su efecto durante el almacenamiento en refrigeración dando lugar a un desequilibrio de los compuestos responsables del aroma y sabor, lo que ocasiona una pérdida de calidad que limita la vida útil y provoca el rechazo por parte del consumidor. Un grupo de enzimas procedentes de microorganismos que pueden estar presentes en el queso son las descarboxilasas, que en presencia de aminoácidos libres son responsables de la formación de aminas biógenas. La acumulación de estos compuestos en el queso puede provocar efectos adversos para la salud del consumidor.

Las altas presiones hidrostáticas se emplean como método de pasteurización no térmica en diferentes alimentos para eliminar los microorganismos patógenos y alterantes, manteniendo en mayor medida las propiedades nutricionales y organolépticas de los alimentos que los tratamientos térmicos. En queso se ha estudiado además el efecto de las altas presiones con el fin de acelerar la maduración. Únicamente en queso fresco se han estudiado los tratamientos de altas presiones con el fin de alargar la vida útil.

En el presente trabajo de investigación se ha evaluado el efecto de la aplicación de altas presiones hidrostáticas sobre la proteolisis, lipolisis y catabolismo de aminoácidos y ácidos grasos libres en cuatro variedades de queso (queso azul, Torta del Casar, Brie y Arzúa-Ulloa) a lo largo de un prolongado periodo de maduración y almacenamiento en refrigeración, con el fin de alargar la vida útil y evitar la acumulación de aminas biógenas.

En queso azul de leche pasteurizada de oveja, madurado con *Penicillium roqueforti* en su interior, se aplicaron 400 y 600 MPa a las 3 (400-3S y 600-3S), 6 (400-6S y 600-6S) y 9 semanas (400-9S y 600-9S) de maduración. Los análisis se realizaron inmediatamente después de los tratamientos y a los 3, 6, 9 y 12 meses. Los tratamientos de altas presiones redujeron los niveles de microorganismos, llegándose a niveles no detectables de *P. roqueforti* en los quesos tratados con 600 MPa. La fuerte proteolisis primaria característica de esta variedad de queso no pudo evitarse con los tratamientos de altas presiones y únicamente los tratamientos de 600 MPa aplicados a las 3 y 6 semanas consiguieron reducir los niveles de péptidos hidrófilos. Por otro lado, los tratamientos de

600 MPa y el de 400 MPa aplicado a las 3 semanas consiguieron reducir la proteolisis secundaria, con niveles de aminoácidos y proteolisis global similares a los del queso control de 180 días. Las altas presiones redujeron la formación de tiramina, pero no de putrescina, triptamina y β-feniletilamina. Los tratamientos de altas presiones disminuyeron la formación de todos los grupos de ácidos grasos libres, excepto en los quesos 400-6S y 400-9S. En cuanto a los compuestos volátiles, las altas presiones consiguieron reducir los niveles de ésteres, alcoholes, hidrocarburos, compuestos bencénicos y nitrogenados, especialmente en los quesos tratados con 600 MPa, en los cuales además se redujo la formación de cetonas. En los quesos tratados a las 3 semanas disminuyeron los niveles de ácidos volátiles, compuestos azufrados y terpenos. Únicamente el tratamiento de 600 MPa aplicado a las 3 semanas redujo el ligero aumento de intensidad de sabor que se dio en el queso control. Todos los quesos tratados mantuvieron la misma calidad de sabor que el queso control, excepto el queso tratado con 600 MPa a las 3 semanas.

En Torta del Casar elaborada con leche cruda de oveja y cuajo vegetal de cardo (*Cynara cardunculus*), se aplicaron 400 y 600 MPa a las 3 (400-3S y 600-3S) y 5 semanas (400-5S y 600-5S) de maduración. Los análisis se realizaron inmediatamente después de los tratamientos y a los 2, 4, 6 y 8 meses. Las altas presiones redujeron los niveles de microorganismos, en especial los tratamientos de 600 MPa, y evitaron el aumento de pH que se dio en el queso control. Los tratamientos de 600 MPa consiguieron frenar la proteolisis primaria, reduciendo la hidrólisis de caseínas y la formación de péptidos hidrófilos. La proteolisis secundaria únicamente se frenó con los tratamientos de 600 MPa, reduciendo el tratamiento de 600 MPa aplicado a las 3 semanas la formación de aminoácidos libres y los tratamientos de 600 MPa la proteolisis global. Las altas presiones redujeron la actividad tirosina descarboxilasa, causando los tratamientos de 600 MPa un descenso de los niveles de tiramina e histamina, mientras que todos los tratamientos consiguieron reducir los niveles de cadaverina y, excepto el tratamiento de 400 MPa aplicado a las 3 semanas, los de putrescina, triptamina y β-feniletilamina. Todos los tratamientos provocaron una reducción de la actividad esterasa al final del almacenamiento en refrigeración, así como de los niveles de ácidos grasos libres de cadena corta, media y larga, de los ácidos de cadena ramificada y de ácido propanoico.

Por lo que respecta a los compuestos volátiles, las altas presiones consiguieron reducir los niveles de ésteres, aldehídos y especialmente de compuestos azufrados, que alcanzaron niveles muy elevados en el queso control. Los tratamientos de 600 MPa redujeron los niveles de alcoholes y los tratamientos aplicados a las 3 semanas los de ácidos volátiles. Todos los tratamientos evitaron la fuerte pérdida de calidad de sabor y olor que se dio en el queso control, reduciéndose también el aumento de intensidad de olor, olor pútrido y olor rancio. Los tratamientos de 600 MPa frenaron el incremento de intensidad de sabor que se dio en el queso control.

En queso Brie de leche de vaca pasteurizada, con *Penicillium camemberti* en su superficie, se aplicaron 400 y 600 MPa a las 2 (400-2S y 600-2S) y 3 semanas (400-3S y 600-3S) de maduración. Los análisis se realizaron inmediatamente después de los tratamientos y a los 1, 2, 3 y 4 meses. Los tratamientos de altas presiones redujeron los niveles de microorganismos, llegándose a niveles no detectables de *P. camemberti* en los quesos tratados con 600 MPa, y evitaron el fuerte aumento de pH que se dio en el queso control. La proteolisis primaria se vio reducida por los tratamientos de 600 MPa, disminuyendo la hidrólisis de α_S- y β-caseína, evitando todos los tratamientos el fuerte aumento de péptidos hidrófobos que se dio en el queso control. Sin embargo, las altas presiones no consiguieron frenar la proteolisis secundaria, estimada por los niveles de aminoácidos libres y la proteolisis global. Las altas presiones redujeron los niveles de ácidos grasos libres de cadena corta y ramificada. Además, los tratamientos aplicados a las 2 semanas redujeron los niveles de ácidos grasos de cadena media y larga y los tratamientos de 600 MPa los niveles de ácido etanoico. En cuanto a los compuestos volátiles, las altas presiones consiguieron limitar el incremento de cetonas y compuestos bencénicos, nitrogenados y azufrados. Todos los tratamientos evitaron el descenso de elasticidad y firmeza, mientras que sólo los tratamientos aplicados a las 2 semanas limitaron el descenso de fracturabilidad. Las altas presiones evitaron la pérdida de luminosidad y el aumento de la tendencia al rojo y al amarillo en el interior del queso. Las altas presiones provocaron la pérdida de la capa superficial de moho haciendo que los parámetros de color de la superficie fuesen muy diferentes a los del queso control. Todos los tratamientos evitaron el aumento de la intensidad de sabor y olor y de sabor amargo, así como la pérdida de calidad de sabor y olor que se dio en el queso control.

En el queso Arzúa-Ulloa elaborado con leche cruda de vaca, se aplicaron 400 y 600 MPa a las 2 (400-2S y 600-2S) y 3 semanas (400-3S y 600-3S) de maduración. Los análisis se realizaron inmediatamente después de los tratamientos y a los 2, 4, 6 y 8 meses. Los tratamientos de altas presiones redujeron los niveles de microorganismos, especialmente en los quesos tratados con 600 MPa, y evitaron el incremento de pH que se dio en el queso control. Las altas presiones no consiguieron frenar la proteolisis primaria pero sí la secundaria, reduciendo los niveles de aminoácidos libres y la proteolisis global. Las altas presiones disminuyeron la actividad tirosina descarboxilasa y los niveles de tiramina. Además, los tratamientos de 600 MPa redujeron los niveles de putrescina e histamina. Las altas presiones disminuyeron los niveles de ácido etanoico y de los ácidos grasos de cadena corta. Por lo que respecta a los compuestos volátiles, las altas presiones evitaron parcialmente el descenso de ácidos volátiles, éteres y compuestos azufrados. En los quesos tratados con 600 MPa se evitó el descenso de cetonas e hidrocarburos. Los tratamientos de 600 MPa frenaron la pérdida de luminosidad del interior del queso, aunque redujeron la tendencia al rojo y no afectaron a la tendencia al amarillo. Los tratamientos de altas presiones no evitaron el incremento de la intensidad del sabor ni de sabor umami que se dio en el queso control, pero mantuvieron la calidad del sabor.

Financiación para el desarrollo de la presente Tesis Doctoral:

La presente Tesis Doctoral se realizó en el marco del proyecto AGL 2009-07801, "Prevención de la sobremaduración y de la formación de aminas biógenas en quesos mediante tratamientos por altas presiones" del Ministerio de Ciencia e Innovación.

Durante la realización de la presente Tesis, su autor fue beneficiario de una beca F.P.I. (Formación de Personal Investigador) del Ministerio de Ciencia e Innovación, con referencia BES-2010-030444 asociada al proyecto AGL 2009-07801.

ESTRUCTURA DE LA TESIS

La presente tesis doctoral consta de los siguientes apartados:

- Una **Introducción** (Capítulo 1) redactada en español, que incluye una revisión bibliográfica sobre el queso, las aminas biógenas y las altas presiones hidrostáticas, y los **Objetivos** principales del trabajo de investigación.

- Nueve **capítulos temáticos** (Capítulos 2 a 10) redactados en inglés y presentados en formato de publicaciones en revistas científicas, que constituyen el cuerpo de la tesis.

- Una **Discusión general** (Capítulo 11) y una sección de **Conclusiones** (Capítulo 12), redactadas en español.

- Dos **resúmenes ampliados**, uno redactado en español (Capítulo 13) y el segundo redactado en inglés (Capítulo 14).

Capítulo 1. Introducción.

Fotografía: equipo de altas presiones modelo “Hiperbaric 135” empleado para la presurización de los quesos, de Hiperbaric (Burgos).

1.1. El queso

El queso es un sistema complejo en el que se suceden un gran número de reacciones bioquímicas que hacen que en un momento dado se alcance el equilibrio correcto de los compuestos que dan al producto final sus características organolépticas típicas. Los agentes responsables de estos cambios son los microorganismos y enzimas presentes en la leche, los microorganismos y enzimas añadidos (en particular los cultivos o fermentos lácticos y el cuajo u otras enzimas coagulantes) y los microorganismos contaminantes que acceden a la leche o al queso durante la maduración. Factores intrínsecos como el pH y la actividad de agua, y externos como la temperatura y la humedad relativa modulan la actividad de los agentes implicados en la maduración del queso.

1.1.1. Definición, historia y producción

1.1.1.1. Definiciones de queso

Queso es el nombre genérico que se da a un grupo de productos alimenticios basados en la coagulación de la leche y separación del suero. Según el Diccionario de la Lengua de la Real Academia Española el queso se define como "el producto obtenido por maduración de la cuajada de la leche con características propias para cada uno de los tipos según su origen o método de fabricación".

Una definición más completa sería la dada por la FAO en el Codex Alimentarius para Leche y Productos Lácteos, en el apartado 2 de la Norma General para el Queso (CODEX STAN 283-1978), donde se define de la siguiente manera:

> "2.1 Se entiende por queso el producto blando, semiduro, duro y extra duro, madurado o no madurado, y que puede estar recubierto, en el que la proporción entre las proteínas de suero y la caseína no sea superior a la de la leche, obtenido mediante:
>
> a. Coagulación total o parcial de la proteína de la leche, leche desnatada, leche parcialmente desnatada, nata, nata de suero, o suero de mantequilla, o de cualquier combinación de estos materiales, por acción del cuajo u otros coagulantes idóneos, y por escurrimiento parcial del suero que se desprende

como consecuencia de dicha coagulación, respetando el principio de que la elaboración del queso da lugar a una concentración de la proteína láctea (especialmente la porción de caseína) y que por consiguiente, el contenido de proteína del queso deberá ser evidentemente más alto que el de la mezcla de los materiales lácteos ya mencionados en base a la cual se elaboró el queso; y/o

b. Técnicas de elaboración que comportan la coagulación de la proteína de la leche y/o de productos obtenidos de la leche que dan un producto final que posee las mismas características físicas, químicas y organolépticas que el producto definido en el apartado a.

2.1.1 Se entiende por queso sometido a maduración el queso que no está listo para el consumo poco después de la fabricación, sino que debe mantenerse durante cierto tiempo a una temperatura y en unas condiciones tales que se produzcan los cambios bioquímicos y físicos necesarios y característicos del queso en cuestión.

2.1.2 Se entiende por queso madurado por mohos un queso curado en el que la maduración se ha producido principalmente como consecuencia del desarrollo característico de mohos por todo el interior y/o sobre la superficie del queso.

2.1.3 Se entiende por queso sin madurar el queso que está listo para el consumo poco después de su fabricación."

1.1.1.2. Antecedentes históricos

No se sabe con exactitud cuándo o dónde comenzó la elaboración de los primeros quesos. Se supone que esta práctica está relacionada con la historia de la domesticación de animales productores de leche hace 8.000 – 10.000 años, en el Neolítico. Según la leyenda, un mercader árabe que realizaba un largo viaje por el desierto conservó leche en un recipiente fabricado a partir del estómago de un cordero y cuando fue a consumirla vio que estaba coagulada y que se había separado el suero. Los primeros productos lácteos fermentados probablemente se produjeron por una combinación fortuita de sucesos, como la aparición de un grupo de bacterias con la capacidad de crecer en la leche y producir suficiente ácido para bajar el pH hasta el

punto isoeléctrico de las caseínas junto con unas condiciones y temperatura apropiadas para que estas crecieran y dieran lugar al producto fermentado. Originariamente el queso debió elaborarse como método para conservar la leche mediante coagulación ácida espontánea. Cuando el gel de leche coagulada por acidificación se rompía, este se separaba en suero y cuajada, siendo el suero ácido una bebida refrescante, mientras que la cuajada podía tomarse fresca o bien almacenarse para su posterior consumo. Posteriormente se observó que la leche coagulaba en contacto con determinados productos de origen animal o vegetal. Las enzimas proteolíticas están ampliamente distribuidas, pero una primera fuente pudo ser el estómago de los rumiantes. Al encontrar restos de cuajada en el estómago de mamíferos lactantes, después de ser sacrificados, se pudieron empezar a utilizar los estómagos como fuente de estas enzimas, que siguen empleándose en la actualidad. Las ventajas de convertir la leche en queso, tales como su estabilidad durante el almacenamiento y la facilidad de transporte, hicieron que los procesos de elaboración de queso se fueran estableciendo en las antiguas civilizaciones (Fox & McSweeney, 2004). Existen evidencias arqueológicas (Figura 1) sobre la elaboración de queso que datan del año 5500 a.C. en el norte de Europa (Salque *et al.*, 2013). Los primeros testimonios gráficos son el *Friso de la Lechería* (Figura 2), friso sumerio datado en el tercer milenio a.C., y diversos murales de tumbas egipcias de alrededor del año 2000 a.C.

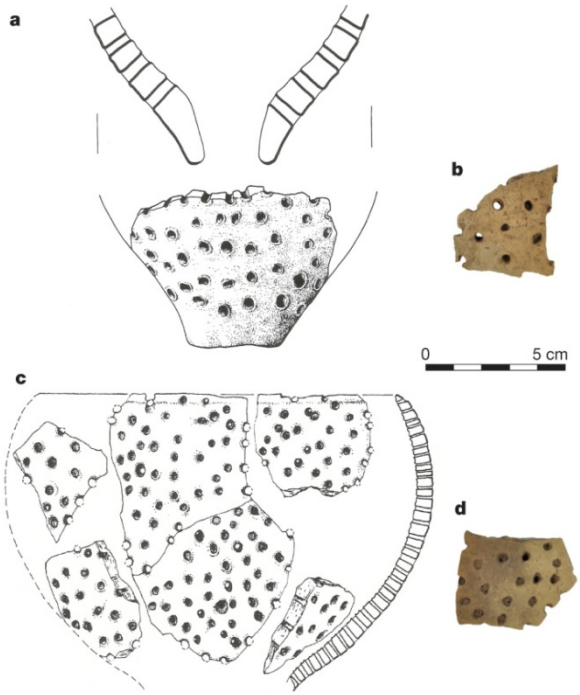


Figura 1. Fragmentos de vasijas para el desuerado (b, d) y esquema de la reconstrucción de los fragmentos de la vasija (a, c). (Salque *et al.*, 2013).

En la antigua Grecia el queso era un alimento habitual entre campesinos y pastores. En el siglo VIII a.C. Homero describe en la Odisea a Polifemo elaborando quesos de leche de oveja y en la Ilíada hace referencia a quesos elaborados con savia de higuera como coagulante. Es de la palabra griega *formos* (cesta donde se depositaba el queso y

Figura 2. Friso de la lechería. (Friso sumerio, 3000 a.C.).

se separaba el suero) de donde viene el nombre que se da al queso en francés, italiano y catalán (fromage, formaggio y formatge). El consumo del queso en Grecia se hacía condimentándolo con miel, hierbas aromáticas y aceite.

En Roma se siguió perfeccionando la forma de hacer queso, como expuso Columella en el siglo I d.C. en el libro VII del tratado "De re rustica". Más tarde Plinio el Viejo en su obra "Naturalis historia", describe la diversidad de quesos de la época, donde describe el queso Lunense, de 100 libras de peso. Aquí el queso era consumido por todas las clases sociales y los soldados romanos lo recibían como parte de su ración. De la palabra latina *caseus* (carente de suero) deriva el nombre de queso en español, queixo en gallego o queijo en portugués.

Los avances en la elaboración del queso en Europa progresaron lentamente en los siglos posteriores a la caída del Imperio Romano. Durante la Edad Media, la mejora y conservación de las prácticas queseras tuvo lugar en los monasterios, de donde proceden muchos de los quesos que conocemos en la actualidad.

La llegada de la Época Renacentista implicó el auge del comercio y el aumento de la población urbana, convirtiendo el queso en producto importante para la economía. En esta época el queso comenzó a comercializarse fuera de las zonas de producción, llegando hasta el Nuevo Mundo con las colonizaciones.

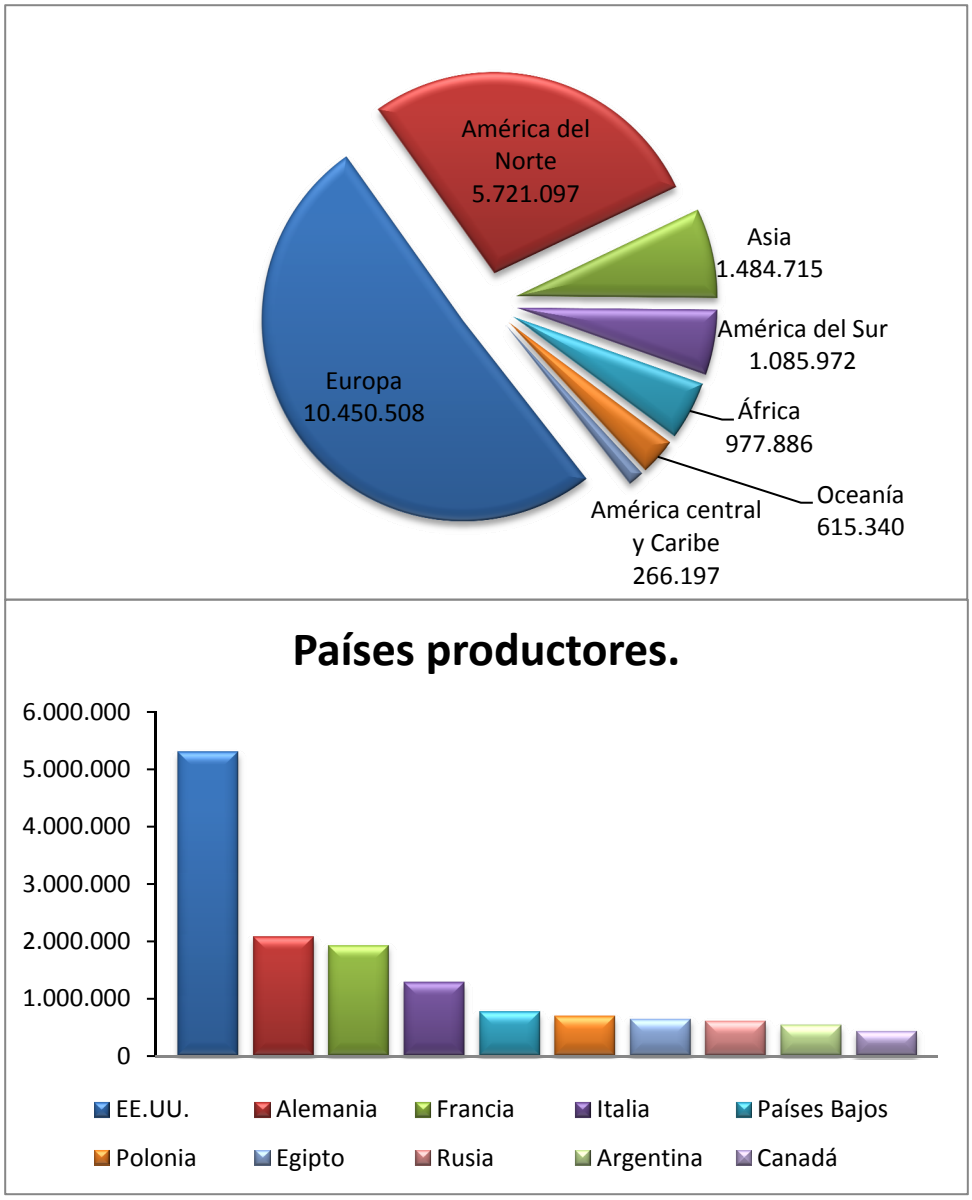
Durante el siglo XIX el queso se convirtió en un producto gastronómico símbolo de exquisitez y distinción. Con la llegada del siglo XX y los avances tecnológicos y bacteriológicos, el sector se industrializó, haciendo posible el aumento de la producción y del comercio y consumo a nivel mundial.

1.1.1.3. Producción y consumo

En la actualidad el queso es uno de los principales productos agroalimentarios, con una producción mundial de aproximadamente 20,6 x 10^6 toneladas en el año 2012 y un

aumento medio anual de alrededor del 1,8 % en los últimos 10 años. Como se recoge en la Figura 3, el continente con mayor producción es Europa, con aproximadamente 10,4 x 10^6 toneladas (50,5 % de la producción mundial) pero el país con mayor producción es Estados Unidos con 5,3 x 10^6 toneladas (25,5 % de la producción mundial).

Figura 3. Producción (en toneladas) de queso durante el año 2012.

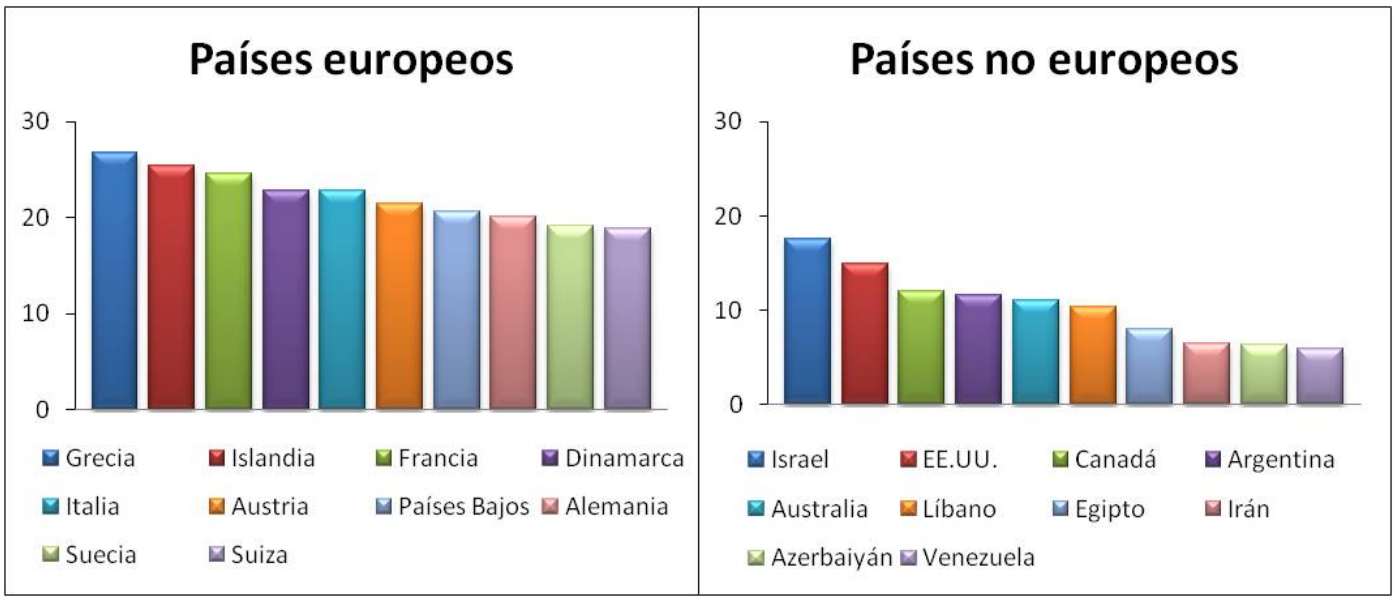


Fuente: FAOSTAT.

En cuanto al consumo per cápita (Figura 4) en el año 2011, la media mundial se estima en 2,9 kg/persona y año, situándose Grecia a la cabeza de consumo con 26,7 kg/persona y año.

En España la producción de queso durante el año 2012 fue de 213.732 toneladas, situándose como decimonoveno productor a nivel mundial (FAOSTAT, 2014). Por otro lado el consumo per cápita en el año 2013 fue de 8,2 kg (MAGRAMA, 2014).

Figura 4. Consumo per cápita (kg/persona y año) de queso en el mundo durante el año 2011.



Fuente: FAOSTAT.

1.1.2. Elaboración del queso

La transformación de la leche en queso comprende una serie de etapas: tratamiento o preparación de la leche, acidificación, coagulación, corte de la cuajada y desuerado, moldeo, prensado, salado y maduración. Dichas etapas pueden diferir según la variedad. A continuación se describen las principales características de la leche y las etapas de la transformación de la leche en queso.

1.1.2.1. La leche

La leche es una dispersión coloidal formada por agua, grasa, proteínas, azúcares, vitaminas y minerales. Su componente mayoritario es el agua. La concentración del resto de los componentes depende de factores como la especie (Tabla 1) y, dentro de una misma especie, de la raza, la alimentación, la estación del año, la fase de lactancia e incluso el número diario de ordeños.

Las proteínas de la leche se clasifican en dos grupos principales, las caseínas y las proteínas del suero. Las caseínas precipitan a 20 ºC cuando se alcanza un pH de 4,6 (su

punto isoeléctrico), mientras que las proteínas del suero permanecen solubles en esas condiciones. Las proteínas del suero representan entre el 14 y el 26 % de la proteína total de la leche y se dividen en dos fracciones, lactoalbúminas y lactoglobulinas. La fracción de las lactoalbúminas está formada principalmente por α-lactoalbúmina (α-LA) y β-lactoglobulina (β-LG), además de proteínas menores. La β-LG representa aproximadamente el 50 % de las proteínas séricas en la leche de vaca y su punto isoeléctrico se alcanza a un pH de aproximadamente 5,2. Su estructura es globular y es muy resistente a la proteolisis. La α-LA representa aproximadamente el 20 % de las proteínas séricas de la leche de vaca, su estructura también es globular y su punto isoeléctrico se alcanza a un pH de aproximadamente 4,8 (Fox, 2009). La fracción de las lactoglobulinas está compuesta principalmente por inmunoglobulinas.

Tabla 1. Composición de la leche de vaca, oveja y cabra.

	Vaca	Oveja	Cabra
Proteína %	2,35-3,80	4,75-7,20	2,61-4,09
Grasa %	3,50-5,02	3,60-9,97	3,48-5,63
Lactosa %	4,40-4,90	4,11-5,51	4,10-4,76
Extracto seco %	11,44-13,72	14,40-20,70	11,60-14,96

Fuente: Malacarne *et al.*, 2002, Park *et al.*, 2007, Raynal-Ljutovac *et al.*, 2008, Yuksel *et al.*, 2012.

La grasa de la leche se encuentra en forma de glóbulos cuyo diámetro puede ir desde 0,1 a 20 μm. Alrededor del 99 % de la grasa de la leche se encuentra compartimentada en estos glóbulos. La membrana que rodea a los glóbulos está formada por fosfolípidos, colesterol, lipoproteínas, glicoproteínas y enzimas. El interior de los glóbulos de grasa está constituido en su mayoría (98 %) por triglicéridos, aunque también contienen diglicéridos, monoglicéridos, ácidos grasos libres, lípidos polares y esteroles, así como trazas de vitaminas liposolubles y β-caroteno. El peso molecular de los triglicéridos puede ir desde 470 a 890 g/mol y la colocación de los ácidos grasos en las posiciones del glicerol no es aleatoria (Gresti *et al.*, 1993, Blasi *et al.*, 2008, Lopez, 2011). Los ácidos grasos de cadena inferior a 10 carbonos suelen localizarse

mayoritariamente en la posición sn-3 mientras que el ácido mirístico tiende preferentemente a colocarse en la posición sn-2. El ácido palmítico suele encontrase distribuido por igual entre las posiciones sn-1 y sn-2 mientras que el oleico, aunque se distribuye entre las tres posiciones, suele encontrarse mayoritariamente en la posición sn-1.

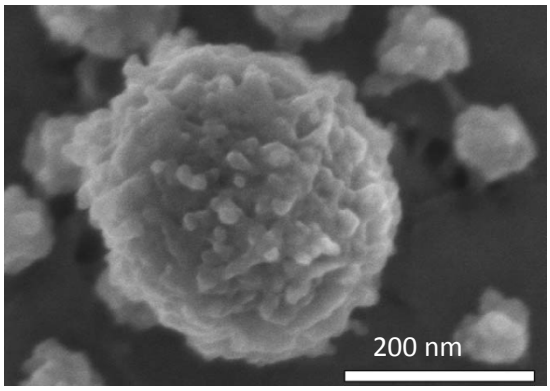


Figura 5. Micela de caseína obtenida por microscopía electrónica de barrido. (Dalgleish *et al.*, 2004).

Las caseínas son las principales proteínas de la leche y se encuentran formando agregados moleculares llamados micelas, cuyo diámetro puede ir desde 50 a 500 nm (Figura 5). Las micelas están formadas aproximadamente por un 92 % de caseínas y un 8 % de compuestos inorgánicos como fosfatos, calcio y otras sales y cationes. Las micelas están formadas por cuatro caseínas principales: la alfa S1 (α_{S1}-CN), alfa S2 (α_{S2}-CN), beta (β-CN) y kappa (κ-CN). La mayoría de los modelos estructurales indican que la κ-CN está situada principalmente en la superficie, formando una capa protectora que proporciona estabilidad a la micela. La cantidad de κ-CN es inversamente proporcional al tamaño de la micela. La β-CN se encuentra distribuida principalmente en el interior, mientras que las α_S-CNs se encuentran distribuidas por toda la micela (Dalgleish *et al.*, 2004, Dalgleish & Corredig, 2012, Bijl *et al.*, 2014). La estabilidad térmica de las caseínas y por tanto la de la leche, así como el color blanco y la coagulación por efecto del cuajo, se deben a la estructura y propiedades de la micela de caseína (Fox & Brodkorb, 2008). Desde el punto de vista de la estabilidad de la micela, la κ-CN es la caseína más importante ya que es la única soluble en presencia de calcio a la concentración que este se encuentra en la leche y es capaz de estabilizar diez veces su peso de caseínas sensibles al calcio (Farrell *et al.*, 2004, Fox & Brodkorb, 2008). Las principales proteínas de la leche (α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, α-LA y β-LG) presentan microheterogeneidades que pueden deberse a cinco factores: variabilidad en el grado de fosforilación, puentes disulfuro, grado de glicosilación, polimorfismo genético e hidrólisis producida por la plasmina. La plasmina

es una enzima capaz de hidrolizar la β-CN dando péptidos C-terminales que son las llamadas gamma caseínas (γ-CN) y péptidos N-terminales que son las proteosa-peptonas. Los fragmentos N-terminales de la hidrólisis de la α_{S1}-CN por la acción de la plasmina son las lambda caseínas (λ-CN). Las variantes debidas al polimorfismo genético ocurren durante la síntesis de estas proteínas por sustitución o deleción de aminoácidos. Existen variantes genéticas en leche de vaca, oveja, cabra y otras las especies. En leche de vaca se han identificado 8 variantes de α_{S1}-CN, 4 de α_{S2}-CN, 12 de β-CN, 11 de κ-CN, 3 de α-LA y 11 de β-LG (Caroli *et al.*, 2009, Fox, 2009). Las cuatro caseínas principales se diferencian entre ellas en el número de residuos aminoacídicos que las forman (199 la α_{S1}-CN, 207 la α_{S2}-CN, 209 la β-CN y 169 la κ-CN), peso molecular (23.612 la α_{S1}-CN, 25.228 la α_{S2}-CN, 23.980 la β-CN y 19.005 la κ-CN), la cantidad de grupos fosfato que llevan unidos, el grado de glicosilación y el punto isoeléctrico (Fox, 2009). Su proporción varía según el tipo de leche, siendo el porcentaje respecto a la caseína total de 37 y 33 % de α_{S1}-CN, 7 y 14 % de α_{S2}-CN, 42 y 30 % de β-CN y 9 y 14 % de κ-CN en leche de vaca y oveja respectivamente (Bramanti *et al.*, 2003). La cantidad de proteína de la leche, así como la proporción de caseínas y la estructura de las micelas de caseína tienen una gran influencia en las características tecnológicas de la leche.

La lactosa, el principal carbohidrato de la leche, es un disacárido formado por una molécula de α- o β-glucosa y otra de β-galactosa. Durante la elaboración del queso la mayor parte de la lactosa se pierde en el suero, mientras que la parte que queda retenida en la cuajada es empleada por las bacterias lácticas para producir ácido láctico. En la leche también se pueden encontrar, aunque en muy baja concentración, glucosa, galactosa y oligosacáridos.

La leche contiene minerales esenciales (potasio, sodio, calcio, magnesio, cloro y ésteres de fosfato), de los cuales sodio, potasio y cloro pueden aparecer como iones libres. Las concentraciones de calcio, magnesio, fosfato y citrato dependen del contenido de caseínas de la leche. La leche también contiene diversas vitaminas liposolubles como la A, D y E, y vitaminas hidrosolubles como la B_1, B_2, B_3, B_5, B_6, B_{12} y C.

Se han descrito más de 70 enzimas endógenas en la leche, algunas de las cuales tienen características tecnológicas reconocidas, mientras que de otras se desconoce

cuál es su papel. Estas enzimas pueden encontrarse libres, asociadas a los glóbulos de grasa o a las micelas de caseína. La leche contiene, entre otras, proteinasas como la plasmina, que es una proteinasa alcalina, o la catepsina D, que es una proteinasa ácida cuya concentración está relacionada con el número de células somáticas (Fox & Kelly, 2006). Otras enzimas con características tecnológicas conocidas son las lipasas y esterasas, entre las que destaca la lipoproteína lipasa. También se encuentran lisozima, xantina oxidorreductasa, catalasa, lactoperoxidasa, amilasa y aldolasa.

1.1.2.2. La coagulación de la leche

Como ya se ha indicado, la elaboración de quesos conlleva una serie de etapas o pasos que dependen del tipo de queso a elaborar (Tabla 2). La coagulación de la leche es la única etapa común a todas las variedades de queso y da como resultado un gel de proteína en el que quedan atrapados los glóbulos de grasa (Fox & McSweeney, 2004).

Tabla 2. Etapas en la elaboración del queso.

1	Preparación de la leche (pasteurización, homogeneización, microfiltración...). Opcional.
2	Adición de cultivo iniciador y/o cultivos adjuntos. Opcional.
3	Coagulación (enzimática, ácida o mixta).
4	Corte de la cuajada.
5	Tratamiento de la cuajada (calentamiento, lavado, salado...). Opcional.
6	Desuerado.
7	Moldeado.
8	Prensado. Opcional
9	Maduración. Opcional

La coagulación de la leche puede ser ácida y/o enzimática. La coagulación ácida se puede realizar por adición de bacterias que fermentan la lactosa a ácido láctico, por adición de ácidos o por adición de glucono-δ-lactona (GDL) que por hidrólisis da ácido glucónico, provocando la acidificación de la leche. La acidificación de la leche provoca cambios en las propiedades fisicoquímicas de las micelas de caseína. A medida que el pH desciende, el fosfato cálcico coloidal de las micelas se va disolviendo provocando la desestabilización de estas y la liberación de las caseínas al medio. Cuando el pH se va aproximando al punto isoeléctrico de las caseínas se van formando agregados hasta

que se obtiene el gel láctico desmineralizado (Lucey & Singh, 1997). El gel formado por acidificación es un gel firme, friable y poco contráctil que retiene mucha agua.

La coagulación enzimática se realiza mediante la adición de proteasas a la leche. Los cuajos tradicionalmente empleados en la elaboración de queso son los de origen animal, que contienen quimosina (EC 3.4.23.4) como principal enzima (80-90 %) y (10-20 %) pepsina (EC 3.4.23.1). Este cuajo se obtiene del estómago de mamíferos lactantes. La quimosina es una proteasa aspártica y su actividad proteolítica comparada con la de otras proteinasas es muy baja, pero es muy activa en la hidrólisis del enlace Phe_{105}-Met_{106} de la κ-caseina (Horne & Banks, 2004). La pepsina es una enzima muy parecida a la quimosina aunque menos específica. Es una proteasa muy ácida, con un pH óptimo de actividad en torno a 2 y que a valores de pH superiores a 6,6 se inhibe. Existen también cuajos microbianos obtenidos de hongos o bacterias (EC 3.4.23.9) como: *Rhizomucor miehei*, *Aspergillus oryzae* o *Irpex lactis* cuya especificidad es comparable a la de la quimosina, pero que tienen mayor estabilidad térmica (Kumar *et al.*, 2010). La quimosina recombinante es una preparación enzimática obtenida de microorganismos modificados genéticamente como *Aspergillus niger*, *Kluyveromyces lactis* y *Escherichia coli*, a los que se les ha introducido el gen de la quimosina. Debido a la limitación en la producción de cuajo natural, al aumento de la producción de quesos y a la aprobación de la quimosina recombinante como GRAS (Generally Recognized as Safe) por la FDA (Food and Drug Administration) de Estados Unidos en los años 90, en la actualidad más del 50 % de los quesos fabricados en el mundo son elaborados con este tipo de quimosina (Kumar *et al.*, 2010). También pueden emplearse proteasas de origen vegetal, de diversas fuentes como hojas o ramas de higuera, frutas (kiwi o melón), raíces (jengibre) y flores, como las cardosinas o cinarasas extraídas del cardo (*Cynara cardunculus*) (Shah *et al.*, 2014). Las cardosinas son proteinasas aspárticas que, al igual que la quimosina, hidrolizan el enlace Phe_{105}-Met_{106} de la κ-caseina (Roseiro *et al.*, 2003). La hidrólisis de la κ-caseína provoca la ruptura de la capa protectora de la micela de caseína en macropéptido y para-κ-caseína. En presencia de suficiente calcio estas micelas modificadas comienzan a unirse unas con otras en largas cadenas ramificadas, que acaban formando una estructura tridimensional que da lugar a la cuajada (Horne & Banks, 2004, Osintsev & Qvist, 2004). La mayor parte del cuajo empleado en la

elaboración del queso se pierde con el suero, mientras que la parte de cuajo que queda retenido en la cuajada continúa con su actividad pudiendo hidrolizar la β-caseína y la α_{S1}-caseína. La α_{S2}-caseína parece ser resistente a la hidrólisis por la quimosina y la para-κ-caseína, a pesar de tener varios enlaces adecuados para su actividad, tampoco parece que se hidrolice con esta enzima.

1.1.2.3. La maduración del queso

Una de las etapas más importantes en el proceso de elaboración de la mayoría de los quesos es la maduración, proceso por el cual el queso alcanza las características de sabor, aroma y textura típicas de cada variedad. Cada tipo de queso tiene un tiempo y unas condiciones específicas de maduración (temperatura y humedad). El tiempo de maduración puede ir desde las 1-2 semanas para el queso Mozzarella hasta 2 años para Parmigiano-Reggiano. La maduración es un proceso complejo que normalmente implica cambios en la microbiota, incluyendo la muerte y lisis del cultivo iniciador, el desarrollo de microbiota endógena de la leche y en algunos casos el crecimiento de microbiota secundaria, añadida o contaminante accidental. La actividad metabólica de la microbiota secundaria influye en el desarrollo del sabor y en algunos casos, como en los quesos con mohos en su superficie, en la textura. La maduración implica a su vez una serie de cambios bioquímicos que pueden deberse a las enzimas del cuajo, las endógenas de la leche, las de los cultivos iniciadores, las de la microbiota secundaria añadida, las de la microbiota de la leche y las enzimas exógenas añadidas para acelerar la maduración (McSweeney, 2004). Las reacciones bioquímicas que se dan durante la maduración se pueden clasificar en tres apartados principales:

1.1.2.3.1. Glicolisis de la lactosa. Catabolismo de lactato y citrato

Durante la formación de la cuajada, la lactosa es fermentada por el cultivo iniciador dando ácido láctico (principalmente el isómero L), lo que provoca una disminución del pH. La acidificación tiene una gran influencia en la textura debido a la desmineralización de la micela de caseína. También tiene influencia en la proteolisis debido a que la micela de caseína desmineralizada es más susceptible a la proteolisis y, además, a pH bajo la cuajada puede retener más quimosina.

La lactosa que queda retenida en la cuajada es rápidamente metabolizada a L-lactato por el cultivo iniciador. Al cabo de unos días apenas se detecta lactosa. El lactato formado es a su vez un importante sustrato para una serie de reacciones (Figura 6) que ocurren durante la maduración del queso (McSweeney & Fox, 2004) y que se describen brevemente a continuación:

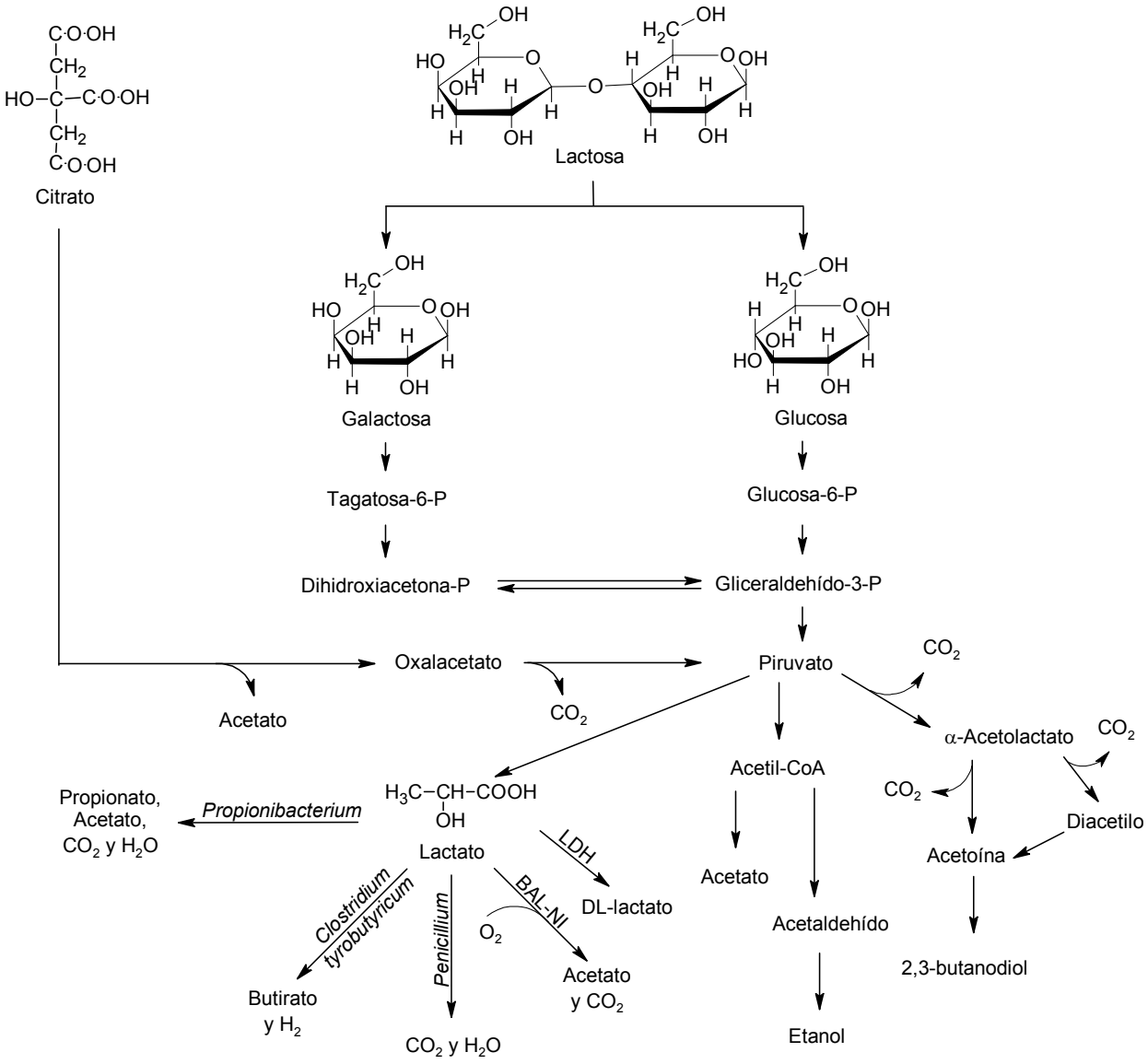


Figura 6. Esquema de la glicolisis y del catabolismo de lactato y citrato (Sarantinopoulos *et al.*, 2001, Marilley & Casey, 2004). (LDH: Lactato deshidrogenasa. BAL-NI: Bacterias lácticas no iniciadoras).

• En la mayoría de los quesos se produce la racemización del L-lactato a D-lactato por la acción de bacterias lácticas de la microbiota secundaria. Esta racemización suele producirse por la acción de la enzima L-lactato deshidrogenasa (LDH), que oxida el L-

lactato formando piruvato, el cual es reducido a D-lactato por efecto de la D-LDH obteniéndose la mezcla racémica de DL-lactato. La racemización probablemente no afecta al sabor final del queso, pero esta mezcla es menos soluble que el L-lactato puro lo que provoca que se formen cristales de lactato cálcico que pueden provocar rechazo por el consumidor.

• En presencia de oxígeno algunas bacterias lácticas pueden oxidar el lactato dando acetato, etanol, formato y/o CO_2 aunque esta ruta no es muy común debido a la limitación de oxígeno que existe en el queso. Las bacterias del género *Pediococcus* en presencia de una elevada concentración de oxígeno pueden formar 1 mol de acetato y 1 mol de CO_2 a partir de 1 mol de lactato y 1 mol de O_2. El pH óptimo para que se dé esta oxidación es entre 5 y 6. Esta reacción depende por tanto de las cepas presentes, así como de la concentración de lactato y de la disponibilidad de oxígeno. El acetato suele encontrarse en la mayoría de los quesos y contribuye al sabor.

• En los quesos madurados con mohos en la superficie (como Camembert o Brie) el catabolismo oxidativo es muy fuerte. Los mohos superficiales (*Penicillium camemberti*) catabolizan rápidamente el lactato a H_2O y CO_2 provocando un aumento del pH en la superficie, lo que causa en el queso un gradiente de pH y la difusión del lactato hacia la superficie.

• El lactato también puede ser catabolizado anaeróbicamente por *Clostridium tyrobutyricum* produciéndose butirato e hidrógeno, lo que provoca el defecto conocido como "hinchazón tardía" que se da en algunos quesos de pasta dura.

• En los quesos tipo Suizo el lactato es metabolizado por *Propionibacterium freudenreichii* subsp. *shermanii* con formación de propionato, acetato, CO_2 y H_2O. El CO_2 migra a través de la cuajada hasta los puntos más débiles en los que se acumula, formándose los característicos ojos de este tipo de quesos.

Los niveles de citrato en la leche son bajos y además la mayor parte se pierde, al igual que la lactosa, en el suero. Las pequeñas cantidades de citrato residual retenido en la cuajada pueden ser metabolizadas por los cultivos iniciadores mesófilos, formándose diacetilo, acetato, acetoína, 2,3-butanodiol y CO_2. El CO_2 producido durante el metabolismo del citrato es el responsable de los característicos ojos de los quesos de

tipo holandés (Edam, Gouda) y de defectos en queso Cheddar y Cottage. Debido a la formación de diacetilo, acetato, acetoína y 2,3-butanodiol el metabolismo del citrato es muy importante en el aroma y sabor de quesos de tipo holandés, Cheddar y Cottage (McSweeney & Fox, 2004).

1.1.2.3.2. Lipolisis y catabolismo de los ácidos grasos libres

La fracción lipídica es esencial para la correcta formación del sabor durante la maduración del queso. La mayor parte de la grasa de la leche son triglicéridos (aproximadamente el 98 %), en los que los ácidos grasos están colocados en posiciones no aleatorias del glicerol. Durante la maduración esta grasa puede ser oxidada o hidrolizada. Los ácidos poliinsaturados son especialmente sensibles a la oxidación, dando aldehídos insaturados que provocan defectos del sabor como la rancidez. La oxidación lipídica no ocurre de manera significativa en el queso, debido probablemente a su bajo potencial redox y a la presencia de antioxidantes naturales. La hidrólisis enzimática de los triglicéridos (lipolisis) ocurre en los quesos durante la maduración dando lugar a ácidos grasos libres, diglicéridos, monoglicéridos y, en última instancia, glicerol (Figura 7). La mayoría de los quesos tienen un grado de lipolisis bajo o moderado y un exceso de ácidos grasos libres provoca defectos de sabor. Únicamente los quesos madurados por mohos o los de pasta dura italianos se caracterizan por un elevado grado de lipolisis.

La lipolisis en el queso se debe a la presencia de enzimas lipolíticas (hidrolasas), que rompen el enlace éster que existe entre el ácido graso y el glicerol, obteniéndose el ácido graso libre. Estas enzimas lipolíticas pueden clasificarse como lipasas y esterasas, que se diferencian por tres características principales: longitud de la cadena del acilléster que hidrolizan, naturaleza físico-química del sustrato y la cinética enzimática.

Las esterasas hidrolizan preferentemente acilésteres de 2 a 8 átomos de carbono, mientras que las lipasas hidrolizan preferentemente los de 10 o más átomos de carbono. Las esterasas hidrolizan sustratos solubles en soluciones acuosas mientras que las lipasas hidrolizan sustratos emulsionados. Las esterasas tienen una cinética de tipo Michaelis-Menten clásica, mientras que las lipasas únicamente se activan cuando se encuentran en una interfase hidrófoba/hidrófila y siguen una cinética de Michaelis-Menten interfacial.

En general las enzimas lipolíticas son específicas de las posiciones exteriores (sn-1 y sn-3) del triglicérido. Inicialmente los triglicéridos son hidrolizados a 1,2- y 2,3-diglicéridos y después a 2-monoglicéridos. Las lipasas del queso pueden proceder de la leche, de algunos preparados de cuajo, del cultivo iniciador, de cultivos adjuntos, de la microbiota secundaria o bien pueden ser lipasas exógenas añadidas.

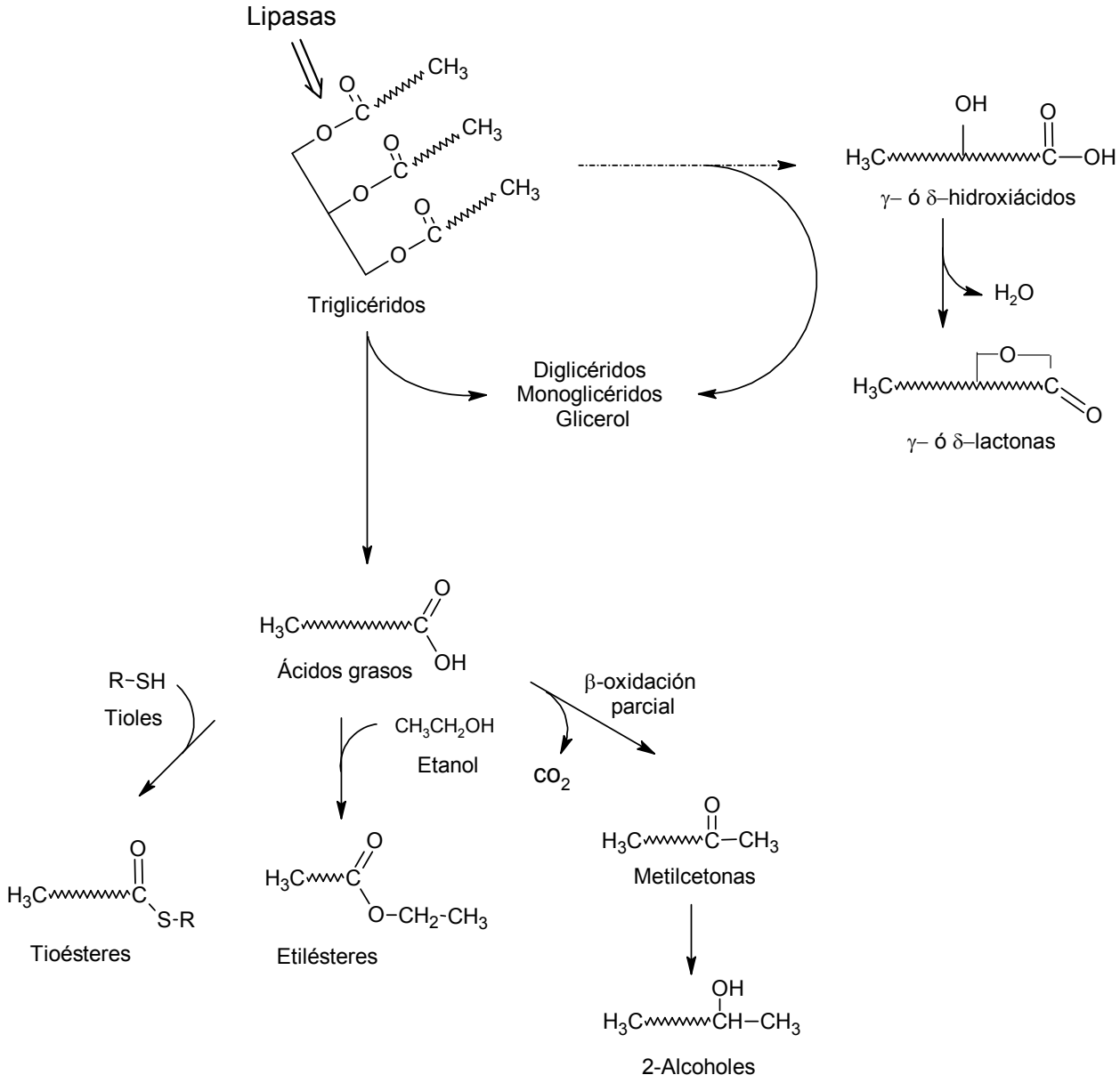


Figura 7. Esquema de la lipolisis y del catabolismo de ácidos grasos libres. (Collins *et al.*, 2003).

La leche contiene una potente lipasa endógena, la lipoproteína lipasa (LPL) que normalmente nunca alcanza su máxima actividad en la leche. La LPL en la leche está asociada con las micelas de caseína y la membrana lipoproteica de los glóbulos de

grasa. Si esta membrana se rompe por agitación, homogeneización o un mal manejo de la leche, puede tener lugar una excesiva lipolisis que da lugar a la aparición de sabores defectuosos. Esta enzima no tiene una gran especificidad en cuanto al tipo de ácido graso, pero tiene mayor tendencia por la hidrólisis de los ácidos grasos situados en las posiciones sn-1 y sn-3 de los mono-, di- y triglicéridos donde suelen encontrarse ácidos grasos de cadena corta o media (Collins *et al.*, 2003). La LPL es más activa en quesos de leche cruda que en quesos de leche pasteurizada. Un tratamiento de 72 ºC durante 15 segundos provoca una considerable inactivación de la LPL, pero puede seguir contribuyendo a la lipolisis en algunos quesos de leche pasteurizada ya que se requiere un tratamiento de 78 ºC durante 10 segundos para su completa inactivación (Deeth, 2006).

Normalmente los cuajos comerciales no tienen actividad lipolítica, pero algunas pastas de cuajo empleadas en la elaboración de algunas variedades de quesos, como los de pasta dura italianos, contienen esterasa pregástrica. Esta esterasa es altamente específica para la esterificación de ácidos grasos de cadena corta situados en la posición sn-3.

Otra fuente de enzimas lipolíticas son los microorganismos, que pueden proceder de cultivos iniciadores, cultivos adjuntos o ser microorganismos contaminantes. Las lipasas y esterasas de las bacterias lácticas son los principales agentes lipolíticos de gran variedad de quesos elaborados con leche pasteurizada. Las bacterias lácticas poseen enzimas capaces de hidrolizar un amplio rango de ésteres de ácidos grasos de los mono-, di- y triglicéridos. Las bacterias lácticas son muy poco lipolíticas en comparación con otros microorganismos, aunque su presencia en gran número en el queso durante un largo periodo de maduración puede hacer que se liberen niveles significativos de ácidos grasos. Las lipasas y esterasas de las bacterias lácticas son en general intracelulares, por lo que deben ser liberadas mediante la lisis de las células para ejercer su actividad, aunque en algún caso se han encontrado esterasas asociadas a la superficie celular (Gobbetti *et al.*, 1997). Las bacterias propiónicas tienen una actividad lipolítica de 10 a 100 veces mayor que las bacterias lácticas. En los quesos con mohos, se ha visto que los del género *Penicillium* tienen una gran actividad lipolítica. Las

lipasas y esterasas procedentes de microorganismos son muy diferentes y dependen de la especie e incluso de la cepa.

Las lipasas exógenas pueden ser de origen animal o microbiano. Estas enzimas se emplean de forma comercial en algunos quesos de pasta dura italianos y de forma experimental en otras variedades, para conseguir alcanzar sabores característicos y acelerar la maduración (Hernández *et al.*, 2009).

Los principales ácidos grasos libres procedentes de la lipolisis se pueden clasificar según el tamaño de la cadena como de cadena corta: butírico ($C_{4:0}$), caproico ($C_{6:0}$) y caprílico ($C_{8:0}$); de cadena media: cáprico ($C_{10:0}$), laúrico ($C_{12:0}$) y mirístico ($C_{14:0}$); y de cadena larga: palmítico ($C_{16:0}$), esteárico ($C_{18:0}$), oleico ($C_{18:1}$), linoleico ($C_{18:2}$) y linolénico ($C_{18:3}$). También se encuentran ácidos carboxílicos que no provienen de la lipolisis como el acético y el propiónico y ácidos ramificados como el isobutírico y el isovalérico. Los ácidos grasos libres pueden contribuir directamente al sabor del queso, especialmente los de cadena corta y cadena media. Así el $C_{4:0}$ aporta sabor rancio y típico de queso, el $C_{6:0}$ aporta sabor picante y típico de queso azul y el $C_{8:0}$ da sabor a cera, jabón, cabra y moho, pero fundamentalmente son importantes sustratos de reacciones que dan lugar a compuestos responsables del aroma y sabor del queso (Curioni & Bosset, 2002).

Los ácidos grasos libres formados durante la maduración del queso pueden dar lugar a cetonas, ésteres, lactonas, aldehídos y alcoholes (Figura 7). La formación de metilcetonas se produce mediante la β-oxidación del ácido graso libre y posterior descarboxilación del correspondiente cetoácido. Por sucesivas reacciones de β-oxidación se pueden obtener metilcetonas de cadena corta a partir de ácidos grasos libres de cadena larga. Estas metilcetonas procedentes del catabolismo de ácidos grasos libres son muy típicas de quesos madurados con mohos, especialmente la 2-heptanona y la 2-nonanona que contribuyen al aroma característico de queso azul y son las principales metilcetonas del queso Camembert. Las metilcetonas también son importantes en variedades de queso no maduradas con mohos. Las metilcetonas pueden a su vez ser reducidas, dando los correspondientes alcoholes secundarios que aportan al queso aromas a fresco, hierba y afrutado (Kinsella & Hwang, 1976, Qian *et al.*, 2002). Los ésteres son compuestos que forman parte del aroma del queso y se forman por reacción de un ácido y un alcohol, por esterificación química o enzimática.

Esta última puede ser llevada a cabo por la enzima carboxilesterasa, que está presente en la mayoría de los microorganismos implicados en la elaboración de queso. Los ésteres se encuentran en gran variedad de quesos y contribuyen aportando aromas afrutados, florales y dulces (Curioni & Bosset, 2002). Las lactonas aportan al queso aromas de melocotón, coco y albaricoque y se forman a partir de los hidroxiácidos de la grasa. Para las δ-lactonas y las γ-lactonas de cadena larga se ha propuesto un mecanismo de formación directa en un solo paso del hidroxiácido que se encuentra esterificado en el triglicérido, mecanismo que estaría únicamente influido por la temperatura (Alewijn *et al.*, 2007). Otro mecanismo para la formación de lactonas podría ser la hidrólisis del hidroxiácido y posterior esterificación cíclica, dependiente de la presencia de microorganismos y de la temperatura (Rehman *et al.*, 2000, Curioni & Bosset, 2002). Los aldehídos lineales pueden formarse por β-oxidación de ácidos grasos libres insaturados y se caracterizan por aportar al queso aromas frescos y herbales, pero cuando su concentración supera cierto límite pueden dar lugar a aromas desagradables (Curioni & Bosset, 2002, Collins *et al.*, 2003).

1.1.2.3.3. Proteolisis y catabolismo de los aminoácidos

La proteolisis es el fenómeno bioquímico más complejo y en la mayoría de variedades el más importante que tiene lugar durante la maduración del queso, influyendo tanto en el sabor como en la textura. Los péptidos de pequeño tamaño y los aminoácidos liberados pueden contribuir directamente al sabor. Además, estos últimos sirven como sustrato de reacciones en las que se forman nuevos compuestos responsables del aroma y sabor. La proteolisis contribuye a la textura mediante la hidrólisis de la matriz de proteínas del queso, disminuyendo la actividad de agua e indirectamente aumentando el pH (Upadhyay *et al.*, 2004). Durante la proteolisis se produce la hidrólisis de las caseínas y de los péptidos de tamaño elevado, dando lugar a péptidos de tamaño intermedio (proteolisis primaria), que a su vez son hidrolizados para dar péptidos pequeños y en última instancia aminoácidos libres (proteolisis secundaria). Este proceso se debe a la acción de enzimas (proteasas y peptidasas) que pueden proceder de la leche, del cuajo residual y de microorganismos (bacterias lácticas del cultivo iniciador, cultivos adjuntos y otras bacterias presentes en la leche), además de enzimas exógenas que pueden ser añadidas para acelerar la maduración.

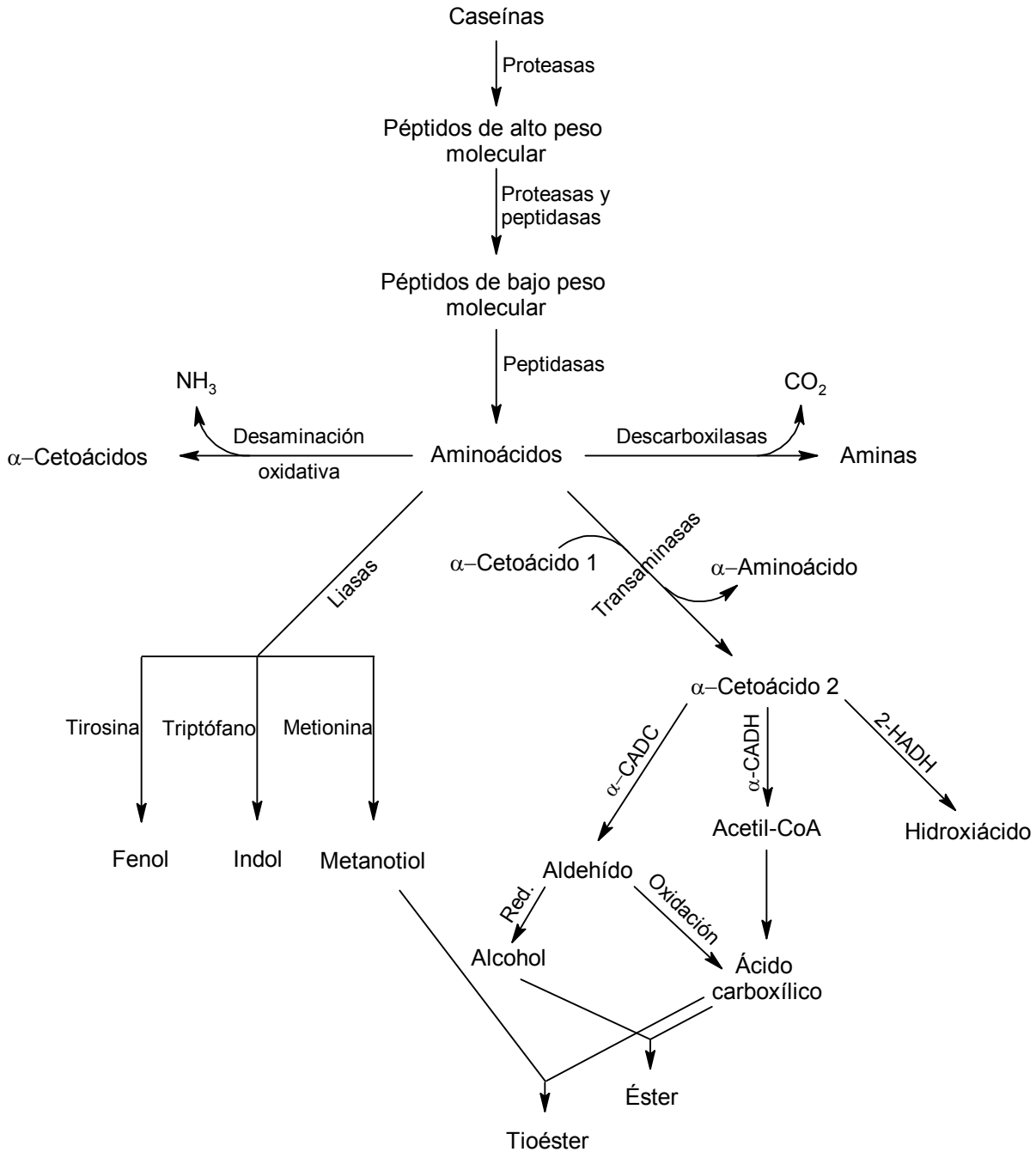


Figura 8. Esquema de la proteolisis y del catabolismo de aminoácidos (Yvon & Rijnen, 2001, McSweeney, 2004). (α-CADC: α-cetoácido descarboxilasa. α–CADH: α-cetoácido deshidrogenasa. 2-HADH: 2-hidroxiácido deshidrogenasa).

De las numerosas enzimas de la leche, la plasmina es la más importante desde el punto de vista de la proteolisis. Aunque tratamientos térmicos intensos pueden provocar la inactivación de la plasmina, con tratamientos HTST o UHT la plasmina mantiene entre un 20 y un 40 % de su actividad (Ismail & Nielsen, 2010). La plasmina es una proteinasa sérica alcalina asociada a la micela de caseína, con un pH y temperatura

óptimos de 7,5 y 37 ºC. La plasmina actúa preferentemente sobre la β-CN, la cual posee de 15 a 17 enlaces (dependiendo de la variante) susceptibles de ser hidrolizados, pero únicamente 3 enlaces son hidrolizados de manera significativa: Lys_{28}-Lys_{29}, Lys_{105}-His_{106} y Lys_{107}-Glu_{108}. La α_{S2}-CN es también un buen sustrato para la plasmina. En tampón la plasmina puede hidrolizar la α_{S2}-CN hasta en 14 enlaces distintos, 12 de los cuales son enlaces Lys-X y 2 son Arg-X. Sin embargo, no está bien establecido qué péptidos resultan de la hidrólisis de la α_{S2}-CN por plasmina en leche o queso debido a la gran variabilidad en el grado de fosforilación (Larsson *et al.*, 2006). La α_{S1}-CN es menos susceptible de ser hidrolizada por la plasmina que la α_{S2}- y la β-CN y se han identificado 12 enlaces Lys-X y 5 Arg-X susceptibles de ser hidrolizados por la plasmina, dando como resultado las λ-CN. La κ-CN y las proteínas séricas son muy resistentes a la hidrólisis por plasmina.

Tras el desuerado parte del cuajo queda retenido en la cuajada jugando un importante papel durante la proteolisis inicial de las caseínas. La β-CN en tampón es hidrolizada en 7 enlaces distintos por la quimosina, mientras que en queso esta hidrólisis es muy limitada y mayoritariamente afecta al enlace Leu_{192}-Tyr_{193}. De todos los enlaces hidrolizados de la α_{S1}-CN por la quimosina, en queso sólo tres sufren esta hidrólisis: el Phe_{23}-Phe_{24} (el más susceptible de ser atacado), Leu_{98}-Leu_{99} y Leu_{101}-Lys_{102}. La α_{S2}-CN parece ser bastante resistente a la quimosina, mientras que la para-κ-CN a pesar de tener varios enlaces susceptibles de ser hidrolizados se ha visto que es muy resistente (Exterkate *et al.*, 1997, Upadhyay *et al.*, 2004).

Las cardosinas se caracterizan por ser más proteolíticas que la quimosina. Estas enzimas vegetales tienen una actividad más inespecífica, hidrolizando más enlaces peptídicos de las caseínas y dando lugar a mayor cantidad de péptidos hidrófobos de sabor amargo (Roseiro *et al.*, 2003).

Otra fuente de enzimas proteolíticas son los microorganismos presentes en el queso. Sus enzimas pueden ser intracelulares y extracelulares. Las extracelulares están asociadas a la pared celular y pueden considerarse como enzimas inmovilizadas (Visser, 1993), mientras que las intracelulares necesitan ser liberadas a la matriz del queso por medio de la lisis celular. Las bacterias lácticas del cultivo iniciador poseen una proteinasa asociada a la pared celular (PrtP), oligoendopeptidasas intracelulares (PepO y

PepF), al menos tres aminopeptidasas generales (PepN, PepC y PepG), glutamil aminopeptidasa (PepA), pirrolidona carboxilil aminopeptidasa (PCP), leucil aminopeptidasa (PepL), prolildipeptidil aminopeptidasa (PepX), prolin aminopeptidasa (PepI), aminopeptidasa P (PepP), prolinasa (PepR), prolidasa (PepQ), dipeptidasas generales (PepV, PepD, PepDA) y tripeptidasas generales (PepT), además de un sistema de transporte de péptidos y aminoácidos. Todo este sistema proteolítico es necesario para que las bacterias lácticas alcancen poblaciones elevadas en la leche. En quesos de leche cruda se encuentran además bacterias lácticas no pertenecientes al cultivo iniciador. Estas bacterias lácticas aportan al queso su actividad proteolítica complementando la de las bacterias lácticas del cultivo iniciador (Sousa *et al.*, 2001, Savijoki *et al.*, 2006). En algunas variedades de queso se añaden también cultivos adjuntos que aportan a los quesos determinadas características. Los microorganismos adjuntos principales son *P. roqueforti* en quesos azules (Roquefort, Gorgonzola), *P. camemberti* en quesos madurados con mohos en su superficie (Camembert, Brie), *Brevibacterium linens* y otros microorganismos corineformes y distintas especies de levaduras en quesos de corteza lavada (Munster, Limburger) y *Propionibacterium freudenreichii* subsp. *shermanii* en los quesos de tipo Suizo. Cada microorganismo aporta al queso sus enzimas, favoreciendo la proteolisis. De *B. linens* se ha aislado una proteinasa extracelular y una aminopeptidasa intracelular. En los quesos de tipo Suizo, las cepas de *Propionibacterium* spp. son muy poco proteolíticas aunque tienen una gran actividad peptidolítica (Sousa *et al.*, 2001). Los mohos del género *Penicillium* se caracterizan por tener dos proteasas extracelulares, de las cuales una es una metaloproteasa y la otra es una proteasa aspártica. Estos mohos también presentan exopeptidasas extracelulares así como metaloaminopeptidasas extracelulares y peptidasas intracelulares. Son considerados como muy proteolíticos debido principalmente a la acción de la proteasa aspártica y de la metaloproteasa (Cantor *et al.*, 2004, Spinnler & Gripon, 2004).

Los aminoácidos liberados durante la proteolisis pasan a ser sustratos de reacciones de formación de compuestos responsables del sabor y aroma del queso (Figura 8). De forma general se considera que estas reacciones pueden darse principalmente a través de dos rutas diferentes. Una de las rutas se inicia mediante una

eliminación catalizada por liasas que cortan la cadena lateral de los aminoácidos. Esta ruta se ha observado en los aminoácidos aromáticos (tirosina y triptófano) y en la metionina, formándose en un solo paso fenol, indol y metanotiol respectivamente. La otra ruta empieza principalmente con una transaminación catalizada por transaminasas y se ha observado en aminoácidos aromáticos, de cadena ramificada y en metionina.

Las transaminasas están ampliamente distribuidas entre los microorganismos y emplean el piridoxal-5'-fosfato como cofactor. Estas enzimas transfieren el grupo amino de un aminoácido a un α-cetoácido (normalmente ácido α-cetoglutárico) formándose el α-cetoácido correspondiente al aminoácido y un nuevo aminoácido correspondiente al α-cetoácido original. Estos α-cetoácidos son productos intermedios de la ruta de la aminotransferasa y pueden dar lugar a hidroxiácidos, aldehídos, alcoholes, ácidos carboxílicos y metanotiol (Yvon & Rijnen, 2001, Smit *et al.*, 2005). La reducción de los α-cetoácidos a hidroxiácidos se ha observado en muchas bacterias lácticas. Se han identificado varias 2-hidroxiácido deshidrogenasas, siendo la más conocida la lactato deshidrogenasa cuya especificidad está principalmente restringida al piruvato. Otro grupo de enzimas son las llamadas hidroxiisocaproato-deshidrogenasas, debido a que tienen preferencia por el α-cetoisocaproato aunque catalizan otros sustratos empleando NADH como donante de hidrógeno. La descarboxilación de los α-cetoácidos da lugar a aldehídos que aportan a los quesos aromas a malta y chocolate. Esta transformación se produce principalmente por la acción de levaduras, aunque se ha visto también en algunas bacterias. La mayoría de las cepas de lactococos producen pequeñas cantidades de aldehídos, lo que sugiere que tienen poca actividad o que estos aldehídos son transformados rápidamente por reducción a alcoholes o por oxidación a ácidos carboxílicos (Yvon & Rijnen, 2001, Smit *et al.*, 2005). La reacción entre ácido y alcohol da como resultado la formación de ésteres, que aportan al queso notas dulces, afrutadas y florales. La descarboxilación oxidativa de los α-cetoácidos da como resultado la formación de ácidos carboxílicos. Esta reacción está catalizada por α-cetoácido deshidrogenasas y genera acetil-CoA, que posteriormente es oxidado, liberándose los ácidos carboxílicos. La formación de ácidos carboxílicos a partir de aminoácidos no parece ser muy común, aunque algunas levaduras, bacterias lácticas y propionibacterias los producen bajo ciertas condiciones (Yvon & Rijnen, 2001). Los α-

cetoácidos de la fenilalanina y de la metionina pueden ser transformados químicamente en benzaldehído y metiltioacetaldehído, respectivamente. El 2-metilpropanal puede formarse mediante una reacción química a partir del α-cetoácido de la leucina. Otra importante reacción química que sufren los aminoácidos es la degradación de Strecker, que da como producto 3-metilbutanal cuando el sustrato es la leucina. Otras rutas del catabolismo de aminoácidos son la desaminación oxidativa del aminoácido, formándose amoniaco y un α-cetoácido, y la descarboxilación, que implica la conversión de un aminoácido en su correspondiente amina biógena liberándose CO_2. Las aminas biógenas generalmente tienen aromas fuertes y desagradables, y son además compuestos con actividad fisiológica que pueden tener efectos tóxicos (Silla Santos, 1996, Curtin & McSweeney, 2004) como posteriormente se describe en la sección 1.3.

1.1.2.3.4. Sobremaduración

La calidad de los quesos viene determinada por su sabor, aroma, textura y apariencia visual. El aroma y el sabor característico de cada variedad de queso se debe al correcto equilibrio y concentración de compuestos responsables del aroma y sabor (Mulder, 1952). Estos compuestos pueden ser volátiles o no volátiles. Los volátiles contribuyen principalmente al aroma, pero también influyen en el sabor y los no volátiles contribuyen de forma significativa al sabor. Durante la maduración se forman estos compuestos debido a la glicolisis, lipolisis, proteolisis y catabolismo del lactato, citrato, aminoácidos y ácidos grasos.

La sobremaduración es un fenómeno producido por un exceso de cualquiera de los procesos que tienen lugar durante la maduración, especialmente la proteolisis, provocando un desequilibrio en la composición de los compuestos responsables del aroma y sabor. Este desequilibrio de compuestos provoca que las cualidades del queso no sean las adecuadas, causando una menor aceptación por parte del consumidor y las consecuentes pérdidas económicas del productor (Wick *et al.*, 2004). Algunas variedades de queso experimentan una intensa proteolisis debido al empleo de enzimas coagulantes de elevada actividad proteolítica, tales como las proteasas extraídas del cardo (*Cynara cardunculus*) empleadas en la elaboración de algunas variedades de queso como la Torta del Casar o el queso de la Serena. En otros quesos, como el azul o el Brie, los responsables de la elevada proteolisis son los mohos pertenecientes al

género *Penicillium* que poseen proteasas y peptidasas de elevada actividad. Una elevada proteolisis también puede provocar defectos de textura debido a que la hidrólisis de las caseínas durante el almacenamiento disminuye las interacciones proteína-proteína provocando una disminución de la firmeza. En la práctica, el tiempo de maduración provoca un aumento de la firmeza debido fundamentalmente a la pérdida de humedad, por lo que el reblandecimiento del queso debido a la sobremaduración sólo tendría lugar en quesos envasados de forma que conserven la humedad (Lucey *et al.*, 2003). Una excesiva lipolisis también puede provocar sobremaduración debido a la liberación de altos niveles de ácidos grasos. Apenas se han realizado trabajos de investigación para controlar este problema más allá del empleo de temperaturas de refrigeración para reducir la actividad enzimática. Durante la maduración en la industria existe un control efectivo de la temperatura, ya que un aumento de temperatura durante esta fase provocaría una maduración acelerada que reduciría la vida útil del producto. La sobremaduración se puede dar durante la conservación del producto en establecimientos comerciales y hogares, donde el control de la temperatura no siempre es el adecuado. El fenómeno de la sobremaduración limita la vida útil del queso y en algunos casos hace que dentro de la vida útil establecida por el fabricante, el queso llegue al consumidor con peor calidad que la deseada por el fabricante (Wick *et al.*, 2004).

1.2. Tipos de quesos

En la actualidad existe una gran diversidad de quesos, habiéndose sugerido la existencia de más de 1000 variedades. En la Universidad de Wisconsin se ha realizado una lista con 1400 variedades de queso (www.cdr.wisc.edu). A nivel de divulgación, en la página web www.mundoquesos.com se encuentran descritas 2420 variedades de quesos. Muchas de estas variedades son muy similares, por lo que podrían considerarse variantes en vez de variedades. La producción de queso tiene una larga historia y tradición, lo que se refleja en la gran variedad de quesos existente.

Para proteger y preservar esta diversidad tradicional, en 1883 se empezó a plantear el concepto de denominación de origen protegida (DOP). Esta denominación se da a los quesos que son elaborados con una materia prima determinada, en una zona

geográfica delimitada y mediante el empleo de unas técnicas dadas (McSweeney *et al.*, 2004). Se han realizado diferentes intentos de clasificación de quesos, basándose en la textura (extraduro, duro, semiduro, semiblando y blando), método de coagulación (coagulación enzimática, ácida o mixta), especie de procedencia de la leche (vaca, oveja, cabra…), tratamiento aplicado a la leche (cruda, pasteurizada, termizada o microfiltrada), tiempo de maduración (tierno, semicurado, curado, viejo y añejo), contenido graso (extragraso, graso, semigraso y desnatado) y en los cultivos iniciadores y secundarios empleados (madurados por mohos en el interior, por mohos en superficie, de corteza lavada…).

A continuación se describen las características principales de los quesos objeto del estudio realizado en la presente Tesis. La elección de estos quesos se ha realizado en base al alto grado de proteolisis y al riesgo de formación de aminas biógenas. Entre los quesos elegidos se encuentra uno de leche de oveja pasteurizada con mohos en su interior (queso azul, Roncari-blue), uno de leche cruda de oveja coagulado con cuajo vegetal (Torta del Casar), uno de leche de vaca pasteurizada con mohos en su superficie (queso Brie) y uno de leche cruda de vaca coagulado con cuajo animal (queso Arzúa-Ulloa).

1.2.1. Queso azul

El queso azul se caracteriza por el crecimiento del moho *Penicillium roqueforti* en su interior, que le confiere su apariencia, aroma y sabor típicos. Muchos países fabrican sus propios tipos de queso azul, cada uno con sus diferentes características. Pueden elaborarse con distintos tipos de leche de vaca, oveja, cabra o mezcla, cruda o pasteurizada. Los quesos azules son en general muy heterogéneos, con pronunciados gradientes de pH, sal y actividad de agua (a_w). Presentan un alto grado de lipolisis y de proteolisis en comparación con otros quesos. La maduración tiene lugar en

Figura 9. Corte de queso azul (Roncari-blue).

su mayor parte a temperaturas entre 6 y 12 ºC y puede durar de 3 a 6 meses. El pH de los quesos azules puede abarcar desde 4,6 hasta 5,3, dependiendo de la variedad. La conversión de la lactosa en ácido láctico se produce por la acción de las bacterias lácticas del cultivo iniciador. Durante la maduración el pH puede aumentar hasta 6,5 en el centro y hasta 5,9 en la corteza. El pH del interior aumenta más rápidamente que en la corteza debido a que el nivel de NaCl es menor, lo cual favorece el crecimiento de *P. roqueforti*. Este aumento de pH se debe al metabolismo del ácido láctico a CO_2 por mohos y levaduras y a la elevada proteolisis seguida de la producción de amoniaco por desaminación de los aminoácidos. El salado se puede hacer por inmersión en salmuera o por aplicación de sal seca sobre la superficie. En ambos casos se produce un gradiente de NaCl de la superficie al interior, que se va equilibrando lentamente durante la maduración. El crecimiento de *P. roqueforti* está muy influenciado por la composición gaseosa del queso, ya que la concentración de O_2 disminuye rápidamente generando un ambiente anaerobio en casi todo el queso excepto en las fisuras y canales de penetración. La especie *P. roqueforti* está particularmente bien adaptada para crecer en este ambiente de baja concentración de O_2 combinado con una alta concentración de CO_2.

La lipolisis en los quesos azules es muy intensa. En otras variedades una lipolisis excesiva puede causar la aparición de rancidez, pero en los quesos azules los ácidos grasos libres son neutralizados cuando aumenta el pH. Por lo general, el nivel de ácidos grasos libres totales aumenta con la maduración, especialmente después de la esporulación de los mohos, aunque se ha observado en ocasiones un descenso al final de la maduración (Prieto *et al.*, 2000). Este descenso puede deberse a la conversión de los ácidos grasos en metilcetonas. Normalmente los niveles de ácidos grasos de tamaño entre $C_{12:0}$ – $C_{18:3}$ en los quesos azules son más elevados que los de tamaño $C_{4:0}$ – $C_{10:0}$ (Larsen & Jensen, 1999). La degradación de lípidos en los quesos azules es debida principalmente a las enzimas de *P. roqueforti* que produce dos lipasas extracelulares, una ácida y otra alcalina. El pH óptimo de la lipasa ácida es 6 pero mantiene una estabilidad máxima a pH comprendido entre 3,7 y 6,0. Su temperatura óptima es de 35-40 ºC pero mantiene un 37 % de su actividad máxima a 5 ºC. El pH óptimo para la lipasa alcalina es de 8,8-9,0 a 30 ºC, y de 9,0-10,0 a 20 ºC, pero sigue

teniendo una considerable actividad a niveles de pH entre 4,5 y 11,0. Aunque *P. roqueforti* domina por lo general en la degradación de los lípidos, existen otros agentes lipolíticos. La actividad lipolítica de las bacterias lácticas es generalmente baja y no tiene mucha influencia en los quesos azules pero también puede influir en la lipolisis la presencia de levaduras, ya que las levaduras que aparecen en quesos azules tienen al menos actividad esterasa y pueden hidrolizar los ácidos grasos de cadena corta de los triglicéridos.

La proteolisis en los quesos azules también es muy intensa en comparación con otros tipos de quesos. Las caseínas son hidrolizadas en más enlaces y en mayor proporción, por lo que apenas quedan caseínas intactas al final de la maduración. Se forma mayor número de péptidos diferentes que en los quesos de pasta semidura y se libera una mayor cantidad de aminoácidos como resultado de las peptidasas de *P. roqueforti* y de las levaduras y en menor grado de las bacterias lácticas. Inicialmente la proteolisis es debida, al igual que en otros quesos, al cuajo residual, plasmina y enzimas del cultivo iniciador. Después de un tiempo la proteolisis es dominada por *P. roqueforti*, que produce dos proteasas extracelulares: una metaloproteasa y una proteasa aspártica. La actividad de estas dos enzimas es máxima en el momento en que el moho empieza a esporular. La metaloproteasa es activa a pH entre 4,5 y 8,5 y su pH óptimo para la hidrólisis de caseínas es de 5,5. La metaloproteasa tiene una especificidad muy amplia, pudiendo hidrolizar la α_{S1}-CN y la β-CN en tampón dando lugar a 8 y 9 péptidos diferentes, respectivamente. Además la metaloproteasa se caracteriza por hidrolizar la β-CN en el enlace Pro_{90}-Glu_{91}, lo cual no es muy común. La proteasa aspártica es estable a pH comprendido entre 3,5 y 6,0, y presenta dos valores de pH óptimos para la hidrólisis de caseínas que son 3,5 y 5,5. La proteasa aspártica, al igual que la quimosina, tiene preferencia por el enlace Phe_{23}-Phe_{24} de la α_{S1}-CN, y posteriormente el péptido C-terminal formado es hidrolizado para dar 4 ó 5 nuevos péptidos. La β-CN puede ser hidrolizada por esta enzima para dar 5 péptidos diferentes. Se ha establecido una relación entre el desarrollo de *P. roqueforti*, la actividad de la proteasa aspártica y la mayor liberación de péptidos amargos. *Penicillium roqueforti* produce una metaloaminopeptidasa alcalina extracelular con un pH óptimo de 8,0 que es específica de los aminoácidos hidrófobos y por tanto su actividad reduce el amargor. Además este

moho posee varias exopeptidasas, una carboxipeptidasa ácida extracelular con amplia especificidad que libera aminoácidos básicos, ácidos e hidrófobos y que también contribuye a la reducción del amargor. *Penicillium roqueforti* tiene también gran número de peptidasas intracelulares que pueden ser alcalinas, carboxi- y aminopeptidasas. La actividad proteolítica así como el nivel de proteasas y peptidasas varía según la cepa de *P. roqueforti* empleada. En los quesos azules el catabolismo de los aminoácidos es llevado a cabo por las bacterias lácticas y los mohos. Los aminoácidos más comunes en este tipo de quesos son el ácido glutamico, leucina, valina y lisina. La desaminación oxidativa de los aminoácidos es debida a *P. roqueforti* en el interior del queso y a otros microorganismos en la superficie. Esta actividad produce amoniaco, que contribuye al sabor del queso azul. La concentración de aminas varía ampliamente, apareciendo por lo general la tiramina en mayor concentración que la triptamina y la histamina (Cantor *et al.*, 2004).

En los quesos azules se producen, principalmente por *P. roqueforti*, un gran número de compuestos volátiles y no volátiles que influyen en el sabor y aroma. El sabor del queso azul deriva principalmente de la degradación de los lípidos. Los ácidos grasos libres dan sabor y aroma al queso y son a su vez transformados a otros compuestos. Los ácidos ($C_{4:0}$-$C_{12:0}$) tienen notas de sabor rancio y picante y suelen encontrarse en los quesos azules en altas concentraciones. El alto pH neutraliza estos ácidos haciendo que estos aporten su sabor pero sin que se dé el defecto de rancidez. Las metilcetonas son los compuestos mayoritarios en los quesos azules y su concentración en el queso está relacionada con la intensidad de sabor a "queso azul". Las metilcetonas mayoritarias en estos quesos son la 2-heptanona y la 2-nonanona, aunque también son importantes la 2-pentanona y la 2-undecanona. En general las metilcetonas aportan al queso aromas florales, afrutados y a moho. Estos compuestos pueden ser reducidos a alcoholes secundarios en condiciones anaerobias. Los principales alcoholes secundarios son 2-heptanol, 2-nonanol y 2-pentanol. Sus sabores son similares a los aportados por las correspondientes metilcetonas, pero en alta concentración pueden dar un exceso de sabor a moho. Otros compuestos que aportan aromas afrutados y florales son los ésteres y las lactonas. Los aldehídos suelen aparecer en baja concentración y no se conoce bien su impacto en el sabor de estos quesos.

1.2.2. Torta del Casar

La Torta del Casar es un queso de pasta blanda, amparado bajo la denominación de origen protegida (DOP) de la Torta del Casar aprobada por el reglamento de la Comisión Europea (EC 1491/2003). Este queso se caracteriza por el empleo en su elaboración de cuajo vegetal natural, obtenido de las flores desecadas del cardo (*Cynara cardunculus*), sin adición de ningún cultivo iniciador. Se elabora en 36 términos municipales de la provincia de Cáceres, a partir de leche cruda de ovejas de raza merina o entrefina. El periodo mínimo de maduración es de 60 días y debe realizarse a temperaturas comprendidas entre 4 y 12 ºC, y entre 75 y 90 % de humedad relativa. El empleo de cuajo vegetal hace que este queso tenga un sabor ligeramente amargo y una textura entre blanda y untuosa. El pH final de estos quesos debe estar comprendido entre 5,2 y 5,9. El salado se puede realizar mediante inmersión en salmuera o por aplicación de sal seca sobre la superficie (BOE, 2002). El empleo de leche cruda sin adición de un cultivo iniciador hace que los microorganismos presentes en la leche jueguen un importante papel en el proceso de maduración de este queso. Existe una gran variabilidad en el producto final, provocado por las diferencias de microorganismos presentes en la leche cruda. Además de bacterias lácticas, se encuentran en estos quesos enterococos, coliformes y micrococos así como levaduras y mohos (Ordiales *et al.*, 2013a). Debido al empleo de leche cruda también pueden encontrarse microorganismos patógenos como *Listeria*, *Salmonella* o *Staphylococcus aureus*, que normalmente desaparecen al cabo de los 60 días de maduración.

Figura 10. Cuña de Torta del Casar.

Los agentes responsables de la lipolisis en estos quesos son la LPL endógena de la leche y las lipasas y esterasas de los microorganismos presentes en la leche. La LPL no tiene una gran especificidad en cuanto al tipo de ácido graso, pero tiene mayor tendencia por la hidrólisis de los ácidos grasos situados en las posiciones sn-1 y sn-3 de los mono-, di- y triglicéridos donde suelen encontrarse ácidos grasos de cadena corta o

media (Collins *et al.*, 2003). La diversidad de microorganismos hace que el grado de lipolisis en estos quesos sea muy variado. Por lo general las bacterias lácticas tienen poca actividad lipolítica, pero al encontrarse en gran número pueden tener un efecto considerable. Por otro lado, la aportación de otros microorganismos que se encuentren en menor número puede ser importante debido a una mayor capacidad lipolítica. La liberación de ácidos grasos contribuye al sabor y aroma del queso, por su presencia y por ser sustratos de posteriores reacciones.

La proteolisis de esta variedad de queso viene dada por la acción de la plasmina, de las enzimas de los microorganismos presentes y, principalmente, de las cardosinas del cuajo retenido. El cuajo vegetal empleado en la elaboración de estos quesos se caracteriza por tener menor actividad coagulante y mayor actividad proteolítica. Esta actividad proteolítica es menos específica que la de la quimosina, pudiendo hidrolizar α_{S1}-CN, β-CN y al menos una de las γ-CN, dando mayor número de péptidos diferentes. La actividad proteolítica del extracto de las flores del cardo depende de la variedad, fase de maduración de la flor, parte de la flor, tiempo de secado y contenido final de humedad. Este extracto contiene dos enzimas, cardosina A y cardosina B. La cardosina A se asemeja en su actividad a la quimosina, mientras que la cardosina B se asemeja a la pepsina (Verissimo *et al.*, 1995). Estas proteinasas tienen mayor afinidad por las zonas hidrófobas de las caseínas, provocando una mayor liberación de péptidos hidrófobos que dan al queso su característico sabor amargo. La α_{S1}-CN puede ser atacada en 9 enlaces distintos, siendo el enlace Phe_{23}-Val_{24} el más susceptible. La α_{S2}-CN se hidroliza principalmente en los enlaces Phe_{88}-Tyr_{89} y Ser_{9}-Ser_{10}, mientras que la β-CN lo hace en los enlaces Leu_{127}-Thr_{128} y Leu_{190}-Tyr_{191}.

Además de los compuestos volátiles más comunes en los quesos como los alcoholes, cetonas, aldehídos y esteres, en la Torta del Casar cabe destacar la presencia de los ácidos 2-metilpropanoico y 3-metilbutanoico, procedentes de la desaminación de la valina y de la leucina respectivamente, y también la presencia de los ácidos acético y propiónico procedentes de la actividad microbiana, siendo estos de mayor importancia en el aroma de este tipo de quesos que los ácidos procedentes de la lipolisis (Delgado *et al.*, 2010, Ordiales *et al.*, 2013b).

1.2.3. Queso Brie

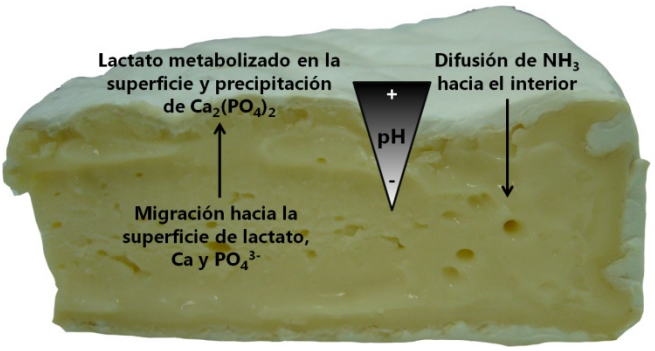


Figura 11. Esquema de migración y gradientes en queso Brie.

El queso Brie pertenece, al igual que el Camembert, al grupo de quesos madurados por mohos en superficie. Se caracteriza por estar recubierto de una capa blanca, formada por el micelio del moho *Penicillium camemberti*. La presencia de este moho confiere al queso una apariencia visual y un aroma y sabor característicos. Este queso se elabora con leche de vaca, que puede ser cruda o pasteurizada. La maduración se realiza a temperaturas comprendidas entre 11 y 13 ºC y en torno al 90 % de humedad relativa. Se añade a la leche un cultivo iniciador de bacterias lácticas y *P. camemberti* y opcionalmente se pueden añadir como cultivos adjuntos *Geotrichum candidum* y *Kluyveromyces lactis* (Spinnler & Gripon, 2004). Las bacterias lácticas fermentan la lactosa, formándose ácido láctico y disminuyendo el pH. El descenso del pH junto a la presencia de oxígeno permite al moho *P. camemberti* colonizar la superficie degradando el lactato y produciendo CO_2, con la consiguiente elevación del pH en la superficie. Este aumento del pH superficial hace que se produzca un gradiente de pH y una migración del lactato hacia la corteza. El pH de la superficie puede aumentar hasta 7,0 al final de la maduración, mientras que en el interior el aumento es menor. El elevado pH de la superficie permite que los microorganismos sensibles al pH ácido se puedan establecer en la corteza y favorece la actividad de las enzimas. Un efecto del gradiente de pH es la migración de calcio y fosfatos hacia la superficie del queso, donde por efecto del elevado pH se forma fosfato cálcico insoluble. Estos gradientes y la migración de minerales provocan cambios reológicos que dan a este queso su textura característica.

La lipolisis en quesos Camembert y Brie es consecuencia principalmente de las enzimas de *P. camemberti*, acompañado de las lipasas y esterasas de las bacterias lácticas del cultivo iniciador. *Penicillium camemberti* produce grandes cantidades de una lipasa extracelular alcalina, cuyo pH óptimo está en torno a 9,0 aunque mantiene un

50 % de su actividad a pH 6,0. Esta enzima es el principal agente lipolítico en queso Brie y es más activa en triglicéridos con ácidos de bajo peso molecular. *Geotrichum candidum* sintetiza dos lipasas, una de las cuales libera preferentemente ácido oleico, y la otra ácidos insaturados de 18 carbonos situados en la posición sn-2 del triglicérido.

Aunque estos quesos sufren una intensa proteolisis, esta no llega a ser tan intensa como en los quesos azules. La proteolisis es producida por tres agentes, el cuajo, la plasmina y las proteinasas de los microorganismos, siendo las de *P. camemberti* las más activas. En estos quesos se ha observado una fuerte degradación de la α_{S1}-CN en todo el queso, mientras que la β-CN sólo se degrada fuertemente en la parte externa. *Penicillium camemberti* tiene una gran actividad proteolítica debido a la producción de endo- y exopeptidadasas extracelulares y sintetiza también grandes cantidades de una metaloproteinasa y de una proteinasa aspártica. Durante la maduración esta actividad es muy baja en el centro del queso, mientras que en la parte externa se produce un gran aumento después de 6-7 días de maduración, cuando *P. camemberti* empieza a crecer. La metaloproteinasa y la aspartil proteinasa alcanzan su concentración máxima en el queso a los 15 días y después empiezan a descender. Debido a que el moho sólo se encuentra en la superficie, el grado de proteolisis es mayor en esta zona que en el interior. El moho produce grandes cantidades de aminoácidos debido a la síntesis de exopeptidasas extracelulares. Por otro lado *G. candidum* sintetiza proteinasas intra- y extracelulares, cuya producción depende mucho de la cepa. La actividad enzimática de *G. candidum* y de las bacterias lácticas es mucho menor que la de *P. camemberti*, siendo este último el principal responsable de la proteolisis.

Al igual que en queso azul, las metilcetonas juegan un papel importante en el aroma de los quesos madurados por mohos en superficie. El principal agente en la formación de las metilcetonas es un sistema enzimático de *P. camemberti*, que mediante la β-oxidación de los ácidos grasos libres da lugar a la formación este tipo de compuestos. El 1-octen-3-ol es un compuesto característico de este tipo de quesos, que aporta aroma a champiñón crudo y puede llegar a representar entre el 5-10 % de los compuestos volátiles en queso Camembert. Los ácidos grasos tienen también un importante papel en estos quesos, sobre todo los de cadena media y corta. Al igual que en el queso azul también se pueden encontrar lactonas, que aportan al queso notas

afrutadas. También se pueden encontrar, aunque en menor concentración, otros compuestos responsables del aroma tales como aldehídos, compuestos azufrados, aminas, ésteres y terpenos.

1.2.4. Queso Arzúa-Ulloa

El queso Arzúa-Ulloa es un queso gallego, elaborado con leche de vaca, que se encuentra amparado bajo la DOP de Arzúa-Ulloa aprobada por el reglamento de la Comisión Europea (UE 20/2010). La zona de producción comprende 32 municipios de las provincias de A Coruña, Lugo y Pontevedra. La leche empleada en la elaboración de este queso debe ser de las razas frisona, pardo alpina, rubia gallega y/o sus cruces. Puede emplearse cruda o pasteurizada. Para su coagulación se emplea extracto de cuajo animal y fermentos lácticos. La cuajada obtenida debe ser lavada con agua, con el fin de reducir la acidez. El salado de estos quesos puede realizarse sobre la cuajada directamente en la cuba o introduciendo los quesos en una solución de salmuera. Los quesos Arzúa-Ulloa se caracterizan por ser quesos de pasta semiblanda, de sabor suave con aroma lácteo que recuerda a la mantequilla. Dentro de la DOP de Arzúa-Ulloa se distinguen tres tipos de queso: Arzúa-Ulloa, Arzúa-Ulloa de granja y Arzúa-Ulloa curado. El de granja se caracteriza por que la leche debe proceder de vacas que se encuentran en la propia explotación en la que se elabora el queso. El periodo de maduración debe ser como mínimo de 6 días, excepto en el queso curado que debe ser como mínimo de 6 meses. La maduración se realiza a temperaturas inferiores a 15 ºC y entre un 75 y 90 % de humedad relativa. El pH de estos quesos debe de estar comprendido entre 5,0 y 5,5.

Figura 12. Cuña de queso Arzúa-Ulloa.

En general se considera que los quesos de esta variedad elaborados con leche cruda suelen tener un sabor más amargo y una mayor intensidad que los de leche pasteurizada (Menéndez *et al.*, 2000). El queso Arzúa-Ulloa de leche cruda tiene gran variabilidad de sabor debido a las diferencias en la microbiota de la leche. La proteolisis

y lipolisis en estos quesos depende del tipo de leche empleada. En los quesos de leche pasteurizada son las enzimas del cultivo iniciador junto con las enzimas resistentes a la pasteurización de la leche las responsables de la maduración. En los quesos de leche cruda además de estas enzimas se encuentran las enzimas de los microorganismos inicialmente presentes en la leche, como cepas de *Enterococcus* y *Micrococcus* que presentan actividad proteolítica y lipolítica (Centeno *et al.*, 1995).

Los quesos Arzúa-Ulloa de leche cruda, además de sufrir una mayor proteolisis y lipolisis, tienen mayor número y concentración de compuestos volátiles relacionados con el aroma y sabor que los elaborados con leche pasteurizada. Los compuestos más abundantes suelen ser los alcoholes, seguidos de cetonas y ésteres en los quesos de leche cruda y las cetonas y aldehídos en los de leche pasteurizada. El hecho de que los ácidos grasos y los ésteres sólo se encuentren en los quesos de leche cruda y que la abundancia de cetonas y alcoholes secundarios sea mayor, está en consonancia con la lipolisis más intensa en estos quesos. Además, la mayor abundancia de aldehídos de cadena ramificada y de alcoholes en los quesos de leche cruda indica que el catabolismo de aminoácidos de cadena ramificada es mayor en estos quesos, como consecuencia de la actividad de los microorganismos procedentes de la leche (Rodríguez-Alonso *et al.*, 2009).

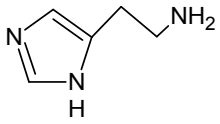
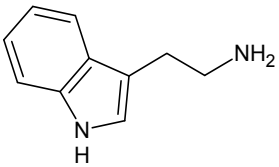
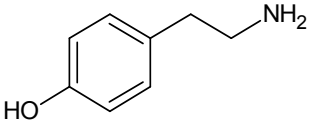
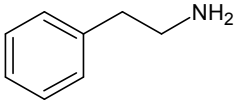
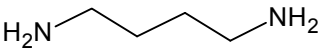
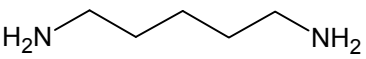
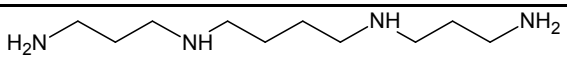
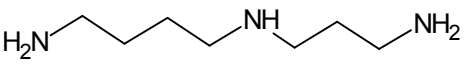
1.3. Aminas biógenas

Las aminas biógenas son compuestos básicos nitrogenados de bajo peso molecular, con actividad biológica. Su síntesis y degradación forma parte del metabolismo normal de animales, plantas y microorganismos (ten Brink *et al.*, 1990). Las aminas (Tabla 3) pueden ser alifáticas (cadaverina, putrescina, espermina y espermidina), aromáticas (tiramina y β-feniletilamina) o heterocíclicas (histamina y triptamina).

Se pueden clasificar según el número de grupos amino que contienen como mono-, di- o poliaminas (Silla Santos, 1996). La putrescina, espermidina y espermina se pueden considerar como un grupo diferenciado, debido a las funciones que desempeñan en el organismo y a la participación de la putrescina en la síntesis de la espermidina y espermina (Kalač, 2009).

A pesar de formar parte del metabolismo normal y de regular diversas funciones indispensables en el organismo, la ingesta de niveles elevados de aminas biógenas puede tener efectos tóxicos, tales como aumento de la presión sanguínea, dolores de cabeza, calambres abdominales y urticaria.

Tabla 3. Características de las principales aminas biógenas.

Aminoácido precursor	Amina biógena	Fórmula	Estructura química	Categoría
Histidina	Histamina	$C_5H_9N_3$		Monoamina heterocíclica
Triptófano	Triptamina	$C_{10}H_{12}N_2$		Monoamina heterocíclica
Tirosina	Tiramina	$C_8H_{11}NO$		Monoamina aromática
Fenilalanina	β-feniletilamina	$C_8H_{11}N$		Monoamina aromática
Ornitina	Putrescina	$C_4H_{12}N_2$		Diamina alifática
Lisina	Cadaverina	$C_5H_{14}N_2$		Diamina alifática
Ornitina y Arginina	Espermina	$C_{10}H_{26}N_4$		Poliamina alifática
Ornitina y Arginina	Espermidina	$C_7H_{19}N_3$		Poliamina alifática

Las aminas biógenas pueden estar presentes como componentes endógenos de alimentos frescos, como frutas y verduras. También pueden aparecer y acumularse como resultado de la actividad enzimática microbiana, que en algunos casos se asocia a técnicas de manipulación poco higiénicas (ten Brink *et al.*, 1990). En los alimentos las

aminas biógenas se forman principalmente por descarboxilación de aminoácidos (Loizzo *et al.*, 2013). Entre los alimentos con mayores niveles de aminas biógenas se encuentran el pescado y productos derivados del pescado, productos lácteos, carne y productos cárnicos, vegetales fermentados y bebidas alcohólicas como el vino y la cerveza (Ladero *et al.*, 2010).

1.3.1. Aminas biógenas en alimentos

La mayor incidencia de casos de intoxicación por aminas biógenas está asociada al consumo de pescado y productos derivados (Silla Santos, 1996). En el pescado la amina más abundante es la histamina, aunque también se han encontrado putrescina, cadaverina, tiramina, espermina y espermidina, además de β-feniletilamina y triptamina en bajas concentraciones. La familia de los *Scombridae*, familia a la que pertenecen el atún y el bonito, es la más habitualmente implicada en casos de intoxicación por histamina (escombroidosis). Estos pescados se caracterizan por tener una musculatura rica en histidina libre, entre 1 g/kg en el arenque y 15 g/kg en el atún, que puede ser convertida en histamina (Shalaby, 1996). Parte de la microbiota habitual del pescado tiene la capacidad de producir histamina a bajas temperaturas. El contenido de aminas biógenas en pescado se ha propuesto como índice de frescura, contaminación o deterioro del producto (Visciano *et al.*, 2012).

El queso es, tras el pescado y sus derivados, el alimento más frecuentemente asociado a intoxicaciones por aminas biógenas y por lo tanto también uno de los más estudiados (Stratton *et al.*, 1991). Como ya se ha indicado, el resultado final de la proteolisis es la formación de aminoácidos libres, que junto a la presencia de microorganismos con actividad descarboxilasa puede llevar a la acumulación de grandes cantidades de aminas biógenas (Silla Santos, 1996). Debido a la gran variedad de quesos que existe, los niveles de aminas biógenas son muy variables. Un motivo de la gran variabilidad de aminas biógenas en los quesos es el empleo de leche cruda o pasteurizada, ya que la leche cruda puede contener microorganismos, tanto gram positivos como gram negativos, con actividad descarboxilasa que son eliminados mediante los tratamientos de pasteurización. Algunos cultivos iniciadores pueden contener cepas de bacterias lácticas con actividad descarboxilasa (Burdychova & Komprda, 2007). Otro factor que provoca variabilidad en los niveles de aminas en el

queso es la diferencia en el tiempo de maduración cada variedad de queso: a mayor tiempo de maduración mayor concentración de aminas biógenas se puede alcanzar (Komprda *et al.*, 2008a). Como se puede observar en la Tabla 4, la concentración de aminas biógenas en queso puede abarcar desde cantidades traza hasta niveles de 2101 mg/kg de cadaverina, 1585 mg/kg de tiramina y 129 mg/kg de triptamina en queso azul de leche pasteurizada (Novella-Rodríguez *et al.*, 2003), 1042 mg/kg de histamina y 876 mg/kg de putrescina en queso azul de leche cruda (Fernández *et al.*, 2007a), 202 mg/kg de espermidina y 40 mg/kg de espermina en queso Cheddar (Bardócz *et al.*, 1993) y 54,3 mg/kg de β-feniletilamina en queso de tipo holandés (Komprda *et al.*, 2007).

La carne y los productos cárnicos son un sustrato rico en aminoácidos libres. Las aminas biógenas están presentes en la carne fresca de forma natural, pero su presencia en altas concentraciones es un índice de incorrecta manipulación (Ruiz-Capillas & Jiménez-Colmenero, 2004a). El procesado de la carne, cortado, loncheado o picado, puede favorecer la contaminación por microorganismos con actividad descarboxilasa, lo que junto con el almacenamiento a temperaturas poco adecuadas (> 5 ºC) o durante largos periodos puede permitir la formación de aminas biógenas. El contenido en aminas biógenas de la carne se ha propuesto como índice de calidad y frescura del producto (Ruiz-Capillas & Jiménez-Colmenero, 2004a). Los productos cárnicos con mayor concentración de aminas biógenas suelen ser los productos fermentados. La variabilidad observada en este tipo de productos depende de la adición o no de cultivos iniciadores de bacterias lácticas, del empleo de distintos ingredientes como sal y aditivos, y de los tiempos y condiciones de fermentación (Shalaby, 1996). En los productos cárnicos fermentados la amina biógena más abundante suele ser la tiramina, pudiendo llegar a concentraciones de más de 600 mg/kg en chorizo (Hernández-Jover *et al.*, 1997). Además de tiramina se puede encontrar histamina, putrescina, cadaverina, triptamina, β-feniletilamina, espermidina y espermina. La putrescina y la cadaverina llegan a alcanzar niveles de hasta 415 mg/kg y 658 mg/kg respectivamente en chorizo, debido probablemente a la contaminación con microorganismos gram negativos (Hernández-Jover *et al.*, 1997, Latorre-Moratalla *et al.*, 2012).

En frutas y verduras frescas, al igual que en otros alimentos frescos, las aminas biógenas se suelen encontrar en concentraciones bajas. La presencia de altas concentraciones está asociada a contaminación microbiana, largos periodos de almacenamiento o mala manipulación (Moret *et al.*, 2005). Los productos vegetales fermentados tienen una gran variabilidad de concentraciones de aminas biógenas, según el microorganismo implicado en la fermentación y el tiempo y condiciones de fermentación. Se han observado niveles de hasta 951 y 529 mg/kg de tiramina y putrescina respectivamente en chucrut (Kalač *et al.*, 1999) y de 592, 550 y 486 mg/kg de histamina, cadaverina y espermidina respectivamente en salsa de soja (Lu *et al.*, 2009).

Las bebidas fermentadas como el vino y la cerveza pueden contener aminas biógenas. Las aminas biógenas más abundantes en el vino son la histamina, tiramina y putrescina. Su formación y acumulación depende de las condiciones de elaboración, tiempo de maceración con la piel, duración y condiciones de la fermentación, y crecimiento de bacterias y levaduras endógenas en el mosto (Gardini *et al.*, 2005, Beneduce *et al.*, 2010). Las aminas se pueden formar durante la fermentación alcohólica por acción de las levaduras, aunque sus niveles suelen ser bajos al finalizar esta fase. Sin embargo, se puede producir un aumento considerable durante la fermentación maloláctica por parte de las bacterias lácticas y durante el almacenamiento y añejado (Lonvaud-Funel, 2001, Ancin-Azpilicueta *et al.*, 2008). El rango de concentraciones de aminas biógenas en vino puede abarcar desde niveles no detectables hasta valores de 47,3 mg/L de putrescina (Landete *et al.*, 2005).

Tabla 4. Concentración (mg/kg) de aminas biógenas en diferentes alimentos.

Alimento	Tiramina	Histamina	Triptamina	Putrescina	Cadaverina	Feniletilamina	Espermina	Espermidina	Referencia
Productos lácteos									
Queso azul (LP)	0 - 1585	0 - 377	0 - 129	3 - 257	0 - 2101	0 - 40	0 - 19	0 - 72	(Novella-Rodríguez *et al.*, 2003)
Queso curado (LC)	0 - 609	0 - 391	0 - 34	0 - 670	1 - 369	0 - 30	0 - 22	0 - 40	
Queso azul (LC)	0 - 1052	0 - 1042	nd	0 - 876	0 – 757	0 - 27	-	nd	(Fernández *et al.*, 2007a)
Cheddar (LP)	nd	nd	nd	654,9	nd	nd	40,0	202,2	(Bardócz *et al.*, 1993)
Queso tipo holandés (LP)	299,8	1,8	-	60,8	2,0	54,3	0,2	0,3	(Komprda *et al.*, 2007)
Emmental (LP)	19,3	19,6	nd	16,8	12,6	nd	nd	nd	(Mayer *et al.*, 2010)
Gouda (LP)	27,7	26,1	104,4	nd	nd	7,0	16,3	28,8	(Mayer *et al.*, 2010)
Pescado									
Atún fresco	0 – 10,6	0 – 9,5	0 – 5,8	0 – 4,8	0 – 9,5	0 – 1,7	7,3 – 37,0	1,2 – 11,7	(Veciana Nogues *et al.*, 1997)
Atún enlatado	0 – 40,5	0 – 40,5	0 – 12,9	0 – 2,2	0 – 12,0	0 – 7,3	2,2 -35,2	1,5 – 9,9	
Atún	nd	25,6	nd	1,7	-	nd	nd	nd	(Du *et al.*, 2002)
Rihaakuru	0 - 50,8	0 - 5478,1	< 5,00	0 - 290,1	0 - 291,1	0 - 23,9	0 - 379,6	0 - 79,9	(Naila *et al.*, 2011)
Pulpo	0 – 14,5	1,3 – 9,1	0 – 3,4	2,8 – 94,1	0,1 – 164	nd	0 – 4,2	0 – 1,6	(Hu *et al.*, 2012)
Calamar	0 – 24,3	0 – 7,4	0 – 4,0	2,7 – 22,1	1,9 – 17,3	nd	1,3 – 6,4	0 – 1,8	(Hu *et al.*, 2012)
Productos cárnicos									
Salchichón	53 - 513	0 - 151	0 - 65	5 - 400	0 - 342	0 - 35	7 - 42	1 – 14	(Hernández-Jover *et al.*, 1997)
Fuet	32 – 743	0 - 358	0 - 88	2 - 222	5 - 51	0 - 34	9 - 30	1 - 11	
Jamón curado	4,87	1,73	nd	0,92	5,52	nd	44,28	5,44	(Ruiz-Capillas & Jiménez-Colmenero, 2004b)
Chorizo	214,45	15,53	nd	185,14	229,33	nd	39,94	8,15	

nd: no determinado; -: no detectado; LP: leche pasteurizada; LC: leche cruda.

En la cerveza la putrescina, espermidina, espermina y agmatina, suelen considerarse como constituyentes naturales, ya que estas aminas biógenas se encuentran en la malta. Histamina, tiramina y cadaverina se consideran procedentes de la actividad de bacterias lácticas contaminantes durante la elaboración de la cerveza. La mayor formación de aminas biógenas en la cerveza se produce durante la fermentación, aunque también pueden producirse durante otras etapas de la elaboración (Bokulich & Bamforth, 2013) y durante el almacenamiento en botella, debido a una mala pasteurización (Kalač & Křížek, 2003). En algunos tipos de cerveza especiales la fermentación secundaria en botella puede dar lugar a altas concentraciones de aminas biógenas. La mayor variabilidad en el contenido en aminas biógenas se ha observado en cervezas elaboradas por fermentación espontánea, abarcando niveles de tiramina que van desde menos de 1 mg/L hasta 68 mg/L (Izquierdo-Pulido *et al.*, 1996b, Romero *et al.*, 2003, Loret *et al.*, 2005).

1.3.2. Formación de aminas biógenas

Las monoaminas (histamina, tiramina, triptamina y β-feniletilamina) y las diaminas (cadaverina y putrescina) se forman principalmente como resultado de la descarboxilación directa de aminoácidos. Las reacciones de descarboxilación están mediadas por enzimas descarboxilasa, e implican la eliminación del grupo carboxilo del aminoácido precursor, dando así lugar a la amina correspondiente. (Figura 13 y Figura 14). Las enzimas que median esta reacción son la histidina descarboxilasa (HDC; EC 4.1.1.22), tirosina descarboxilasa (TDC; EC 4.1.1.25), triptófano descarboxilasa (TrDC; EC 4.1.1.28), fenilalanina descarboxilasa (FDC; EC 4.1.1.53), lisina descarboxilasa (LDC; EC 4.1.1.18) y ornitina descarboxilasa (ODC; EC 4.1.1.17), que dan lugar a histamina, tiramina, triptamina, β-feniletilamina, cadaverina y putrescina, respectivamente. Existe además otra ruta alternativa para la formación de putrescina, llamada "deureación" (Komprda et al., 2008b), en la que la arginina es descarboxilada por la enzima arginina descarboxilasa (ADC; EC 4.1.1.19) dando lugar a la agmatina (Figura 14), la cual por acción de la agmatina ureohidrolasa (agmatinasa; EC 3.5.3.11) pierde urea dando lugar a la putrescina (Hillary & Pegg, 2003). La arginina puede convertirse en ornitina por acción de la arginasa (EC 3.5.3.1). Las enzimas descarboxilasa pertenecen al grupo de enzimas piridoxal-fosfato-dependientes, pero algunas de ellas pueden emplear como

cofactor piruvato en vez de piridoxal-5'-fosfato (Coton *et al.*, 1998; Recsei & Snell, 1985; Kamath *et al.*, 1991). En presencia de pequeñas cantidades de ornitina, la ornitina descarboxilasa puede formar cadaverina mediante la descarboxilación de la lisina (Karovičová & Kohajdová, 2005).

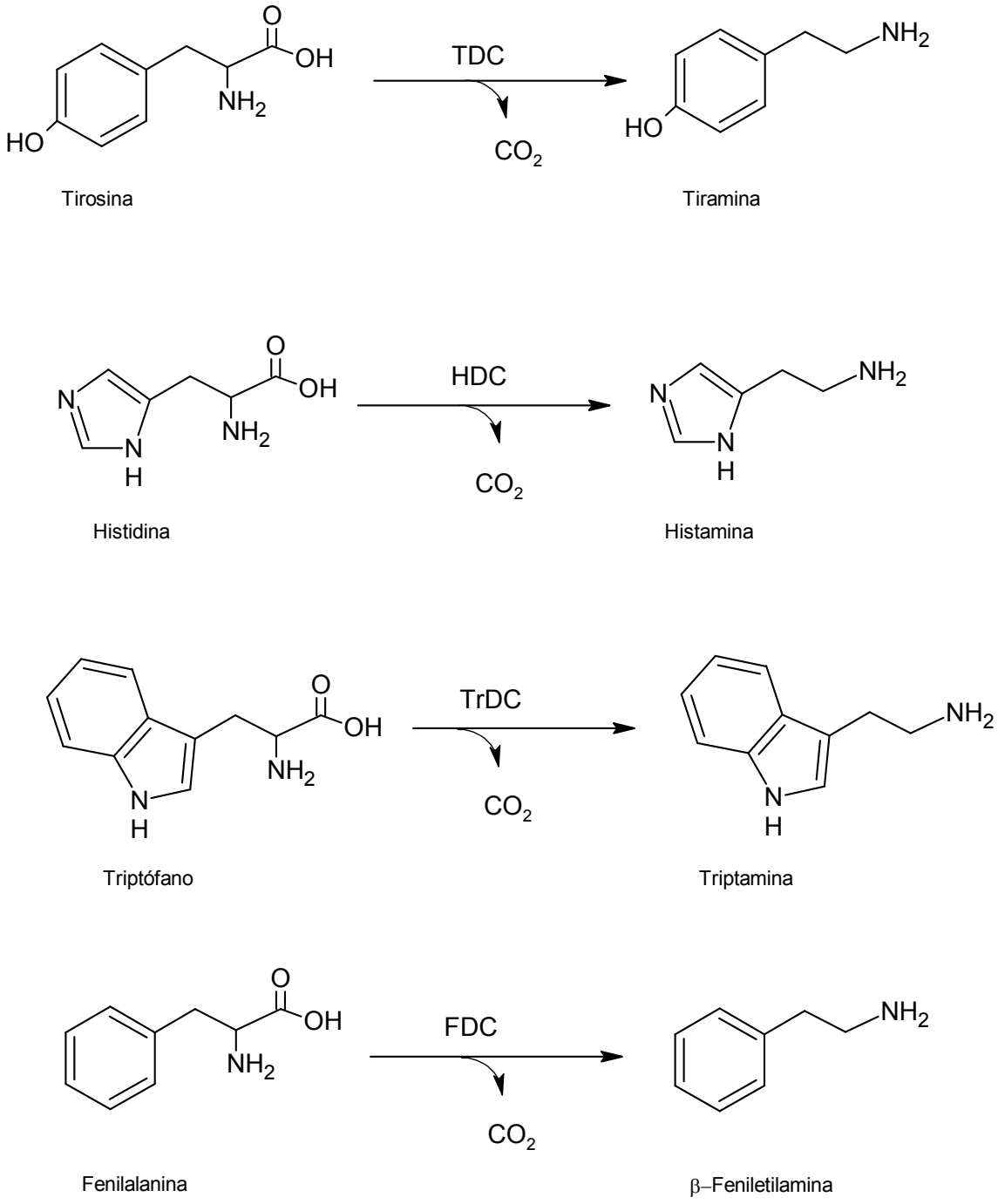


Figura 13. Reacciones de formación de las aminas biógenas. (TDC: tirosina descarboxilasa. HDC: histidina descarboxilasa. TrDC: triptófano descarboxilasa. FDC: fenilalanina descarboxilasa).

La síntesis de poliaminas (espermidina y espermina) es un proceso más complejo en el cual intervienen también reacciones de descarboxilación (Bardócz, 1995). Dicha síntesis se produce a partir de metionina y putrescina. La metionina proporciona los grupos aminopropilo necesarios para convertir la putrescina en espermidina, y esta a su vez en espermina.

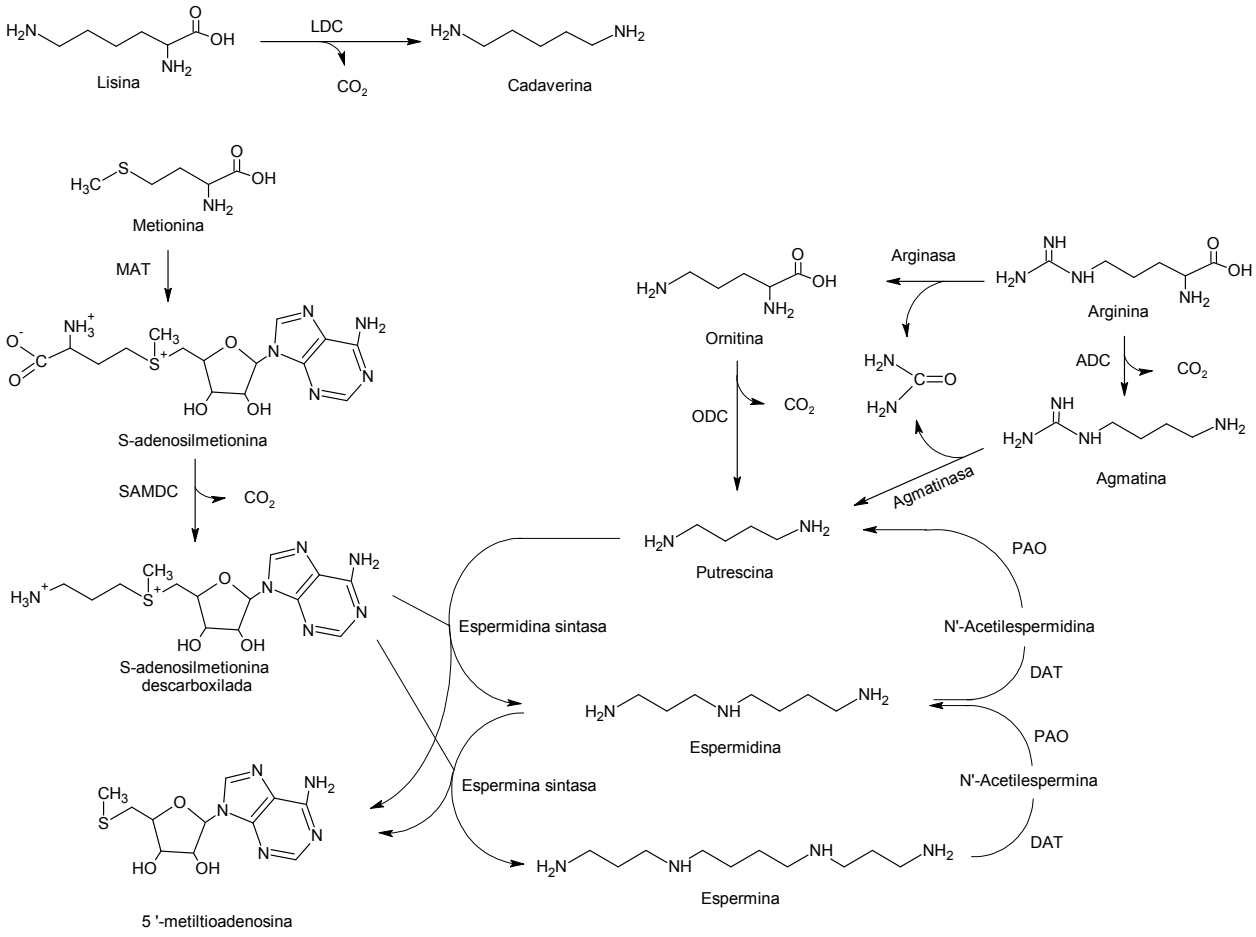


Figura 14. Ruta y reacciones de formación de diaminas y poliaminas. (LDC: lisina descarboxilasa. MAT: metionina adenosiltransferasa. SAMDC: S-adenosil-L-metionina descarboxilasa. ADC: arginina descarboxilasa. ODC: ornitina descarboxilasa. PAO: poliamino oxidasa. DAT: diamino N-acetiltransferasa).

Mediante la acción de la enzima metionina adenosiltransferasa y posterior descarboxilación la metionina se transforma en S-adenosilmetionina descarboxilada, la cual en presencia de putrescina y de espermidina sintasa da lugar a espermidina, y en presencia de espermidina y de espermina sintasa da lugar a la espermina. Esta reacción puede sufrir un proceso de retroconversión (Figura 14) mediante la enzima diamino N-acetiltranferasa (DAT; EC 2.3.1.57) dando N'-acetilespermina o N'-acetilespermidina que

mediante la acción de una poliamino oxidasa (PAO) dan lugar a espermidina y putrescina, respectivamente (Hillary & Pegg, 2003, Wallace *et al.*, 2003).

1.3.3. Factores que influyen en la formación de aminas biógenas

La presencia de microorganismos descarboxilasa positivos es determinante para la formación de aminas biógenas en alimentos. No obstante, otros muchos factores como la disponibilidad de aminoácidos libres, pH, NaCl, temperatura y presencia del cofactor piridoxal-5'-fosfato, pueden también influir sobre la síntesis de aminas biógenas (Giuffrida *et al.*, 2006). La disponibilidad de aminoácidos es un factor limitante para la síntesis de aminas biógenas, ya que como se ha descrito anteriormente las aminas biógenas se forman por descarboxilación de aminoácidos (Tabla 3, Figuras 13 y 14). El pH del medio puede afectar tanto al crecimiento microbiano como a la actividad enzimática de las descarboxilasas (cuyo pH óptimo es de 5,0), influyendo además en la regulación de la transcripción de los genes de la descarboxilasa (*tdc*A) y del transportador (*tyr*P) que se expresan únicamente a valores de pH ácidos (Linares *et al.*, 2009, Linares *et al.*, 2011). La formación de aminas biógenas por los microorganismos podría ser un mecanismo para contrarrestar el pH del medio cuando se alcanzan condiciones ácidas (Koessler *et al.*, 1928, Bover Cid *et al.*, 2008, Loizzo *et al.*, 2013). Así la acidificación del medio favorece la actividad descarboxilasa provocando un aumento de la concentración de aminas (Chander *et al.*, 1988, Chander *et al.*, 1989, Fernández *et al.*, 2007b), aunque otros estudios indican una reducción de estas debido a un menor crecimiento microbiano (Maijala, 1994, Gardini *et al.*, 2001, Tabanelli *et al.*, 2012). La presencia de altas concentraciones de NaCl en el medio causa una disminución del crecimiento microbiano y de la formación de aminas biógenas (Chander *et al.*, 1988, Chander *et al.*, 1989, Sumner *et al.*, 1990, Gardini *et al.*, 2001, Tabanelli *et al.*, 2012). La temperatura tiene un marcado efecto sobre la formación de aminas biógenas, ya que influye en el crecimiento y multiplicación microbianos y en la actividad enzimática de las descarboxilasas (Chander *et al.*, 1988, Chander *et al.*, 1989, Gardini *et al.*, 2001, Tabanelli *et al.*, 2012). Así por ejemplo en un estudio realizado con queso madurado durante 56 días a 16 ºC se obtuvo una concentración total de aminas de 800 mg/kg, mientras que después 112 días a 10 ºC la concentración de aminas fue de 350 mg/kg (Pachlová *et al.*, 2012). En otro estudio realizado con extracto de histidina descarboxilasa libre de células,

se obtuvo la máxima actividad descarboxilasa a temperaturas próximas a 50 ºC (Tabanelli *et al.*, 2012). Las descarboxilasas son enzimas piridoxal-fosfato-dependientes, que requieren de este compuesto como cofactor para poder ejercer su actividad (Curtin & McSweeney, 2004). Sin embargo, hay algunos estudios en los que este cofactor tuvo menor influencia sobre la formación de aminas que el resto de factores estudiados (Moreno-Arribas & Lonvaud-Funel, 1999, Gardini *et al.*, 2005, Marcobal *et al.*, 2006).

Un factor indispensable para la formación y acumulación de aminas biógenas en alimentos es la presencia de microorganismos con actividad descarboxilasa. Son muchos los microorganismos capaces de producir aminas en alimentos, incluyendo bacterias gram positivas, gram negativas, levaduras y mohos. En queso las aminas biógenas, especialmente tiramina e histamina, son formadas principalmente por las bacterias lácticas (Linares *et al.*, 2011), que pueden formar parte del cultivo iniciador o pertenecer a la microbiota endógena de la leche (Burdychova & Komprda, 2007, Komprda *et al.*, 2008b). En un caso de intoxicación por histamina debido al consumo de queso Suizo se identificó una cepa de *Lactobacillus buchneri* como la principal responsable (Sumner *et al.*, 1990). En un estudio de quesos de tipo holandés se aislaron 17 cepas de *Lactobacillus* y 19 de *Enterococcus*, y se vio que 14 de las cepas de *Enterococcus* eran tirosina descarboxilasa positivas, pertenecientes a las especies *Enterococcus durans*, *faecalis*, *faecium* y *casseliflavus*. De las cepas de *Lactobacillus* aisladas 3 fueron histidina descarboxilasa positivas, entre las cuales se identificó una cepa de *Lactobacillus helveticus* que pertenecía al cultivo iniciador (Burdychova & Komprda, 2007). En queso Montasio se aislaron 1237 bacterias lácticas y 200 enterobacterias, de las cuales 182 bacterias lácticas y 181 enterobacterias mostraron capacidad de descarboxilar al menos uno de los aminoácidos ensayados (Marino *et al.*, 2008). En queso azul (Marino *et al.*, 2000) se aislaron 104 cepas de *Enterobacteriaceae* y todas ellas mostraron capacidad para descarboxilar al menos dos aminoácidos. Se observó además que la mayoría de estos aislados pertenecían al género *Enterobacter* (especies *gergoviae*, *cloacae* y *aerogenes*), seguido de *Serratia liquefaciens* y *Escherichia coli*. Las cepas de *Enterobacter aerogenes* fueron las que produjeron mayor cantidad de cadaverina, y las de *Enterobacter cloacae* de putrescina. Histamina y tiramina se produjeron en menor cantidad y únicamente un aislado de *Arizona* spp. produjo una

concentración de tiramina superior a 100 ppm. En un estudio realizado con diferentes variedades de quesos españoles se obtuvieron 694 aislados, de los cuales 125 se clasificaron como bacterias gram positivas con actividad descarboxilasa y 44 como gram negativas con actividad descarboxilasa. De las 125 bacterias gram positivas con actividad descarboxilasa, se vio que 117 eran formadoras de tiramina y únicamente 10 de histamina, identificándose 63 como *Enterococcus faecalis*. De los 44 aislados de bacterias gram negativas con actividad descarboxilasa, 43 eran formadoras de histamina y únicamente 2 de tiramina, identificándose 20 de estos aislados como *Hafnia alvei*. Además se aislaron 2 mohos formadores de histamina, uno de los cuales fue identificado como *Geotrichum candidum* (Roig-Sagués *et al.*, 2002). En leche y quesos de distintas regiones de Francia se aislaron 173 bacterias gram negativas, de las cuales el 64 % presentó capacidad de producir al menos una amina biógena, principalmente putrescina o cadaverina. Los principales microorganismos productores de histamina fueron *Morganella morganii*, *Hafnia alvei*, *Klebsiella* spp. y *Enterobacter* spp., mientras que los principales productores de tiramina fueron fundamentalmente especies de los géneros de *Enterobacter*, *Proteus* y *Pseudomonas* (Coton *et al.*, 2012). En general se considera que la mayoría de las bacterias gram negativas descritas como contaminantes de la leche, como *Escherichia coli*, *Hafnia alvei*, *Morganella morganii* o *Pseudomonas*, pueden tener la capacidad de producir histamina, putrescina o cadaverina, mientras que los principales microorganismos formadores de aminas biógenas en queso son las bacterias gram positivas, siendo las bacterias lácticas las principales formadoras de histamina y tiramina (Linares *et al.*, 2012). Algunas levaduras han sido descritas como productoras de aminas biógenas. Así, el empleo de las levaduras *Pichia jadinii*, *Yarrowia lipolytica* o *Debaryomyces hansenii* en la elaboración de queso Raclette provocó un aumento en la concentración de aminas biógenas (Wyder *et al.*, 1999). En queso Pecorino Crotonese se aislaron cepas de *Debaryomyces hansenii* capaces de descarboxilar ornitina e histidina, así como cepas de *Yersinia lipolytica* capaces de descarboxilar ornitina, tirosina, lisina y fenilalanina (Gardini *et al.*, 2006)

En pescado los principales microorganismos productores de histamina son *Morganella morganii*, *Klebsiella pneumoniae* y *Hafnia alvei* (López-Sabater *et al.*, 1994b, Özoğul, 2004). Se han aislado además cepas capaces de producir aminas biógenas

pertenecientes a los géneros *Enterobacter*, *Staphylococcus*, *Proteus*, *Acinetobacter*, *Rahnella*, *Plesiomonas*, *Pseudomonas*, *Serratia*, *Shewanella* y *Aeromonas* (López-Sabater *et al.*, 1994a, Tsai *et al.*, 2004, Allen *et al.*, 2005, Dalgaard *et al.*, 2006). Algunos de estos estudios se han realizado sobre muestras implicadas en episodios de intoxicación, como por ejemplo atún ahumado en frío en Dinamarca en 2004 (Emborg & Dalgaard, 2006) o iwashi maruboshi (sardina secada) en Japón en 2002 (Kanki *et al.*, 2004).

En carne y productos cárnicos se debe distinguir entre productos frescos y fermentados. En carne fresca, la cadaverina se ha asociado principalmente a la presencia de *Enterobacteriaceae*, mientras que la putrescina se ha asociado con altos recuentos de microorganismos aerobios totales (Ruiz-Capillas & Jiménez-Colmenero, 2004a). En productos cárnicos frescos se han realizado distintos estudios en función del tipo de envasado durante distintos tiempos de conservación, con el fin de identificar los microorganismos productores de aminas biógenas. En estos estudios se han aislado e identificado diferentes cepas de *Pseudomonas, Serratia, Staphylococcus, Enterobacter, Yersinia, Escherichia coli, Morganella, Proteus, Klebsiella, Citrobacter y Hafnia* (Durlu-Özkaya *et al.*, 2001, Balamatsia *et al.*, 2006, Curiel *et al.*, 2011). Por otro lado, en productos cárnicos fermentados los principales microorganismos productores de aminas biógenas suelen ser las bacterias lácticas, siendo la tiramina la amina más abundante (Ruiz-Capillas & Jiménez-Colmenero, 2004a, Latorre-Moratalla *et al.*, 2012). Se han aislado microorganismos productores de aminas biógenas procedentes del cultivo iniciador o de la microbiota endógena de la carne, que fueron identificados como *Lactobacillus, Lactococcus, Leuconostoc, Weissella, Enterococcus, Staphylococcus, Carnobacterium, Citrobacter, Oenococcus, Enterobacter, Klebsiella, Proteus, Serratia* y *Morganella*, e incluso *Pseudomonas* (Bover-Cid *et al.*, 2001, Suzzi & Gardini, 2003, de las Rivas *et al.*, 2008, Latorre-Moratalla *et al.*, 2010).

En los productos vegetales también debemos distinguir entre producto fresco y fermentado. En vegetales frescos las aminas biógenas se pueden formar principalmente por la presencia de distintas cepas de *Enterobacteriaceae* y *Pseudomonas*. Se han aislado e identificado distintas cepas de *Klebsiella, Pantoea, Hafnia, Enterobacter, Serratia, Morganella* y *Aeromonas* con capacidad de producir histamina, putrescina y/o cadaverina durante el almacenamiento en refrigeración de vegetales (Halász *et al.*, 1994,

Lavizzari *et al.*, 2010). En productos fermentados los principales productores de aminas biógenas suelen ser las bacterias lácticas, como *Lactobacillus* y *Leuconostoc*, que pueden pertenecer al cultivo iniciador o formar parte de la microbiota endógena del producto fresco (Shalaby, 1996, Penas *et al.*, 2010).

El vino puede contener aminas biógenas que, al igual que en otros productos fermentados, suelen formarse por la actividad enzimática de las bacterias lácticas responsables de la fermentación maloláctica. Uno de los principales microorganismos formadores de aminas en vino es *Oenococcus oeni*, junto con cepas de *Lactobacillus*, *Leuconostoc* y *Pediococcus* (Gardini *et al.*, 2005, Landete *et al.*, 2005, Beneduce *et al.*, 2010, Spano *et al.*, 2010). En la cerveza la formación de aminas biógenas sucede principalmente por la actividad de las bacterias lácticas durante la fermentación, pero también pueden estar implicadas algunas cepas de *Enterobacteriaceae* y de *Saccharomyces* (Bokulich & Bamforth, 2013). Se han identificado cepas de *Lactobacillus* y *Pediococcus* con actividad descarboxilasa en diversos tipos de cerveza (Izquierdo-Pulido *et al.*, 1996a, Kalač & Křížek, 2003).

1.3.4. Papel fisiológico y toxicología de las aminas biógenas

Las aminas biógenas juegan importantes papeles en la fisiología de animales, plantas y microorganismos. Las aminas biológicamente más activas son la histamina y la tiramina, y en menor grado las poliaminas (espermidina y espermina).

La histamina está normalmente presente en gran variedad de tejidos en mamíferos y organismos invertebrados. En humanos se puede encontrar en distintas concentraciones en cerebro, pulmones, estómago, intestino delgado y grueso, útero y uréteres (Ladero *et al.*, 2010). La histamina es principalmente sintetizada por mastocitos, basófilos, plaquetas, neuronas histaminérgicas y células enterocromafines, almacenándose intracelularmente en vesículas y pudiendo secretarse al medio (Maintz & Novak, 2007). La histamina modula distintas funciones, interactuando con receptores específicos (H1, H2, H3 y H4) en células diana. Los receptores H1 se encuentran principalmente en el sistema nervioso central, células del musculo liso y células endoteliales. Están implicados en la vasodilatación, broncoconstricción, permeabilidad vascular, procesos cognitivos y de atención, y pueden inducir la liberación de adrenalina y noradrenalina, siendo además responsables de la percepción del dolor y el picor en

picaduras de insectos (Hill *et al.*, 1997, Coruzzi *et al.*, 2001, Bongers *et al.*, 2011). Los receptores H2 están ampliamente distribuidos en diferentes tejidos, entre los que se encuentran el cerebro, mucosa gástrica y tejido cardiaco. Su función principal es la secreción de ácido gástrico, así como la relajación del músculo liso uterino, vascular y de las vías aéreas. También pueden inhibir algunas funciones del sistema inmune (Hill *et al.*, 1997, Bongers *et al.*, 2011). Los receptores H3 se encuentran principalmente en el sistema nervioso central y en menor medida en el periférico. En el sistema nervioso central provocan la inhibición de la liberación y formación de histamina, mientras que en el periférico inhiben la liberación de diversos neurotransmisores como serotonina, noradrenalina, acetilcolina y dopamina, e inhiben además la secreción de ácido gástrico en el estómago (Coruzzi *et al.*, 2001, Bongers *et al.*, 2011). Los receptores H4, los más recientemente identificados, se han encontrado en mastocitos y leucocitos, y están implicados en funciones relacionadas con procesos inflamatorios y alérgicos (Huang & Thurmond, 2008, Bongers *et al.*, 2011).

La tiramina y la β-feniletilamina se encuentran en el organismo en cantidades traza y están implicadas en el mantenimiento de la actividad neuronal (Silla Santos, 1996). La tiramina y la β-feniletilamina provocan aumento de la presión sanguínea y aumento de la frecuencia cardiaca de forma indirecta mediante la liberación de noradrenalina de las vesículas en las que se encuentra almacenada (Knoll *et al.*, 1996, Khwanchuea *et al.*, 2008). La tiramina puede aumentar el nivel de glucosa en sangre (Ladero *et al.*, 2010).

La putrescina, espermidina y espermina son factores esenciales para el crecimiento y funcionamiento normal celular, regulando la expresión génica mediante la modificación de la estructura del ADN y la modulación de las rutas de las señales de transducción (Kalač, 2009, Ladero *et al.*, 2010). Estas aminas intervienen en el desarrollo, maduración y mantenimiento del tracto intestinal (Kalač, 2009), participando además en la protección del epitelio gástrico inhibiendo la secreción de ácido (Medina *et al.*, 2003).

Las aminas biógenas están implicadas en diversas funciones fisiológicas en humanos y animales, pero su ingesta en niveles elevados puede tener efectos tóxicos. La aparición de los síntomas suele ser rápida, entre 5 minutos y 2 horas tras la ingesta, y los efectos no suelen durar más de 12 horas.

En mamíferos existe un eficiente sistema de detoxificación en el tracto intestinal y en condiciones normales las aminas exógenas son rápidamente detoxificadas por acción de las amino oxidasas. No obstante, en individuos con un sistema de detoxificación deficiente, bajo tratamiento con fármacos inhibidores de las amino oxidasas o debido a un consumo excesivo de aminas biógenas, pueden darse episodios de intoxicación (Silla Santos, 1996). La principal vía de detoxificación es la oxidación, aunque también puede producirse por metilación o acetilación (Ladero *et al.*, 2010). Las enzimas responsables de la oxidación son la monoamino oxidasa (MAO) y la diamino oxidasa (DAO). Existen dos isoformas primarias de la MAO conocidas como MAO-A y MAO-B. La MAO-A desamina la serotonina en el sistema nervioso central y las monoaminas ingeridas en el tracto intestinal, mientras que la MAO-B se encuentra principalmente en hígado y músculo, y desamina la dopamina y la β-feniletilamina. La histamina y las diaminas son desaminadas por la DAO en el tracto intestinal, proporcionando protección frente a pequeñas cantidades (McCabe-Sellers *et al.*, 2006).

El efecto tóxico de las aminas se manifiesta con síntomas generales como dolor de cabeza, migraña, hipertensión o hipotensión, taquicardia, urticaria, enrojecimiento y calambres abdominales. La histamina provoca la dilatación de los vasos sanguíneos periféricos provocando hipotensión, enrojecimiento y dolores de cabeza, y también provoca la contracción del músculo liso intestinal dando lugar a calambres abdominales, diarrea y vómitos (Silla Santos, 1996). Los síntomas de la intoxicación por histamina pueden confundirse con reacciones alérgicas ya que induce aumento de secreciones en las vías aéreas, enrojecimiento facial, urticaria, dificultad respiratoria, taquicardia y espasmos bronquiales (Ladero *et al.*, 2010). La tiramina y la β-feniletilamina pueden provocar migrañas y en ocasiones naúseas, vómitos y problemas respiratorios. En casos extremos pueden desembocar en una crisis hipertensiva, hemorragia cerebral y fallo cardiaco (Shalaby, 1996, Ladero *et al.*, 2010). Además de estos efectos tóxicos hay estudios que indican que podrían estar implicadas en otros procesos patológicos. Así, la tiramina puede favorecer la adherencia de *Escherichia coli* O157:H7 al epitelio intestinal (Lyte, 2004). Las diaminas putrescina y cadaverina podrían estar implicadas en procesos neoplásicos y cancerígenos a nivel colo-rectal. En cuanto a las poliaminas espermidina y espermina, son potenciadores del efecto tóxico de las

otras aminas biógenas, aumentando la permeabilidad de la pared intestinal y favoreciendo una mayor absorción de las aminas. Otro problema que plantean las poliaminas y diaminas es su capacidad para formar N-nitrosaminas, agentes mutagénicos, carcinogénicos y teratogénicos (Shalaby, 1996, Ladero *et al.*, 2010), en presencia de nitritos y bajo las condiciones ácidas del estómago. La putrescina y la cadaverina pueden transformarse por efecto de la temperatura durante el cocinado en pirrolidina y piperidina respectivamente, que pueden reaccionar con nitritos dando N-nitropirrolidina y N-nitropiperidina. La formación de nitrosaminas se ve por tanto favorecida por la abundancia de aminas y nitratos o nitritos, pH ácido, temperaturas altas y ciertos tratamientos o procesado del alimento como asado, fritura o ahumado. Existen diversos estudios sobre la toxicidad de las aminas biógenas (Naranjo, 1966, Til *et al.*, 1997), pero debido a la gran cantidad de factores que pueden influir sobre la toxicidad y efecto de estos compuestos, como la presencia de otras aminas, la presencia de inhibidores o potenciadores y la eficiencia del mecanismo de detoxificación individual, resulta muy difícil establecer un límite máximo de seguridad para el consumo de aminas en alimentos. No existe una legislación que regule el límite máximo de aminas biógenas en alimentos, excepto para los niveles de histamina en pescado. La legislación europea (EC 2073/2005) establece un límite máximo de histamina de 200 mg/kg en pescado fresco y de 400 mg/kg en productos curados derivados del pescado, mientras que la FDA de Estados Unidos establece 50 ppm, como nivel orientativo de pescado en mal estado (FDA, 2011).

1.3.5. Control de la formación de aminas biógenas

La acumulación de aminas biógenas en alimentos puede dar lugar a alteraciones (olores y sabores desagradables) y a reacciones tóxicas y adversas. Se han desarrollado distintos métodos con el fin de prevenir la formación de aminas biógenas en alimentos. El más empleado, no sólo para evitar la formación de aminas sino también para evitar otros riesgos microbiológicos, es el control o mantenimiento de temperaturas de conservación entre 0 y 5 ºC, lo que reduce la tasa de crecimiento de los microorganismos productores de aminas biógenas y disminuye la actividad descarboxilasa. En algunos casos, como en atún de aleta amarilla, a estas temperaturas sigue existiendo actividad descarboxilasa y se puede producir un ligero aumento de

aminas biógenas (Du *et al.*, 2002, Naila *et al.*, 2010). En productos fermentados la temperatura necesaria para su elaboración puede favorecer la formación de aminas biógenas. El empleo de técnicas correctas de manipulación y de medidas higiénicas durante el procesado del producto, contribuyen a prevenir la formación de aminas biógenas al reducir la contaminación del producto por microorganismos descarboxilasa positivos. El empleo de cultivos iniciadores que carezcan de actividad descarboxilasa también es un procedimiento útil. En el caso del queso, la pasteurización de la leche elimina los microorganismos descarboxilasa positivos, pero en algunas variedades de queso es necesario el empleo de leche cruda para mantener sus cualidades características.

Determinados conservantes y aditivos (como el sorbato potásico, ácido cítrico, ácido succínico, D-sorbitol y ácido málico) son capaces de inhibir el crecimiento de microorganismos descarboxilasa positivos, controlando así la formación de aminas biógenas en el alimento (Naila *et al.*, 2010). Otros aditivos que resultan también eficaces son el NaCl, que limita el crecimiento microbiano y la formación de aminas biógenas (Gardini *et al.*, 2001, Tabanelli *et al.*, 2012), y la glucono-δ-lactona, que disminuye el pH y limita el crecimiento microbiano (Maijala *et al.*, 1993). El nitrito sódico es capaz de limitar el crecimiento microbiano y la formación de aminas (Kurt & Zorba, 2009), pero puede aumentar el riesgo de formación de nitrosaminas en el alimento. Se ha propuesto también el empleo de ingredientes naturales con actividad antimicrobiana como el ajo y el jengibre para el control de aminas biógenas en alimentos (Naila *et al.*, 2010).

Mediante el envasado a vacío, en atmósfera modificada o en envases activos, se puede también controlar el crecimiento microbiano y la formación de aminas biógenas (Balamatsia *et al.*, 2006). Otras técnicas más complejas incluyen la irradiación y la aplicación de altas presiones hidrostáticas. La irradiación permite alargar la vida útil de los alimentos al reducir la carga microbiana, limitando así también la formación de aminas. Dosis de 20 KGrays sobre disoluciones acuosas de aminas inducen su descomposición hasta un 95 %, pero se requieren más estudios sobre los compuestos de degradación resultantes y los posibles efectos sobre los alimentos (Kim *et al.*, 2004). Las altas presiones hidrostáticas, como se describirá más adelante en detalle,

constituyen un método de pasteurización no térmica de los alimentos, que logra reducir la carga microbiana y alargar la vida útil del producto en función de la intensidad de presurización (Huang *et al.*, 2014), y que limita también la formación de aminas biógenas.

Además de las técnicas anteriormente descritas, basadas principalmente en el control de los microorganismos productores de aminas, existen también métodos basados en la degradación de las aminas biógenas presentes en el alimento. Se ha propuesto el empleo de cepas de bacterias lácticas, *Brevibacterium*, *Geotrichum* o *Micrococcus,* capaces de oxidar aminas a aldehídos, dando lugar a peróxido de hidrógeno y otras aminas o amoniaco. Estos microorganismos pueden incorporarse al alimento durante su procesado o bien emplearse como parte del cultivo iniciador en productos fermentados (Leuschner *et al.*, 1998, Herrero-Fresno *et al.*, 2012).

1.4. Altas presiones hidrostáticas

La aplicación de altas presiones hidrostáticas como procedimiento para conservar la leche y reducir el número de bacterias presentes en la misma se describió ya a finales del siglo XIX (Hite, 1899). Sin embargo, debido a su alto coste y dificultades técnicas su empleo en alimentos no adquirió importancia hasta la década de los 80. El procesado de alimentos con altas presiones (HPP, High Pressure Processing) implica someter al producto una vez sumergido en un líquido transmisor, generalmente agua debido a su baja compresibilidad, a presiones de entre 100 y 600 MPa (aunque pueden alcanzarse niveles de hasta 1400 MPa), con o sin aplicación de calor (Bermúdez-Aguirre & Barbosa-Cánovas, 2011, Mújica-Paz *et al.*, 2011).

Esta tecnología se fundamenta en dos principios:

· La ley de Pascal o principio isostático, por la que una presión externa aplicada sobre un fluido incompresible confinado se transmite de forma instantánea y uniforme en todas las direcciones. Según esto, la presión se transmite de forma instantánea y uniforme a todas las zonas del producto, independientemente de su tamaño y forma, haciendo que todo él reciba el mismo tratamiento (Martínez-Rodríguez *et al.*, 2012, Huang *et al.*, 2014).

· El principio de Le Chatelier, que indica que un sistema en equilibrio al sufrir un cambio (de concentración, presión, volumen o temperatura) se ajusta para contrarrestar dicho cambio. Así, al aumentar la presión se verán favorecidas las reacciones que impliquen una reducción de volumen (Eisenmenger & Reyes-De-Corcuera, 2009).

Según su funcionamiento, los equipos de altas presiones pueden ser:

· Equipos de tipo discontinuo. Estos equipos se emplean para presurizar productos líquidos o sólidos y para ello el producto se introduce en la cámara de presurización envasado en un material flexible. La forma de generar la presión en la cámara puede ser directa (Figura 15 A), mediante la compresión del fluido por un pistón, o indirecta (Figura 15 B), mediante una bomba de alta presión o un intensificador que introduce agua en la cámara provocando el aumento de la presión (Yaldagard *et al.*, 2008). Una vez terminado el tratamiento, la cámara se despresuriza y se retira el producto. Este tipo de equipos tienen la ventaja de que el producto se trata envasado, evitando así su contaminación posterior.

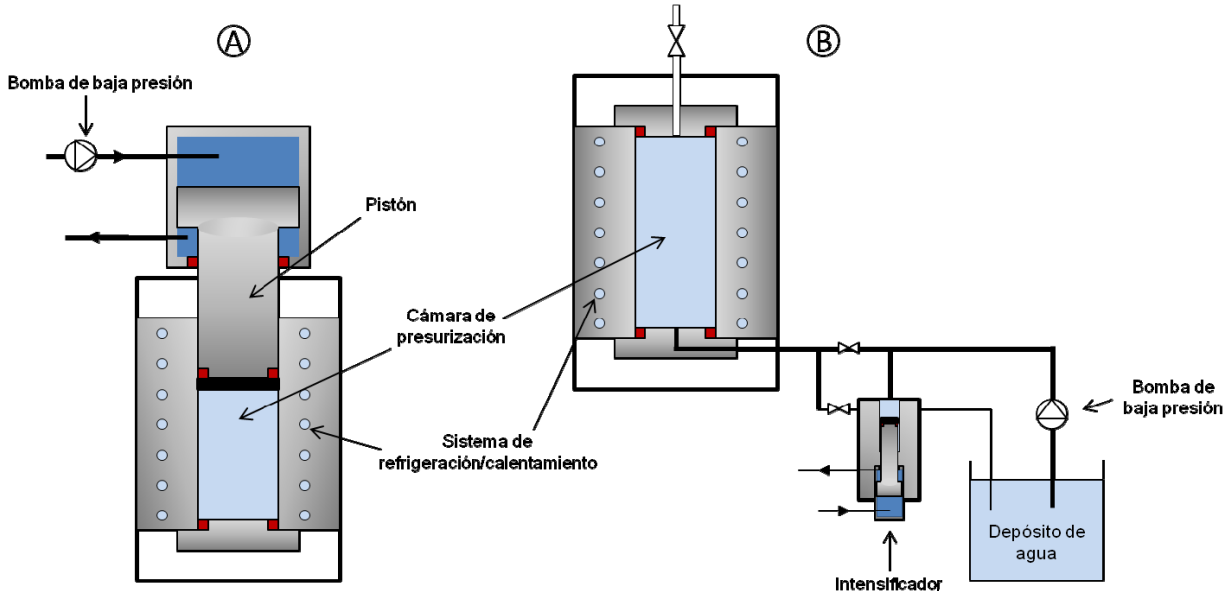


Figura 15. Esquema de sistemas de presurización directa (A) e indirecta (B).

· Equipos de tipo semicontinuo. Estos equipos se emplean para el tratamiento de productos líquidos, que pueden ser bombeados a la cámara de presurización. En este tipo de equipos la presión se genera de forma directa y una vez finalizado el tratamiento el producto es bombeado a un tanque estéril para su posterior envasado (Chawla *et al.*, 2011).

Existe además un sistema, no empleado en alimentos, en el que mediante el calentamiento del fluido de transmisión en la cámara se consigue aumentar la presión (San Martin *et al.*, 2002).

Figura 16. Ejemplo de algunos productos comerciales tratados por altas presiones.

Las altas presiones afectan a enlaces no covalentes, como fuerzas de Van der Waals, interacciones electrostáticas y puentes de hidrógeno (Stewart *et al.*, 2006, Chawla *et al.*, 2011, Mújica-Paz *et al.*, 2011). Por ello, la estructura de moléculas de alto peso molecular, como proteínas o carbohidratos, puede ser alterada por las altas presiones, mientras que moléculas más pequeñas, como compuestos volátiles, pigmentos y vitaminas, no resultan afectadas (Huang *et al.*, 2014). Los tratamientos por altas presiones logran la pasteurización no térmica del producto, así como la inactivación de enzimas, obteniéndose un producto microbiológicamente seguro que mantiene sus

características nutricionales y organolépticas mejor que los sometidos a tratamientos térmicos (Martínez-Rodríguez *et al.*, 2012).

El empleo de las altas presiones como método no térmico de pasteurización en alimentos ha sido aprobado por la FDA y el Departamento de Agricultura de Estados Unidos (Huang *et al.*, 2014). Además, el empleo de tratamientos combinados de altas presiones y temperatura ha sido aprobado por la FDA como método para la esterilización comercial de alimentos de baja acidez (Bermúdez-Aguirre & Barbosa-Cánovas, 2011, Mújica-Paz *et al.*, 2011). Durante la presurización, debido al trabajo de compresión contra las fuerzas intermoleculares, se produce un calentamiento adiabático que aumenta la temperatura del producto y del fluido transmisor (así por ejemplo, el agua sufre un aumento de 3 ºC por cada 100 MPa). Se produce también un desplazamiento del pH, generalmente hacia valores ácidos, que sin embargo una vez retirada la presión vuelve a su valor inicial (Bermúdez-Aguirre & Barbosa-Cánovas, 2011, Mújica-Paz *et al.*, 2011). Los tratamientos por altas presiones presentan también la ventaja de reducir costes y tiempo de procesado frente a los tratamientos térmicos convencionales, además de la mejora en la calidad del producto (Martínez-Rodríguez *et al.*, 2012).

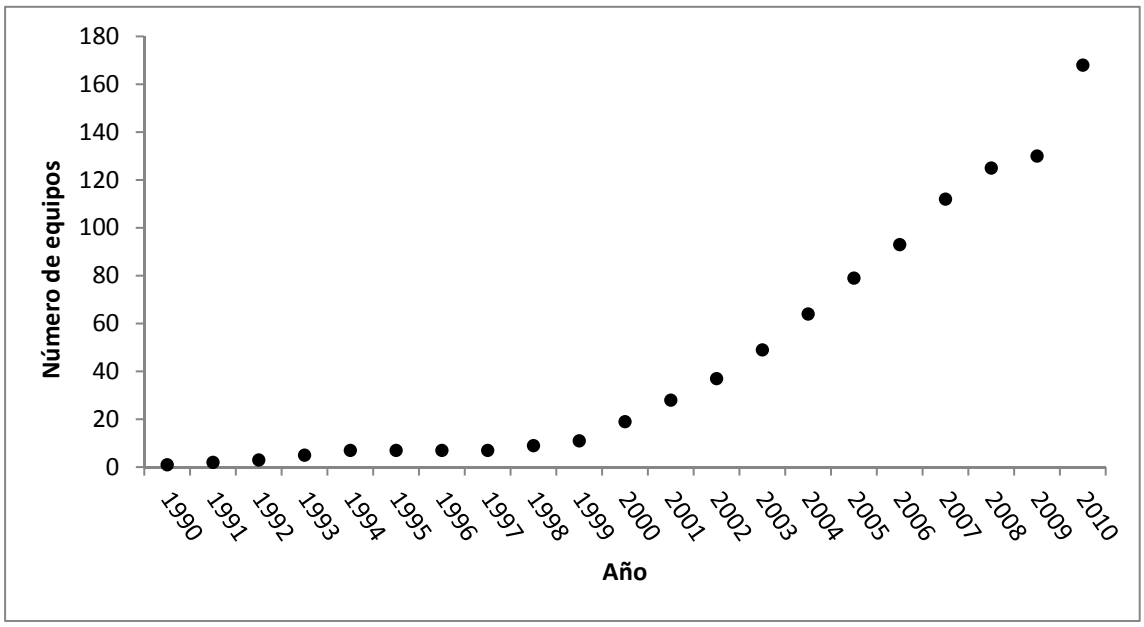


Figura 17. Aumento del número de equipos industriales de altas presiones distribuidos en el mundo.

Existe una amplia gama de productos tratados por altas presiones que se comercializan en todo el mundo (Figura 16). En los últimos años esta tecnología ha

adquirido gran importancia, como se puede ver por el número de equipos distribuidos a nivel mundial (Figura 17).

1.4.1. Modo de acción sobre microorganismos y enzimas

El aumento de la vida útil de los alimentos tratados por altas presiones se debe principalmente a la inactivación de los microorganismos y las enzimas presentes en los mismos. El efecto de las altas presiones sobre los microorganismos depende del nivel de presión alcanzado, del tiempo de aplicación, de las características de los microorganismos, de la temperatura del proceso y de la composición del medio (Stewart *et al.*, 2006). La inactivación de los microorganismos se produce por la acumulación de daños en distintas partes de la célula que cuando exceden la capacidad de reparación, acaba dando lugar a la lisis microbiana. En algunos casos el daño celular puede ser reparado si las condiciones del medio son favorables (Rendueles *et al.*, 2011). Uno de los primeros sitios afectados por las altas presiones es la membrana celular. La presurización induce una disminución de fluidez de los lípidos y fosfolípidos de membrana, llegando incluso a su solidificación, provocando una disminución de la permeabilidad y la pérdida de funcionalidad e integridad de la membrana. Esto mismo sucede en el interior de la célula, en la membrana de algunos orgánulos (Rendueles *et al.*, 2011, Huang *et al.*, 2014). Las altas presiones provocan la disociación de los ribosomas, causando una reducción o inhibición de la síntesis de proteínas y enzimas (Abe, 2007). Las altas presiones pueden causar además daños estructurales y morfológicos, como la separación entre la membrana y la pared celular y la compresión de las vacuolas. Las altas presiones también afectan a la funcionalidad del material genético, inhibiendo los mecanismos de replicación y transcripción del ADN (Huang *et al.*, 2014). Otro efecto importante inducido por las altas presiones es la desnaturalización de proteínas, incluyendo proteínas de membrana y enzimas. La desnaturalización de las proteínas de membrana puede limitar el flujo de protones, impidiendo la regulación del pH intracelular. La desnaturalización de las proteínas por las altas presiones se debe a la modificación de su estructura y su efecto depende del nivel estructural de la molécula. La estructura primaria viene determinada por la secuencia de aminoácidos unidos mediante enlaces covalentes, que no se ven afectados por las altas presiones. La estructura secundaria viene determinada por la formación de

puentes de hidrógeno, que son poco susceptibles a las presiones pero pueden verse afectados a presiones superiores a 300 MPa, siendo el efecto irreversible por encima de 700 MPa. Se ha visto además que la β-lámina es más barorresistente que la α-hélice. La estructura terciaria está determinada por interacciones hidrofóbicas, puentes disulfuro y fuerzas de Van der Waals, que son enlaces muy susceptibles a la presión, de tal modo que presiones superiores a 200 MPa provocan ya cambios significativos. La estructura cuaternaria aparece en proteínas oligoméricas y se debe a la presencia de distintas interacciones, como puentes de hidrógeno, puentes disulfuro e interacciones electrostáticas. Los puentes de hidrógeno presentan una alta resistencia a la presión, pero no así los puentes disulfuro y las interacciones electrostáticas, por lo que la estructura cuaternaria resulta muy susceptible a la presurización (Rendueles *et al.*, 2011, Huang *et al.*, 2014). El efecto de las altas presiones sobre las enzimas es altamente dependiente del tipo de enzima y de las condiciones del medio (San Martin *et al.*, 2002). Las enzimas pueden activarse o inactivarse, dependiendo de la intensidad de la presión aplicada y del tipo de enzima (Yaldagard *et al.*, 2008, Huang *et al.*, 2014). Como ya se ha descrito, los puentes de hidrógeno pueden llegar a verse afectados por las altas presiones, lo que puede resultar en cambios de la estabilidad y actividad de la enzimas. De este modo, algunas enzimas pueden ver incrementada su actividad por la modificación de la velocidad del paso limitante o la modulación de la especificidad y así, por ejemplo, la tripsina sometida a un tratamiento de 300 MPa durante 300 minutos ve incrementada su actividad en un 36% (Eisenmenger & Reyes-De-Corcuera, 2009, Kim *et al.*, 2013). Sin embargo, en general se considera que presiones superiores a 400 MPa pueden inducir cambios de la estructura tridimensional, alterando el centro activo y provocando la inactivación de la enzima. Se ha descrito que algunas enzimas, como la termolisina, se inactivan hasta un 50 % con presiones de 100 MPa (Kim *et al.*, 2013), mientras que otras, como la polifenoloxidasa de ciruela, resisten hasta 900 MPa (Weemaes *et al.*, 1998).

La resistencia de los microorganismos a las altas presiones es muy variable y depende principalmente del tipo de microorganismo, la temperatura y la composición de la matriz en la que se encuentra (Stewart *et al.*, 2006). Valores bajos de actividad de agua ejercen un efecto baroprotector en los microorganismos (Morales *et al.*, 2006).

Además a temperaturas de 4 ºC algunos microorganismos son menos resistentes a la presión que a 20 ºC (San Martin *et al.*, 2002). Las altas presiones normalmente tienen mayor efecto en microorganismos con mayor grado de organización y complejidad estructural. Los microorganismos procariotas son generalmente más resistentes que los eucariotas. Los protozoos y los parásitos son especialmente sensibles a la presión, y presiones relativamente bajas logran su inactivación (Rivalain *et al.*, 2010). Los mohos y levaduras tienen una resistencia intermedia, siendo los micelios de los mohos muy sensibles, mientras que las esporas son más resistentes (Martínez-Rodríguez *et al.*, 2014). Los virus poseen un amplio rango de resistencia a las altas presiones debido su gran diversidad estructural, siendo los poliovirus los más barorresistentes, resistiendo presiones de hasta 600 MPa (Grove *et al.*, 2008). La resistencia de las formas vegetativas de las bacterias depende de su fisiología, morfología y fase en la que se encuentran, siendo en general las gram positivas más resistentes que las gram negativas, los cocos más resistentes que los bacilos, y las bacterias en fase estacionaria más resistentes que las bacterias en fase exponencial (Chawla *et al.*, 2011, Demazeau & Rivalain, 2011b). Se ha visto además que los microorganismos que poseen mayor proporción de ácidos grasos insaturados en la membrana celular son más resistentes a la presión (Rendueles *et al.*, 2011). Por otro lado, las esporas bacterianas son extremadamente resistentes a las altas presiones, por lo que se han estudiado distintas estrategias para su destrucción, como la eliminación directa mediante la combinación de altas presiones con otros parámetros (como altas temperaturas) o la inducción de la germinación de las esporas seguida de la inactivación de las formas vegetativas (Nguyen Thi Minh *et al.*, 2010, Demazeau & Rivalain, 2011a, Reineke *et al.*, 2011).

1.4.2. Efecto de las altas presiones en queso

El empleo de altas presiones en la elaboración del queso se enfocó inicialmente al tratamiento de la leche para eliminar los microorganismos presentes en esta. Posteriormente, las altas presiones empezaron a aplicarse directamente sobre la cuajada o el queso, con el fin inactivar microorganismos no deseados (patógenos o alterantes), prolongar el tiempo de conservación del queso y/o modificar el proceso de maduración. Dependiendo de la variedad de queso y de la intensidad del tratamiento de presurización, la maduración puede ser acelerada mediante la lisis de los

microorganismos y liberación al medio de sus enzimas o frenada mediante la lisis de los microorganismos y la inactivación de las enzimas (Martínez-Rodríguez *et al.*, 2012), alargando la vida útil como se ha visto en queso fresco (Evert-Arriagada *et al.*, 2012, Evert-Arriagada *et al.*, 2014).

1.4.2.1. Efecto sobre los microorganismos

La capacidad de las altas presiones para inactivar microorganismos depende del tipo de microorganismo y del medio en el que se encuentre. Debido a las diferencias físico-químicas que se dan entre las distintas variedades de queso y a los cambios que experimenta cada variedad durante el proceso de maduración, el efecto de la presurización sobre los microorganismos es muy diverso. La capacidad de las altas presiones para reducir la presencia de microorganismos contaminantes del queso ha sido demostrada por diversos autores (Rendueles *et al.*, 2011, Martínez-Rodríguez *et al.*, 2012, Mota *et al.*, 2013). En pasta de queso Cheddar (100 g de queso y 42 g de solución salina), tratamientos de 400 MPa a una temperatura de 10 ºC lograron reducciones de los niveles de *P. roqueforti* de 2 ud. log, mientras que aplicados a 20 ºC o 30 ºC alcanzaron reducciones de 6 ud. log (O'Reilly *et al.*, 2000). En este mismo estudio, se observaron reducciones de *S. aureus* y *E. coli* desde niveles de 10^7 ufc/g hasta niveles no detectables mediante tratamientos de 400 MPa a 30 ºC o de 600 MPa a 20 ºC. En quesos de leche cruda (Arqués *et al.*, 2006), se han conseguido reducciones de bacterias coliformes de hasta 5,5 ud. log mediante tratamientos de 400 MPa aplicados 2 días después de la fabricación, así como reducciones de 4,0 ud. log de bacterias gram negativas, de 1,8 ud. log de *Micrococcaceae* y de 1,5 ud. log de *Staphylococcus* coagulasa positivos. La presurización a 300 o 400 MPa aplicada 50 días después de la fabricación indujo reducciones de 2,3 o 6,4 ud. log de bacterias gram negativas, respectivamente; además, el tratamiento de 400 MPa redujo por debajo del límite de detección los niveles de bacterias coliformes. En otro estudio, la aplicación de 600 MPa al día siguiente de la fabricación consiguió reducir en 4,8 ud. log los niveles de bacterias psicrótrofas, en 2,6 ud. log los de *Enterobacteriaceae* y *Listeria* spp. y en 2,4 ud. log los de *Micrococcaceae* (Delgado *et al.*, 2012). Por otro lado, tratamientos de 200 o 300 MPa aplicados a queso de 1 día lograron reducciones de *Enterobacteriaceae* de entre 1 y 2 ud. log, mientras que 400 o 500 MPa redujeron los niveles entre 3 y 4 ud. log;

tratamientos superiores a 300 MPa aplicados a queso de 15 días consiguieron niveles no detectables de *Enterobacteriaceae* (Juan *et al.*, 2007b).

La presurización no sólo afecta a los microorganismos patógenos y alterantes del queso sino también a los cultivos iniciadores y cultivos secundarios, pudiendo emplearse para controlar la maduración. Se ha observado que tratamientos de 405 MPa durante 3 minutos aplicados a quesos Cheddar elaborados con distintas concentraciones de sal, dieron como resultado reducciones de entre 1 y 4 ud. log de las bacterias lácticas del cultivo iniciador (Ozturk *et al.*, 2013). Tratamientos de 400 y 600 MPa, en queso azul de 42 días, consiguieron reducir los niveles de *P. roqueforti*, levaduras, *Enterococcus*, *Lactobacillus* y bacterias lácticas secundarias, alcanzando reducciones de hasta 3 ud. log para *P. roqueforti* y bacterias lácticas secundarias con el tratamiento de 600 MPa (Voigt *et al.*, 2010). En queso Cheddar de 1 mes (Wick *et al.*, 2004), tratamientos superiores a 300 MPa consiguieron reducir los niveles de *Lactococcus lactis* del cultivo iniciador y tratamientos superiores a 200 MPa redujeron los niveles de *Lactobacillus*. En el caso de los quesos presurizados a 500 y 800 MPa, los niveles de *Lactobacillus* se redujeron por debajo del límite de detección. Durante el almacenamiento posterior de los quesos, los niveles de *Lactococcus lactis* aumentaron únicamente en los quesos presurizados a 400 MPa, mientras que los niveles de *Lactobacillus* aumentaron en los quesos presurizados a 200 y 300 MPa. Sin embargo, en queso Cheddar de 4 meses fueron necesarios tratamientos superiores a 500 MPa para conseguir reducciones significativas de los niveles de *Lactococcus lactis*. En quesos de leche de oveja, la presurización a 200 o 300 MPa aplicada a queso de 1 día indujo reducciones en los niveles de *Lactococcus* de tan sólo 1 ud. log, mientras que tratamientos con 400 y 500 MPa lograron reducciones de 2 y 5 ud. log respectivamente, para *Lactococcus*, y de 2 ud. log para *Lactobacillus*. Estos mismos tratamientos de 400 y 500 MPa aplicados a queso de 15 días, redujeron los niveles de *Lactobacillus* en 2 y 5 ud. log respectivamente, y en 2 ud. log los niveles de *Lactococcus* (Juan *et al.*, 2007b). Tratamientos con 300 MPa en quesos de leche de oveja, aplicados a queso de 1 o 15 días, indujeron reducciones en los niveles de *Lactobacillus* de 3 y 1 ud. log respectivamente (Juan *et al.*, 2007a). Tratamientos de 400 y 600 MPa aplicados a quesos de 1 o 30 días, redujeron significativamente los niveles de bacterias lácticas en

quesos de leche cruda de cabra (Delgado *et al.*, 2012). En quesos de leche cruda de oveja tratados con altas presiones a los 2 días de la fabricación, se obtuvieron reducciones de bacterias lácticas, lactobacilos y enterococos de 1,4, 0,6 y 2,1 ud. log, respectivamente, para el tratamiento de 300 MPa, y de 2,0, 1,6 y 2,7 ud. log, respectivamente, para 400 MPa (Arqués *et al.*, 2006). En quesos en salmuera la aplicación de 200 MPa a queso de 15 días no produjo reducciones significativas, mientras que con 500 MPa se consiguió reducir en 8, 2 y 4 ud. log los niveles de *Lactococcus*, *Lactobacillus* y bacterias lácticas secundarias, respectivamente (Moschopoulou *et al.*, 2010). Las altas presiones también se han empleado con el objetivo de atenuar los cultivos iniciadores empleados en la elaboración de queso. De esta forma, mediante el empleo de 200 MPa se consiguió que cultivos de *Lactococcus lactis*, empleados como adjuntos, no acidificasen durante la elaboración de queso Cheddar y sí aumentasen la proteolisis secundaria durante la maduración (Upadhyay *et al.*, 2007). Estos mismos resultados se obtuvieron también en queso Feta al tratar un cultivo indicador formado por diferentes cepas de bacterias lácticas (Maniou *et al.*, 2013).

1.4.2.2. Efecto sobre las enzimas

El control de la maduración mediante las altas presiones no depende únicamente de la presencia de microorganismos viables, sino también de la actividad de las enzimas presentes, que pueden proceder de la leche o de microorganismos lisados. A una temperatura de 8 ºC, tratamientos inferiores a 800 MPa y 60 minutos, no produjeron ningún efecto sobre la plasmina en queso Cheddar, así como tampoco tratamientos inferiores a 400 MPa aplicados a 20 ºC. Tratamientos de 600 y 800 MPa a 20 ºC, aplicados entre 15 y 60 minutos consiguieron una leve inactivación (≤ 15 %) de la plasmina, mientras que con estos mismos tratamientos aplicados a 30 ºC se consiguió una inactivación de entre el 31 y 48 % (Huppertz *et al.*, 2004, Rynne *et al.*, 2008). En quesos de leche de oveja, la actividad de la plasmina no se vio afectada por tratamientos de hasta 500 MPa (Juan *et al.*, 2007b) y al ser tratada en tampón la plasmina no se vio afectada por presiones de hasta 800 MPa (Malone *et al.*, 2003). La quimosina en queso Cheddar no se vio afectada por presiones inferiores a 400 MPa, independientemente de la temperatura a la que se aplicase el tratamiento, mientras que

tratamientos de 600 y 800 MPa provocaron una inactivación de entre el 87 y el 96 % (Huppertz *et al.*, 2004, Rynne *et al.*, 2008). Tratamientos superiores a 400 MPa aplicados a queso de 1 día de leche de oveja provocaron una importante reducción de la actividad de la quimosina (Juan *et al.*, 2007b), mientras que en tampón esta misma enzima no se vio afectada por presiones de hasta 400 MPa, manteniendo cerca de un 50 % de su actividad a presiones de 800 MPa (Malone *et al.*, 2003). En un estudio con diferentes tipos de quesos (Gouda, Camembert y Kurpiowski), diferentes presiones (200 a 800 MPa) y diferentes tiempos de maduración, se observó que la actividad proteolítica disminuyó con la aplicación de tratamientos superiores a 200 MPa y que la actividad amino- y endopeptidásica se redujeron con tratamientos de 600 MPa. La enzima lisina-aminopeptidasa mostró mayor resistencia a las altas presiones en queso Camembert. En los quesos Camembert y Gouda se encontró actividad caseinolítica residual después de ser tratados con 800 MPa a las 2 semanas de maduración (Reps *et al.*, 2003). En queso Edam se observó un ligero aumento de la actividad proteolítica en los quesos presurizados a 200 MPa, mientras que los tratados a 400 MPa mostraron un ligero descenso de esta actividad (Iwańczak & Wiśniewska, 2005). La actividad lactato deshidrogenasa (LDH) en quesos de leche de oveja aumentó con la aplicación de 300 y 400 MPa un día después de la fabricación, mientras que la aplicación de 500 MPa provocó un descenso de la actividad debido a la inactivación parcial de esta enzima (Juan *et al.*, 2007b, 2008). Igualmente, en quesos en salmuera, la actividad LDH aumentó con tratamiento a 200 MPa y se redujo con tratamiento a 500 MPa (Moschopoulou *et al.*, 2010). En quesos de leche cruda de oveja, la actividad aminopeptidásica se redujo significativamente con tratamientos de 300 y 400 MPa aplicados 2 días después de la fabricación, mientras que la aplicación de 300 MPa a los 50 días provocó un aumento significativo respecto al control (Garde *et al.*, 2007). La aplicación de tratamientos de entre 200 y 500 MPa un día después de la fabricación en quesos de leche de oveja redujo la actividad aminopeptidásica, mientras que estos mismos tratamientos aplicados a los 15 días provocaron un aumento de actividad, excepto en los quesos tratados con 500 MPa (Juan *et al.*, 2007b). La aplicación de tratamientos de 200 y 500 MPa a quesos en salmuera 15 días después de su fabricación no provocó ningún efecto significativo sobre la actividad aminopeptidásica (Moschopoulou *et al.*, 2010). En queso Hispánico de 15 días de maduración tratado con 400 MPa disminuyó la actividad

aminopeptidasa (Ávila *et al.*, 2006), aunque no se vio ninguna diferencia significativa de actividad esterasa respecto al control (Ávila *et al.*, 2007). Tratamientos de 600 MPa a temperatura ambiente consiguieron reducir la actividad de la lipasa en tampón, pero fueron necesarios 700 MPa a 45 ºC para conseguir su total inactivación (Seyderhelm *et al.*, 1996). Mediante estudios en tampón, se ha visto que tratamientos a partir de 400 MPa provocan la inactivación de la proteasa asociada a la envoltura celular, mientras que tratamientos de 100 MPa aumentan su actividad. Las aminopeptidasas se ven afectadas de distinta manera por las altas presiones. Así, se ha visto que en tampón tratamientos de 400 MPa son capaces de inactivar la aminopeptidasa N, mientras que la aminopeptidasa A no se ve afectada por tratamientos de hasta 800 MPa y la aminopeptidasa C aumenta su actividad con tratamientos de hasta 700 MPa (Malone *et al.*, 2003).

1.4.2.3. Efecto sobre la textura y la microestructura

Las altas presiones pueden provocar cambios en la estructura de la red de caseínas del queso, modificando su textura. La aplicación de tratamientos de 405 MPa a quesos Cheddar elaborados con distintas concentraciones de sal, hizo que los valores de dureza y fracturabilidad de los quesos tratados fuesen menores que los de sus respectivos controles no tratados (Ozturk *et al.*, 2013). En quesos de leche de oveja, la aplicación de tratamientos de 300 MPa a diferentes tiempos de maduración provocó que los quesos tratados el día 1 después de la fabricación presentaran valores más altos de deformabilidad que los quesos no tratados y que los presurizados el día 15 (Juan *et al.*, 2008). En quesos de leche cruda de cabra, tratados con 400 y 600 MPa a diferentes tiempos de maduración, se observó que únicamente los tratados a día 1 sufrieron reducciones significativas de dureza, gomosidad y masticabilidad respecto al control, no apreciándose diferencias entre la aplicación de 400 y 600 MPa (Delgado *et al.*, 2012). En quesos de leche cruda de oveja, tratados con 300 y 400 MPa 2 días después de la fabricación, los valores de fracturabilidad y elasticidad se vieron significativamente incrementados, mientras que la dureza únicamente aumentó en los quesos tratados con 400 MPa (Garde *et al.*, 2007). En quesos Cheddar tratados con 345 y 483 MPa durante tiempos de 3 y 7 minutos, los valores de firmeza fueron significativamente menores que los del queso control; los quesos tratados durante 3 minutos con 345 MPa y los

tratados 7 minutos con 483 MPa presentaron valores de elasticidad inferiores al control y no se vio afectada la cohesividad (Serrano *et al.*, 2005). En estos quesos se analizó además su microestructura mediante microscopía electrónica de barrido. En el queso control se observó una red porosa de proteínas, con aspecto de esponja, en la que se englobaban glóbulos grasos de diferentes formas y tamaños, distribuidos sin uniformidad. La matriz de proteína de los quesos tratados fue sin embargo más compacta y continua, más semejante a la de los quesos curados. Estos cambios de estructura se relacionaron con la intensidad y el tiempo de los tratamientos, observándose zonas compactas y zonas esponjosas en los quesos tratados con 345 MPa y obteniéndose una estructura en general más compacta en el queso tratado con 483 MPa durante 7 minutos. Cuando se aplicaron tratamientos de entre 50 y 400 MPa durante 5 o 15 minutos a quesos en salmuera, se observó que los tratamientos de 50 y 100 MPa no afectaron a la textura ni a la microestructura de los quesos, mientras que los quesos presurizados a 200 y 400 MPa presentaron valores de firmeza, elasticidad y gomosidad inferiores a los del queso control (Koca *et al.*, 2011). Mediante el análisis de la microestructura por microscopía confocal de barrido por láser, se pudo apreciar una red porosa de proteínas, con glóbulos de grasa de diferentes tamaños y formas tanto en el queso control como en los tratados con 50 y 100 MPa. Los quesos tratados con 200 y 400 MPa mostraron una red de proteínas más compacta, con glóbulos de grasa de menor tamaño y distribuidos de forma más uniforme. Por otro lado, la aplicación de las altas presiones durante 15 minutos dio lugar a una estructura más densa y compacta que su aplicación durante 5 minutos.

1.5. Bibliografía

Abe, F. (2007). Exploration of the effects of high hydrostatic pressure on microbial growth, physiology and survival: Perspectives from piezophysiology. *Bioscience Biotechnology and Biochemistry* **71**, 2347-2357.

Alewijn, M., Smit, B. A., Sliwinski, E. L. & Wouters, J. T. M. (2007). The formation mechanism of lactones in Gouda cheese. *International Dairy Journal* **17**, 59-66.

Allen, D. G., Green, D. P., Bolton, G. E., Jaykus, L. A. & Cope, W. G. (2005). Detection and identification of histamine-producing bacteria associated with harvesting and processing mahimahi and yellowfin tuna. *Journal of Food Protection* **68**, 1676-1682.

Ancin-Azpilicueta, C., Gonzalez-Marco, A. & Jimenez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition* **48**, 257-275.

Arqués, J. L., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (2006). Inactivation of microbial contaminants in raw milk La Serena cheese by high-pressure treatments. *Journal of Dairy Science* **89**, 888-891.

Ávila, M., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy science* **89**, 2882-2893.

Ávila, M., Calzada, J., Garde, S. & Nuñez, M. (2007). Effect of a bacteriocin-producing *Lactococcus lactis* strain and high-pressure treatment on the esterase activity and free fatty acids in Hispánico cheese. *International Dairy Journal* **17**, 1415-1423.

Balamatsia, C. C., Paleologos, E. K., Kontominas, M. G. & Savvaidis, I. N. (2006). Correlation between microbial flora, sensory changes and biogenic amines formation in fresh chicken meat stored aerobically or under modified atmosphere packaging at 4 degrees C: possible role of biogenic amines as spoilage indicators. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **89**, 9-17.

Bardócz, S., Grant, G., Brown, D. S., Ralph, A. & Pusztai, A. (1993). Polyamines in food - implications for growth and health. *Journal of Nutritional Biochemistry* **4**, 66-71.

Bardócz, S. (1995). Polyamines in food and their consequences for food quality and human health. *Trends in Food Science & Technology* **6**, 341-346.

Beneduce, L., Romano, A., Capozzi, V., Lucas, P., Barnavon, L., Bach, B., Vuchot, P., Grieco, F. & Spano, G. (2010). Biogenic amine in wines. *Annals of Microbiology* **60**, 573-578.

Bermúdez-Aguirre, D. & Barbosa-Cánovas, G. V. (2011). An update on high hydrostatic pressure, from the laboratory to industrial applications. *Food Engineering Reviews* **3**, 44-61.

Bijl, E., de Vries, R., van Valenberg, H., Huppertz, T. & Van Hooijdonk, T. (2014). Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of kappa-casein. *International Dairy Journal* **34**, 135-141.

Blasi, F., Montesano, D., De Angelis, M., Maurizi, A., Ventura, F., Cossignani, L., Simonetti, M. S. & Damiani, P. (2008). Results of stereospecific analysis of triacylglycerol fraction from donkey, cow, ewe, goat and buffalo milk. *Journal of Food Composition and Analysis* **21**, 1-7.

BOE. (2002). Orden APA/1144/2002. *Boletín Oficial del Estado del 23 de Mayo de 2002.* **123**, 18578-18586.

Bokulich, N. A. & Bamforth, C. W. (2013). The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews* **77**, 157-172.

Bongers, G., de Esch, I. & Leurs, R. (2011). Molecular pharmacology of the four histamine receptors. *Advances in experimental medicine and biology* **709**, 11-19.

Bover Cid, S., Miguélez-Arrizado, M. J., Becker, B., Holzapfel, W. H. & Vidal-Carou, M. C. (2008). Amino acid decarboxylation by *Lactobacillus curvatus* CTC273 affected by the pH and glucose availability. *Food Microbiology* **25**, 269-277.

Bover-Cid, S., Hugas, M., Izquierdo-Pulido, M. & Vidal-Carou, M. C. (2001). Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. *International Journal of Food Microbiology* **66**, 185-189.

Bramanti, E., Sortino, C., Onor, M., Beni, F. & Raspi, G. (2003). Separation and determination of denatured alpha(s1)-, alpha(s2)-, beta- and kappa-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. *Journal of Chromatography A* **994**, 59-74.

Burdychova, R. & Komprda, T. (2007). Biogenic amine-forming microbial communities in cheese. *Fems Microbiology Letters* **276**, 149-155.

Cantor, M. D., van den Tempel, T., Hansen, T. K. & Ardö, Y. (2004). Blue cheese. In Cheese: Chemistry, Physics and Microbiology, pp. 175-198: Academic Press.

Caroli, A. M., Chessa, S. & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science* **92**, 5335-5352.

Centeno, J. A., Cepeda, A. & Rodríguez-Otero, J. L. (1995). Identification and preliminary characterization of strains of enterococci and micrococci isolated from Arzúa raw cows'-milk cheese. *Nahrung-Food* **39**, 55-62.

Chander, H., Batish, V. K., Babu, S. & Bhatia, K. L. (1988). Studies on optimal conditions for amine production by *E. coli*. *Milchwissenschaft-Milk Science International* **43**, 90-91.

Chander, H., Batish, V. K., Babu, S. & Singh, R. S. (1989). Factors affecting amine production by a selected strain of *Lactobacillus bulgaricus*. *Journal of Food Science* **54**, 940-942.

Chawla, R., Patil, G. R. & Singh, A. K. (2011). High hydrostatic pressure technology in dairy processing: a review. *Journal of Food Science and Technology-Mysore* **48**, 260-268.

CODEX STAN 238-1978. Norma general del codex para el queso. CODEX ALIMENTARIUS.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal* **13**, 841-866.

Coruzzi, G., Morini, G., Adami, M. & Grandi, D. (2001). Role of histamine H-3 receptors in the regulation of gastric functions. *Journal of Physiology and Pharmacology* **52**, 539-553.

Coton, E., Rollan, G. C. & Lonvaud-Funel, A. (1998). Histidine carboxylase of Leuconostoc oenos 9204: Purification, kinetic properties, cloning and nucleotide sequence of the hdc gene. *Journal of Applied Microbiology* **84**, 143-151.

Coton, M., Delbes-Paus, C., Irlinger, F., Desmasures, N., Le Fleche, A., Stahl, V., Montel, M. C. & Coton, E. (2012). Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiology* **29**, 88-98.

Curiel, J. A., Ruiz-Capillas, C., de las Rivas, B., Carrascosa, A. V., Jiménez-Colmenero, F. & Muñoz, R. (2011). Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration. *Meat Science* **88**, 368-373.

Curioni, P. M. G. & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal* **12**, 959-984.

Curtin, Á. C. & McSweeney, P. L. H. (2004). Catabolism of amino acids in cheese during ripening. In Cheese: Chemistry, Physics and Microbiology, pp. 435-454: Academic Press.

Dalgaard, P., Madsen, H. L., Samieian, N. & Emborg, J. (2006). Biogenic amine formation and microbial spoilage in chilled garfish (Belone belone belone) - effect of modified atmosphere packaging and previous frozen storage. *Journal of Applied Microbiology* **101**, 80-95.

Dalgleish, D. G., Spagnuolo, P. A. & Goff, H. D. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal* **14**, 1025-1031.

Dalgleish, D. G. & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology, Vol 3* **3**, 449-467.

de las Rivas, B., Ruiz-Capillas, C., Carrascosa, A. V., Curiel, J. A., Jímenez-Colmenero, F. & Muñoz, R. (2008). Biogenic amine production by Gram-positive bacteria isolated from Spanish dry-cured "chorizo" sausage treated with high pressure and kept in chilled storage. *Meat Science* **80**, 272-277.

Deeth, H. C. (2006). Lipoprotein lipase and lipolysis in milk. *International Dairy Journal* **16**, 555-562.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J. & Ramírez, R. (2010). Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. *Food Chemistry* **118**, 182-189.

Delgado, F. J., González-Crespo, J., Cava, R. & Ramírez, R. (2012). Changes in microbiology, proteolysis, texture and sensory characteristics of raw goat milk cheeses treated by high-pressure at different stages of maturation. *LWT-Food Science and Technology* **48**, 268-275.

Demazeau, G. & Rivalain, N. (2011a). High hydrostatic pressure and biology: a brief history. *Applied Microbiology and Biotechnology* **89**, 1305-1314.

Demazeau, G. & Rivalain, N. (2011b). The development of high hydrostatic pressure processes as an alternative to other pathogen reduction methods. *Journal of Applied Microbiology* **110**, 1359-1369.

Du, W. X., Lin, C. M., Phu, A. T., Cornell, J. A., Marshall, M. R. & Wei, C. I. (2002). Development of biogenic amines in yellowfin tuna (Thunnus albacares): Effect of storage and correlation with decarboxylase-positive bacterial flora. *Journal of Food Science* **67**, 292-301.

Durlu-Özkaya, F., Ayhan, K. & Vural, N. (2001). Biogenic amines produced by *Enterobacteriaceae* isolated from meat products. *Meat Science* **58**, 163-166.

Eisenmenger, M. J. & Reyes-De-Corcuera, J. I. (2009). High pressure enhancement of enzymes: A review. *Enzyme and Microbial Technology* **45**, 331-347.

EC 1491/2003. Reglamento (CE) Nº 1491/2003 de la comisión de 25 de agosto de 2003 por el que se completa el Reglamento (CE) nº 2400/96 (Ficodindia dell'Etna, Monte Etna, Colline di Romagna, Pretuziano delle Colline Teramane, Torta del Casar, Manzana de Girona or Poma de Girona). Diario Oficial de la Unión Europea. 26-8-2003. L214/6 - L214/7.

EC 2073/2005. Reglamento (CE) nº 2073/2005 de la comisión de 15 de noviembre de 2005 relativo a los criterios microbiológicos aplicables a los productos alimenticios. Diario Oficial de la Unión Europea. 22-12-2005. L338/1 – L338/26.

Emborg, J. & Dalgaard, P. (2006). Formation of histamine and biogenic amines in cold-smoked tuna: An investigation of psychrotolerant bacteria from samples implicated in cases of histamine fish poisoning. *Journal of Food Protection* **69**, 897-906.

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

Evert-Arriagada, K., Hernández-Herrero, M. M., Guamis, B. & Trujillo, A. J. (2014). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *LWT-Food Science and Technology* **55**, 498-505.

Exterkate, F. A., Lagerwerf, F. M., Haverkamp, J. & van Schalkwijk, S. (1997). The selectivity of chymosin action on alpha(s1)- and beta-caseins in solution is modulated in cheese. *International Dairy Journal* **7**, 47-54.

FAOSTAT (2014). http://faostat3.fao.org/faostat-gateway/go/to/home/E.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T. & other authors (2004). Nomenclature of the proteins of cows' milk - Sixth revision. *Journal of Dairy Science* **87**, 1641-1674.

FDA. (2011). Scombrotoxin (histamine) formation. In Fish and Fishery Products Hazards and Controls Guidance. *U. S. Department of Health and Human Services*. pp. 113-152.

Fernández, M., Linares, D. M., del Río, B., Ladero, V. & Alvarez, M. A. (2007a). HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. *Journal of Dairy Research* **74**, 276-282.

Fernández, M., Linares, D. M., Rodríguez, A. & Alvarez, M. A. (2007b). Factors affecting tyramine production in *Enterococcus durans* IPLA 655. *Applied Microbiology and Biotechnology* **73**, 1400-1406.

Fox, P. F. & McSweeney, P. L. H. (2004). Cheese: An Overview. In Cheese: Chemistry, Physics and Microbiology, pp. 1-18: Academic Press.

Fox, P. F. & Kelly, A. L. (2006). Indigenous enzymes in milk: Overview and historical aspects - Part 1. *International Dairy Journal* **16**, 500-516.

Fox, P. F. & Brodkorb, A. (2008). The casein micelle: Historical aspects, current concepts and significance. *International Dairy Journal* **18**, 677-684.

Fox, P. F. (2009). Milk: An overview. In Milk proteins: From expression to food, pp. 1-54. San Diego: Academic Press.

Garde, S., Arqués, J. L., Gaya, P., Medina, M. & Nuñez, M. (2007). Effect of high-pressure treatments on proteolysis and texture of ewes' raw milk La Serena cheese. *International Dairy Journal* **17**, 1424-1433.

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E. & Suzzi, G. (2001). Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *International Journal of Food Microbiology* **64**, 105-117.

Gardini, F., Zaccarelli, A., Belletti, N., Faustini, F., Cavazza, A., Martuscelli, M., Mastrocola, D. & Suzzi, G. (2005). Factors influencing biogenic amine production by a strain of *Oenococcus oeni* in a model system. *Food Control* **16**, 609-616.

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., Guerzoni, M. E. & Lanciotti, R. (2006). Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiology* **23**, 641-648.

Giuffrida, D., Ziino, M., Verzera, A., Condurso, C. & Romeo, V. (2006). Biogenic amines in a typical "pasta filata" Italian cheese. *Acta Alimentaria* **35**, 435-443.

Gobbetti, M., Smacchi, E. & Corsetti, A. (1997). Purification and characterization of a cell surface-associated esterase from Lactobacillus fermentum DT41. *International Dairy Journal* **7**, 13-21.

Gresti, J., Bugaut, M., Maniongui, C. & Bezard, J. (1993). Composition of molecular species of triacylglycerols in bovine milk fat. *Journal of Dairy Science* **76**, 1850-1869.

Grove, S. F., Forsyth, S., Wan, J., Coventry, J., Cole, M., Stewart, C. M., Lewis, T., Ross, T. & Lee, A. (2008). Inactivation of hepatitis A virus, poliovirus and a norovirus surrogate by high pressure processing. *Innovative Food Science & Emerging Technologies* **9**, 206-210.

Halász, A., Baráth, A., Simon-Sarkadi, L. & Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends in Food Science & Technology* **5**, 42-49.

Hernández, I., Barrón, L. J. R., Virto, M., Pérez-Elortondo, F. J., Flanagan, C., Rozas, U., Nájera, A. I., Albisu, M., Vicente M. S. & de Renobales, M. (2009). Lipolysis, proteolysis and sensory properties of ewe's raw milk cheese (Idiazabal) made with lipase addition. *Food Chemistry* **116**, 158-166.

Hernández-Jover, T., Izquierdo-Pulido, M., Veciana-Nogués, M. T., Mariné-Font, A. & Vidal-Carou, M. C. (1997). Biogenic amine and polyamine contents in meat and meat products. *Journal of Agricultural and Food Chemistry* **45**, 2098-2102.

Herrero-Fresno, A., Martínez, N., Sánchez-Llana, E., Díaz, M., Fernández, M., Cruz Martin, M., Ladero, V. & Alvarez, M. A. (2012). *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental model. *International Journal of Food Microbiology* **157**, 297-304.

Hill, S. J., Ganellin, C. R., Timmerman, H., Schwartz, J. C., Shankley, N. P., Young, J. M., Schunack, W., Levi, R. & Haas, H. L. (1997). International union of pharmacology .13. Classification of histamine receptors. *Pharmacological Reviews* **49**, 253-278.

Hillary, R. A. & Pegg, A. E. (2003). Decarboxylases involved in polyamine biosynthesis and their inactivation by nitric oxide. *Biochimica Et Biophysica Acta-Proteins and Proteomics* **1647**, 161-166.

Hite, B. H. (1899). The effect of pressure in the preservation of milk. *Bulletin West Virginia University Agricultural Experiment Station* **58**, 15 - 35.

Horne, D. S. & Banks, J. M. (2004). Rennet-induced Coagulation of Milk. In Cheese: Chemistry, Physics and Microbiology, pp. 47-70: Academic Press.

Hu, Y., Huang, Z. Y., Li, J. & Yang, H. (2012). Concentrations of biogenic amines in fish, squid and octopus and their changes during storage. *Food Chemistry* **135**, 2604-2611.

Huang, H. W., Lung, H. M., Yang, B. B. & Wang, C. Y. (2014). Responses of microorganisms to high hydrostatic pressure processing. *Food Control* **40**, 250-259.

Huang, J. F. & Thurmond, R. L. (2008). The new biology of histamine receptors. *Current Allergy and Asthma Reports* **8**, 21-27.

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research* **71**, 496-499.

Ismail, B. & Nielsen, S. S. (2010). Invited review: Plasmin protease in milk: Current knowledge and relevance to dairy industry. *Journal of Dairy Science* **93**, 4999-5009.

Iwańczak, M. & Wiśniewska, K. (2005). Effect of high pressures on the process of Edam cheese proteolysis. *High Pressure Research* **25**, 43-50.

Izquierdo-Pulido, M., Font-Fábregas, J., Carceller-Rosa, J. M., Mariné-Font, A. & Vidal-Carou, C. (1996a). Biogenic amine changes related to lactic acid bacteria during brewing. *Journal of Food Protection* **59**, 175-180.

Izquierdo-Pulido, M., Hernández-Jover, T., Mariné-Font, A. & Vidal-Carou, M. C. (1996b). Biogenic amines in European beers. *Journal of Agricultural and Food Chemistry* **44**, 3159-3163.

Juan, B., Barron, L. J. R., Ferragut, V., Guamis, B. & Trujillo, A. J. (2007a). Changes in the volatile composition of a semihard ewe milk cheese induced by high-pressure treatment of 300 MPa. *Journal of Agricultural and Food Chemistry* **55**, 747-754.

Juan, B., Ferragut, V., Buffa, M., Guamis, B. & Trujillo, A. J. (2007b). Effects of high pressure on proteolytic enzymes in cheese: Relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science* **90**, 2113-2125.

Juan, B., Ferragut, V., Guamis, B. & Trujillo, A. J. (2008). The effect of high-pressure treatment at 300 MPa on ripening of ewes' milk cheese. *International Dairy Journal* **18**, 129-138.

Kalač, P., Špička, J., Křížek, M., Steidlová, S. & Pelikánová, T. (1999). Concentrations of seven biogenic amines in sauerkraut. *Food Chemistry* **67**, 275-280.

Kalač, P. & Křížek, M. (2003). A review of biogenic amines and polyamines in beer. *Journal of the Institute of Brewing* **109**, 123-128.

Kalač, P. (2009). Recent advances in the research on biological roles of dietary polyamines in man. *Journal of Applied Biomedicine* **7**, 65-74.

Kamath, A. V., Vaaler, G. L. & Snell, E. E. (1991). Pyridoxal phosphate-dependent histidine descarboxylases - cloning, sequencing, and expression of genes from *Klebsiella planticola* and *Enterobacter aerogenes* and properties of the overexpressed enzymes. *Journal of Biological Chemistry* **266**, 9432-9437.

Kanki, M., Yoda, T., Ishibashi, M. & Tsukamoto, T. (2004). Photobacterium phosphoreum caused a histamine fish poisoning incident. *International Journal of Food Microbiology* **92**, 79-87.

Karovičová, J. & Kohajdová, Z. (2005). Biogenic amines in food. *Chemical Papers* **59**, 70-79.

Khwanchuea, R., Mulvany, M. J. & Jansakul, C. (2008). Cardiovascular effects of tyramine: Adrenergic and cholinergic interactions. *European Journal of Pharmacology* **579**, 308-317.

Kim, J. H., Ahn, H. J., Jo, C., Park, H. J., Chung, Y. J. & Byun, M. W. (2004). Radiolysis of biogenic amines in model system by gamma irradiation. *Food Control* **15**, 405-408.

Kim, N., Maeng, J. S. & Kim, C. T. (2013). Effects of medium high pressure treatments on protease activity. *Food Science and Biotechnology* **22**, 289-294.

Kinsella, J. E. & Hwang, D. (1976). Biosynthesis of flavors by Pe*nicillium roqueforti*. *Biotechnology and Bioengineering* **18**, 927-938.

Knoll, J., Miklya, N., Knoll, B., Markó, R. & Rácz, D. (1996). Phenylethylamine and tyramine are mixed-acting sympathomimetic amines in the brain. *Life Sciences* **58**, 2101-2114.

Koca, N., Balasubramaniam, V. M. & Harper, W. J. (2011). High-pressure effects on the microstructure, texture, and color of white-brined cheese. *Journal of Food Science* **76**, E399-E404.

Koessler, K. K., Hanke, M. T. & Sheppard, M. (1928). Production of histamine, tyramine, bronchospastic and arteriospastic substances in blood broth by pure cultures of microorganisms. *Journal of Infectious Diseases* **43**, 363-377.

Komprda, T., Smělá, D., Novická, K., Kalhotka, L., Šustová, K. & Pechová, P. (2007). Content and distribution of blogenic amines in Dutch-type hard cheese. *Food Chemistry* **102**, 129-137.

Komprda, T., Burdychová, R., Dohnal, V., Cwiková, O. & Sládková, P. (2008a). Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese. *European Food Research and Technology* **227**, 29-36.

Komprda, T., Burdychová, R., Dohnal, V., Cwiková, O., Sládková, P. & Dvořáčková, H. (2008b). Tyramine production in Dutch-type semi-hard cheese from two different producers. *Food Microbiology* **25**, 219-227.

Kumar, A., Grover, S., Sharma, J. & Batish, V. K. (2010). Chymosin and other milk coagulants: sources and biotechnological interventions. *Critical Reviews in Biotechnology* **30**, 243-258.

Kurt, S. & Zorba, O. (2009). The effects of ripening period, nitrite level and heat treatment on biogenic amine formation of "sucuk" - A Turkish dry fermented sausage. *Meat Science* **82**, 179-184.

Ladero, V., Calles-Enríquez, M., Fernández, M. & Alvarez, M. A. (2010). Toxicological effects of dietary biogenic amines. *Current Nutrition & Food Science* **6**, 145-156.

Landete, J. M., Ferrer, S., Polo, L. & Pardo, I. (2005). Biogenic amines in wines from three Spanish regions. *Journal of Agricultural and Food Chemistry* **53**, 1119-1124.

Larsen, M. D. & Jensen, K. (1999). The effects of environmental conditions on the lipolytic activity of strains of *Penicillium roqueforti*. *International Journal of Food Microbiology* **46**, 159-166.

Larsson, M., Zakora, M., Dejmek, P. & Ardö, Y. (2006). Primary proteolysis studied in a cast cheese made from microfiltered milk. *International Dairy Journal* **16**, 623-632.

Latorre-Moratalla, M. L., Bover-Cid, S., Talon, R., Aymerich, T., Garriga, M., Zanardi, E., Ianeri, A., Fraqueza, M. J., Elias, M., Drosinos, E. H., Lauková, A. & Vidal-Carou, M. C. (2010). Distribution of aminogenic activity among potential autochthonous starter cultures for dry fermented sausages. *Journal of Food Protection* **73**, 524-528.

Latorre-Moratalla, M. L., Bover-Cid, S., Veciana-Nogués, M. T. & Vidal-Carou, M. C. (2012). Control of biogenic amines in fermented sausages: role of starter cultures. *Frontiers in Microbiology* **3**, 169, pp. 1-9.

Lavizzari, T., Breccia, M., Bover-Cid, S., Vidal-Carou, M. C. & Veciana-Nogues, M. T. (2010). Histamine, cadaverine, and putrescine produced in vitro by *Enterobacteriaceae* and *Pseudomonadaceae* isolated from spinach. *Journal of Food Protection* **73**, 385-389.

Leuschner, R. G., Heidel, M. & Hammes, W. P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. *International Journal of Food Microbiology* **39**, 1-10.

Linares, D. M., Fernández, M., Martín, M. C. & Álvarez, M. A. (2009). Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration. *Microbial Biotechnology* **2**, 625-633.

Linares, D. M., Martín, M. C., Ladero, V., Álvarez, M. A. & Fernández, M. (2011). Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition* **51**, 691-703.

Linares, D. M., Del Río, B., Ladero, V., Martínez, N., Fernández, M., Martín, M. C. & Álvarez, M. A. (2012). Factors influencing biogenic amines accumulation in dairy products. *Frontiers in Microbiology* **3**, 180, pp. 1-10.

Loizzo, M. R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U. G. & Restuccia, D. (2013). Technological aspects and analytical determination of biogenic amines in cheese. *Trends in Food Science & Technology* **30**, 38-55.

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters* **199**, 9-13.

Lopez, C. (2011). Milk fat globules enveloped by their biological membrane: Unique colloidal assemblies with a specific composition and structure. *Current Opinion in Colloid & Interface Science* **16**, 391-404.

López-Sabater, E. I., Rodriguez-Jerez, J. J., Hernández-Herrero, M. & Mora-Ventura, M. T. (1994a). Evaluation of histidine decarboxilase activity of bacteria isolated from sardine (*Sardina pilchardus*) by an enzymic method. *Letters in Applied Microbiology* **19**, 70-75.

López-Sabater, E. I., Rodríguez-Jerez, J. J., Roig-Sagués, A. X. & Mora-Ventura, M. A. T. (1994b). Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of tuna handling on presence of histidine decarboxilase bacteria and histamine level. *Journal of Food Protection* **57**, 318-323.

Loret, S., Deloyer, P. & Dandrifosse, G. (2005). Levels of biogenic amines as a measure of the quality of the beer fermentation process: Data from Belgian samples. *Food Chemistry* **89**, 519-525.

Lu, Y. M., Chen, X. H., Jiang, M., Lv, X., Rahman, N., Dong, M. S. & Gujun, Y. (2009). Biogenic amines in Chinese soy sauce. *Food Control* **20**, 593-597.

Lucey, J. A. & Singh, H. (1997). Formation and physical properties of acid milk gels: a review. *Food Research International* **30**, 529-542.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Invited review: Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science* **86**, 2725-2743.

Lyte, M. (2004). The Biogenic amine tyramine modulates the adherence of *Escherichia coli* O157:H7 to intestinal mucosa. *Journal of Food Protection* **67**, 878-883.

MAGRAMA (2014). http://www.magrama.gob.es/

Maijala, R. (1994). Histamine and tyramine production by a *lactobacillus* strain subjected to external pH decrease. *Journal of Food Protection* **57**, 259-262.

Maijala, R. L., Eerola, S. H., Aho, M. A. & Hirn, J. A. (1993). The efffect of GDL-induced pH decrease on the formation of biogenic amines in meat. *Journal of Food Protection* **56**, 125-129.

Maintz, L. & Novak, N. (2007). Histamine and histamine intolerance. *American Journal of Clinical Nutrition* **85**, 1185-1196.

Malacarne, M., Martuzzi, F., Summer, A. & Mariani, P. (2002). Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *International Dairy Journal* **12**, 869-877.

Malone, A. S., Wick, C., Shellhammer, T. H. & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science* **86**, 1139-1146.

Maniou, D., Tsala, A., Moschopoulou, E., Giannoglou, M., Taoukis, P. & Moatsou, G. (2013). Effect of high-pressure-treated starter on ripening of Feta cheese. *Dairy Science & Technology* **93**, 11-20.

Marcobal, A., Martín-Álvarez, P. J., Moreno-Arribas, M. V. & Muñoz, R. (2006). A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. *Research in Microbiology* **157**, 417-424.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology* **90**, 139-159.

Marino, M., Maifreni, M., Moret, S. & Rondinini, G. (2000). The capacity of Enterobacteriaceae species to produce biogenic amines in cheese. *Letters in Applied Microbiology* **31**, 169-173.

Marino, M., Maifreni, M., Bartolomeoli, I. & Rondinini, G. (2008). Evaluation of amino acid-decarboxylative microbiota throughout the ripening of an Italian PDO cheese produced using different manufacturing practices. *Journal of Applied Microbiology* **105**, 540-549.

Martínez-Rodríguez, Y., Acosta-Muñiz, C., Olivas, G. I., Guerrero-Beltrán, J., Rodrigo-Aliaga, D. & Sepulveda, D. R. (2012). High hydrostatic pressure processing of cheese. *Comprehensive Reviews in Food Science and Food Safety* **11**, 399-416.

Martínez-Rodríguez, Y., Acosta-Muñiz, C., Olivas, G. I., Guerrero-Beltrán, J., Rodrigo-Aliaga, D., Mujica-Paz, H., Welti-Chanes, J. & Sepulveda, D. R. (2014). Effect of high hydrostatic pressure on mycelial development, spore viability and enzyme activity of *Penicillium Roqueforti*. *International Journal of Food Microbiology* **168**, 42-46.

Mayer, H. K., Fiechter, G. & Fischer, E. (2010). A new ultra-pressure liquid chromatography method for the determination of biogenic amines in cheese. *Journal of Chromatography A* **1217**, 3251-3257.

McCabe-Sellers, B. J., Staggs, C. G. & Bogle, M. L. (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: A crossroad where medicine, nutrition, pharmacy, and food industry converge. *Journal of Food Composition and Analysis* **19**, S58-S65.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening: Introduction and overview. In Cheese: Chemistry, Physics and Microbiology, pp. 347-360: Academic Press.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In Cheese: Chemistry, Physics and Microbiology, pp. 361-371: Academic Press.

McSweeney, P. L. H., Ottogalli, G. & Fox, P. F. (2004). Diversity of cheese varieties: An overview. In Cheese: Chemistry, Physics and Microbiology, pp. 1-23: Academic Press.

Medina, M. A., Urdiales, J. L., Rodríguez-Caso, C., Ramírez, F. J. & Sánchez-Jiménez, F. (2003). Biogenic amines and polyamines: Similar biochemistry for different physiological missions and biomedical applications. *Critical Reviews in Biochemistry and Molecular Biology* **38**, 23-59.

Menéndez, S., Centeno, J. A., Godínez, R. & Rodríguez-Otero, J. L. (2000). Effects of *Lactobacillus* strains on the ripening and organoleptic characteristics of Arzúa-Ulloa cheese. *International Journal of Food Microbiology* **59**, 37-46.

Morales, P., Calzada, J., Rodríguez, B., de Paz, M., Gaya, P. & Nuñez, M. (2006). Effect of cheese water activity and carbohydrate content on the barotolerance of *Listeria monocytogenes* Scott A. *Journal of Food Protection* **69**, 1328-1333.

Moreno-Arribas, V. & Lonvaud-Funel, A. (1999). Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. *FEMS Microbiology Letters* **180**, 55-60.

Moret, S., Smela, D., Populin, T. & Conte, L. S. (2005). A survey on free biogenic amine content of fresh and preserved vegetables. *Food Chemistry* **89**, 355-361.

Moschopoulou, E., Anisa, T., Katsaros, G., Taoukis, P. & Moatsou, G. (2010). Application of high-pressure treatment on ovine brined cheese: Effect on composition and microflora throughout ripening. *Innovative Food Science & Emerging Technologies* **11**, 543-550.

Mota, M. J., Lopes, R. P., Delgadillo, I. & Saraiva, J. A. (2013). Microorganisms under high pressure - Adaptation, growth and biotechnological potential. *Biotechnology Advances* **31**, 1426-1434.

Mulder, H. (1952). Taste and flavour forming substances in cheese. *Netherlands Milk and Dairy Journal* **6**, 157 - 167.

Mújica-Paz, H., Valdez-Fragoso, A., Samson, C. T., Welti-Chanes, J. & Torres, J. A. (2011). High-pressure processing technologies for the pasteurization and sterilization of foods. *Food and Bioprocess Technology* **4**, 969-985.

Naila, A., Flint, S., Fletcher, G., Bremer, P. & Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *Journal of Food Science* **75**, R139-R150.

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J. & Meerdink, G. (2011). Biogenic amines and potential histamine - Forming bacteria in Rihaakuru (a cooked fish paste). *Food Chemistry* **128**, 479-484.

Naranjo, P. (1966). Toxicity of histamine: lethal doses. In Histamine and Anti-Histaminics, pp. 179-201. Edited by M. Rocha e Silva: Springer Berlin Heidelberg.

Nguyen Thi Minh, H., Dantigny, P., Perrier-Cornet, J.-M. & Gervais, P. (2010). Germination and inactivation of *Bacillus subtilis* spores induced by moderate hydrostatic pressure. *Biotechnology and bioengineering* **107**, 876-883.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Izquierdo-Pulido, M. & Vidal-Carou, M. C. (2003). Distribution of biogenic amines and polyamines in cheese. *Journal of Food Science* **68**, 750-755.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P. & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology* **66**, 4890-4896.

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J. & Córdoba, M. D. (2013a). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control* **33**, 448-454.

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S. & Córdoba, M. D. (2013b). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of Dairy Science* **96**, 5477-5486.

Osintsev, A. M. & Qvist, K. B. (2004). Study of the mechanism of the proteolytic stage of enzymatic coagulation of milk casein. *Colloid Journal* **66**, 192-196.

Özoğul, F. (2004). Production of biogenic amines by *Morganella morganii*, *Klebsiella pneumoniae* and *Hafnia alvei* using a rapid HPLC method. *European Food Research and Technology* **219**, 465-469.

Ozturk, M., Govindasamy-Lucey, S., Jaeggi, J. J., Johnson, M. E. & Lucey, J. A. (2013). The influence of high hydrostatic pressure on regular, reduced, low and no salt added Cheddar cheese. *International Dairy Journal* **33**, 175-183.

Pachlová, V., Buňka, F., Flasarová, R., Válková, P. & Buňková, L. (2012). The effect of elevated temperature on ripening of Dutch type cheese. *Food Chemistry* **132**, 1846-1854.

Park, Y. W., Juarez, M., Ramos, M. & Haenlein, G. F. W. (2007). Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research* **68**, 88-113.

Penas, E., Frias, J., Sidro, B. & Vidal-Valverde, C. (2010). Impact of fermentation conditions and refrigerated storage on microbial quality and biogenic amine content of sauerkraut. *Food Chemistry* **123**, 143-150.

Prieto, B., Franco, I., Fresno, J. M., Bernardo, A. & Carballo, J. (2000). Picon Bejes-Tresviso blue cheese: an overall biochemical survey throughout the ripening process. *International Dairy Journal* **10**, 159-167.

Qian, M., Nelson, C. & Bloomer, S. (2002). Evaluation of fat-derived aroma compounds in Blue cheese by dynamic headspace GC/olfactometry-MS. *Journal of the American Oil Chemists Society* **79**, 663-667.

Raynal-Ljutovac, K., Lagriffoul, G., Paccard, P., Guillet, I. & Chilliard, Y. (2008). Composition of goat and sheep milk products: An update. *Small Ruminant Research* **79**, 57-72.

Recsei, P. A. & Snell, E. E. (1985). Pyruvoyl-dependent histidine decarboxylases - mechanism of cleavage of the proenzyme from *Lactobacillus buchneri*. *Journal of Biological Chemistry* **260**, 2804-2806.

Rehman, S. U., Banks, J. M., Brechany, E. Y., Muir, D. D., McSweeney, P. L. H. & Fox, P. F. (2000). Influence of ripening temperature on the volatiles profile and flavour of Cheddar cheese made from raw or pasteurised milk. *International Dairy Journal* **10**, 55-65.

Reineke, K., Mathys, A. & Knorr, D. (2011). The impact of high pressure and temperature on bacterial spores: Inactivation mechanisms of bacillus subtilis above 500 MPa. *Journal of Food Science* **76**, M189-M197.

Rendueles, E., Omer, M. K., Alvseike, O., Alonso-Calleja, C., Capita, R. & Prieto, M. (2011). Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT-Food Science and Technology* **44**, 1251-1260.

Reps, A., Kolakowski, P., Wisniewska, K. & Krasowska, M. (2003). The effect of high pressures on proteolytic enzymes in ripened cheeses. *Milchwissenschaft-Milk Science International* **58**, 138-140.

Rivalain, N., Roquain, J. & Demazeau, G. (2010). Development of high hydrostatic pressure in biosciences: Pressure effect on biological structures and potential applications in Biotechnologies. *Biotechnology Advances* **28**, 659-672.

Rodríguez-Alonso, P., Centeno, J. A. & Garabal, J. I. (2009). Comparison of the volatile profiles of Arzúa-Ulloa and Tetilla cheeses manufactured from raw and pasteurized milk. *LWT - Food Science and Technology* **42**, 1722-1728.

Roig-Sagués, A. X., Molina, A. P. & Hernández-Herrero, M. M. (2002). Histamine and tyramine-forming microorganisms in Spanish traditional cheeses. *European Food Research and Technology* **215**, 96-100.

Romero, R., Bagur, M. G., Sánchez-Viñas, M. & Gázquez, D. (2003). The influence of the brewing process on the formation of biogenic amines in beers. *Analytical and Bioanalytical Chemistry* **376**, 162-167.

Roseiro, L. B., Barbosa, M., Ames, J. M. & Wilbey, R. A. (2003). Cheesemaking with vegetable coagulants - the use of *Cynara* L. for the production of ovine milk cheeses. *International Journal of Dairy Technology* **56**, 76-85.

Ruiz-Capillas, C. & Jiménez-Colmenero, F. (2004a). Biogenic amines in meat and meat products. *Critical Reviews in Food Science and Nutrition* **44**, 489-499.

Ruiz-Capillas, C. & Jiménez-Colmenero, F. (2004b). Biogenic amine content in Spanish retail market meat products treated with protective atmosphere and high pressure. *European Food Research and Technology* **218**, 237-241.

Rynne, N. M., Beresford, T. P., Guinee, T. P., Sheehan, E., Delahunty, C. M. & Kelly, A. L. (2008). Effect of high-pressure treatment of 1 day-old full-fat Cheddar cheese on subsequent quality and ripening. *Innovative Food Science & Emerging Technologies* **9**, 429-440.

Salque, M., Bogucki, P. I., Pyzel, J., Sobkowiak-Tabaka, I., Grygiel, R., Szmyt, M. & Evershed, R. P. (2013). Earliest evidence for cheese making in the sixth millennium BC in northern Europe. *Nature* **493**, 522-525.

San Martin, M. F., Barbosa-Canovas, G. V. & Swanson, B. G. (2002). Food processing by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition* **42**, 627-645.

Sarantinopoulos, P., Kalantzopoulos, G. & Tsakalidou, E. (2001). Citrate metabolism by *Enterococcus faecalis* FAIR-E 229. *Applied and Environmental Microbiology* **67**, 5482-5487.

Savijoki, K., Ingmer, H. & Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology* **71**, 394-406.

Serrano, J., Velazquez, G., Lopetcharat, K., Ramirez, J. A. & Torres, J. A. (2005). Moderately high hydrostatic pressure processing to reduce production costs of shredded cheese: Microstructure, texture, and sensory properties of shredded milled curd cheddar. *Journal of Food Science* **70**, S286-S293.

Seyderhelm, I., Boguslawski, S., Michaelis, G. & Knorr, D. (1996). Pressure induced inactivation of selected food enzymes. *Journal of Food Science* **61**, 308-310.

Shah, M. A., Mir, S. A. & Paray, M. A. (2014). Plant proteases as milk-clotting enzymes in cheesemaking: a review. *Dairy Science & Technology* **94**, 5-16.

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Research International* **29**, 675-690.

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology* **29**, 213-231.

Smit, G., Smit, B. A. & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *Fems Microbiology Reviews* **29**, 591-610.

Sousa, M. J., Ardo, Y. & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal* **11**, 327-345.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernández, M., Lopez, P., de Palencia, P. F., Corbi, A., Trip, H. & Lolkema, J.S. (2010). Biogenic amines in fermented foods. *European Journal of Clinical Nutrition* **64**, S95-S100.

Spinnler, H. E. & Gripon, J. C. (2004). Surface mould-ripened cheeses. In Cheese: Chemistry, Physics and Microbiology, pp. 157-174: Academic Press.

Stewart, D. I., Kelly, A. L., Gulinee, T. P. & Beresford, T. P. (2006). High pressure processing: review of application to cheese manufacture and ripening. *Australian Journal of Dairy Technology* **61**, 170-178.

Stratton, J. E., Hutkins, R. W. & Taylor, S. L. (1991). Biogenic amines in cheese and other fermented foods: A review. *Journal of Food Protection* **54**, 460-470.

Sumner, S. S., Roche, F. & Taylor, S. L. (1990). Factors controlling histamine production in swiss cheese inoculated with *lactobacillus buchneri*. *Journal of Dairy Science* **73**, 3050-3058.

Suzzi, G. & Gardini, F. (2003). Biogenic amines in dry fermented sausages: a review. *International Journal of Food Microbiology* **88**, 41-54.

Tabanelli, G., Torriani, S., Rossi, F., Rizzotti, L. & Gardini, F. (2012). Effect of chemico-physical parameters on the histidine decarboxylase (HdcA) enzymatic activity in *Streptococcus thermophilus* PRI60. *Journal of Food Science* **77**, M231-M237.

ten Brink, B., Damink, C., Joosten, H. & JHJ., H. i. t. V. (1990). Occurrence and formation of biologically-active amines in foods. *International Journal of Food Microbiology* **11**, 73-84.

Til, H. P., Falke, H. E., Prinsen, M. K. & Willems, M. I. (1997). Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. *Food and Chemical Toxicology* **35**, 337-348.

Tsai, Y. H., Kung, H. D., Lee, T. M., Lin, G. T. & Hwang, D. F. (2004). Histamine-related hygienic qualities and bacteria found in popular commercial scombroid fish fillets in Taiwan. *Journal of Food Protection* **67**, 407-412.

UE 20/2010. Reglamento (UE) Nº 20/2010 de la comisión de 12 de enero de 2010 por el que se inscribe una denominación en el Registro de Denominaciones de Origen Protegidas y de Indicaciones Geográficas Protegidas [Arzúa-Ulloa (DOP)]. Diario Oficial de la Unión Europea. 13-1-2010. L8/1 – L8/2.

Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A. & Fox, P. F. (2004). Proteolysis in cheese during ripening. In Cheese: Chemistry, Physics and Microbiology, pp. 391-VIII: Academic Press.

Upadhyay, V. K., Huppertz, T., Kelly, A. L. & McSweeney, P. L. H. (2007). Use of high pressure treatment to attenuate starter bacteria for use as adjuncts for Cheddar cheese manufacture. *Innovative Food Science & Emerging Technologies* **8**, 485-492.

Veciana Nogues, M. T., Marine Font, A. & Vidal Carou, M. C. (1997). Biogenic amines in fresh and canned tuna. Effects of canning on biogenic amine contents. *Journal of Agricultural and Food Chemistry* **45**, 4324-4328.

Verissimo, P., Esteves, C., Faro, C. & Pires, E. (1995). The vegetable rennet of *Cynara cardunculus* L. contains 2-proteinases with chymosin and pepsin-like specificities. *Biotechnology Letters* **17**, 621-626.

Visciano, P., Schirone, M., Tofalo, R. & Suzzi, G. (2012). Biogenic amines in raw and processed seafood. *Frontiers in Microbiology* **3**, 188, pp. 1-10.

Visser, S. (1993). Proteolytic enzymes and their relation to cheese ripening and flavor: an overview. *Journal of Dairy Science* **76**, 329-350.

Voigt, D. D., Chevalier, F., Qian, M. C. & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavour compounds in mature blue-veined cheese. *Innovative Food Science & Emerging Technologies* **11**, 68-77.

Wallace, H. M., Fraser, A. V. & Hughes, A. (2003). A perspective of polyamine metabolism. *Biochemical Journal* **376**, 1-14.

Weemaes, C., Ludikhuyze, L., Van den Broeck, I. & Hendrickx, M. (1998). High pressure inactivation of polyphenoloxidases. *Journal of Food Science* **63**, 873-877.

Wick, C., Nienabert, U., Anggraeni, O., Shellhammer, T. H. & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial cheddar cheeses treated with high pressure. *Journal of Dairy Research* **71**, 107-115.

Wyder, M. T., Bachmann, H. P. & Puhan, Z. (1999). Role of selected yeasts in cheese ripening: An evaluation in foil wrapped Raclette cheese. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie* **32**, 333-343.

Yaldagard, M., Mortazavi, S. A. & Tabatabaie, F. (2008). The principles of ultra high pressure technology and its application in food processing/preservation: A review of microbiological and quality aspects. *African Journal of Biotechnology* **7**, 2739-2767.

Yuksel, Z., Avci, E., Uymaz, B. & Erdem, Y. K. (2012). General composition and protein surface hydrophobicity of goat, sheep and cow milk in the region of Mount Ida. *Small Ruminant Research* **106**, 137-144.

Yvon, M. & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal* **11**, 185-201.

- **Objetivos de la Tesis**

El presente trabajo se realizó en el Departamento de Tecnología de Alimentos del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), integrado en el proyecto AGL 2009-07801 "Prevención de la sobremaduración y de la formación de aminas biógenas en quesos mediante tratamientos por altas presiones" del Plan Nacional de I+D+I. Durante su realización, su autor fue beneficiario de una beca de Formación de Personal Investigador del Ministerio de Ciencia e Innovación, con referencia BES-2010-030444.

Los fundamentos de la investigación se basan en la existencia de quesos con una intensa proteolisis, debida al empleo de coagulantes que contienen enzimas de elevada actividad proteolítica o a la inoculación de mohos del género *Penicillium* productores de proteinasas extracelulares de elevada actividad proteolítica. Ello da lugar durante la conservación en refrigeración a un exceso de maduración o sobremaduración, responsable de que el producto llegue al consumidor con menor calidad de la deseada por el fabricante, lo que disminuye la aceptabilidad dentro de su vida comercial. Además, en algunos quesos, en particular si se elaboran con leche cruda, se encuentran microorganismos con actividad descarboxilasa capaces de generar niveles elevados de aminas biógenas, con efectos perjudiciales sobre la salud del consumidor.

Para controlar ambos efectos indeseables, se planteó el presente trabajo de investigación con dos **objetivos** principales: prevenir la sobremaduración de los quesos alargando su vida útil y evitar la acumulación de aminas biógenas en los quesos. El instrumento empleado para ello fue la aplicación de tratamientos de altas presiones hidrostáticas (400 y 600 MPa) a cuatro variedades de quesos elaborados en España, en diferentes momentos de su periodo de maduración.

Para poder valorar la consecución de estos objetivos, se monitorizó el efecto de los diferentes tratamientos de altas presiones, a lo largo de un periodo prolongado de conservación, sobre la proteolisis, la lipolisis, la formación de compuestos volátiles, la textura, el color, las características organolépticas y la formación de aminas biógenas en queso azul, Torta del Casar, queso Brie y queso Arzúa-Ulloa.

Capítulo 2.

Proteolysis and biogenic amine buildup in high-pressure treated ovine milk blue-veined cheese.

Fotografía: corte de queso azul (Roncari-blue).

J. Dairy Sci. 96:4816–4829
http://dx.doi.org/10.3168/jds.2012-6409

Proteolysis and biogenic amine buildup in high-pressure treated ovine milk blue-veined cheese

J. Calzada, A. Del Olmo, A. Picon, P. Gaya, and M. Nuñez[1]
Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, 28040 Madrid, Spain

ABSTRACT

Penicillium roqueforti plays an important role in the ripening of blue-veined cheeses, mostly due to lactic acid consumption and to its extracellular enzymes. The strong activity of *P. roqueforti* proteinases may bring about cheese over-ripening. Also, free amino acids at high concentrations serve as substrates for biogenic amine formation. Both facts result in shorter product shelf-life. To prevent over-ripening and buildup of biogenic amines, blue-veined cheeses made from pasteurized ovine milk were high-pressure treated at 400 or 600 MPa after 3, 6, or 9 wk of ripening. Primary and secondary proteolysis, biogenic amines, and sensory characteristics of pressurized and control cheeses were monitored for a 90-d ripening period, followed by a 270-d refrigerated storage period. On d 90, treatments at 400 MPa had lowered counts of lactic acid bacteria and *P. roqueforti* by less than 2 log units, whereas treatments at 600 MPa had reduced lactic acid bacteria counts by more than 4 log units and *P. roqueforti* counts by more than 6 log units. No residual α-casein (CN) or κ-CN were detected in control cheese on d 90. Concentrations of β-CN, para-κ-CN, and γ-CN were generally higher in 600 MPa cheeses than in the rest. From d 90 onwards, hydrophilic peptides were at similar levels in pressurized and control cheeses, but hydrophobic peptides and the hydrophobic-to-hydrophilic peptide ratio were at higher levels in pressurized cheeses than in control cheese. Aminopeptidase activity, overall proteolysis, and free amino acid contents were generally higher in control cheese than in pressurized cheeses, particularly if treated at 600 MPa. Tyramine concentration was lower in pressurized cheeses, but tryptamine, phenylethylamine, and putrescine contents were higher in some of the pressurized cheeses than in control cheese. Differences in sensory characteristics between pressurized and control cheeses were generally negligible, with the only exception of treatment at high pressure level (600 MPa) at an early ripening stage (3 wk), which affected biochemical changes and sensory characteristics.
Key words: blue cheese, high pressure, biogenic amine, proteolysis

INTRODUCTION

Indigenous milk enzymes, coagulant enzymes, lactic acid bacteria (**LAB**) from starter cultures, and other added or contaminating microorganisms and their enzymes degrade milk proteins to peptides and free AA during cheese manufacture and ripening (Visser, 1993). Peptides contribute to cheese flavor, in most cases positively, although high levels of some hydrophobic peptides have been associated with cheese bitterness (Gomez et al., 1997). Free AA are directly involved in cheese taste and serve also as precursors of compounds responsible for flavor and aroma (Yvon and Rijnen, 2001). In blue-veined cheeses, *Penicillium roqueforti* is a major proteolytic agent, indirectly because of lactic acid consumption, which brings about a rise in cheese pH favorable for many chemical reactions, and directly due to its highly active extracellular proteinases (Gripon et al., 1977).

As ripening continues during storage, distribution, and retail, the cheese purchased by the consumer may be of a stronger or different flavor than the manufacturer intended (Wick et al., 2004). Cheeses suffering extensive proteolysis are particularly prone to undesirable effects such as over-ripening associated to off-flavor development, which bring about shorter product shelf-life. In addition, the free AA generated at high levels in strongly proteolyzed cheeses may serve as substrates for bacterial decarboxylases, resulting in the accumulation of biogenic amines (**BA**), a group of compounds of notable public health significance (Silla Santos, 1996). Tyramine is a potent vasoconstrictor with an effect on healthy individuals usually limited to headache or migraine, whereas histamine may cause urticaria, hypotension, headache, flushing, and abdominal cramps (Taylor, 1986; Til et al., 1997). In spite of the wide consensus on their toxicity, acceptable levels of BA in foods have not been established in most countries, partly because of the variable sensitivity of individuals

Received November 23, 2012.
Accepted April 10, 2013.
[1]Corresponding author: nunez@inia.es

to these compounds, which may be affected by factors such as alcohol consumption. Concentrations of 100 to 800 mg of tyramine per kilogram and 30 mg of phenylethylamine per kilogram have been suggested as toxic (ten Brink et al., 1990), but only an upper limit of 100 mg of histamine per kilogram has been set for fish and fish products.

Monoamines tyramine, phenylethylamine, histamine, and tryptamine are respectively formed through the decarboxylation of tyrosine, phenylalanine, histidine, and tryptophan, whereas diamines cadaverine and putrescine derive from lysine and ornithine or arginine via agmatine (Silla Santos, 1996). Enterococci are generally considered the main tyramine formers and heterofermentative lactobacilli the main producers of histamine, but other cheese-borne bacteria may be also involved in BA formation in cheese (Edwards and Sandine, 1981; Joosten and Northolt, 1987; Pircher et al., 2007).

Even though milk hygienization procedures, such as bactofugation and pasteurization, lower the levels of decarboxylase-positive bacteria, postpasteurization contamination of milk and curd by adventitious bacteria-harboring AA decarboxylases, and their subsequent growth and metabolic activity during cheese ripening, usually results in BA buildup. High-pressure (**HP**) treatments efficiently inactivate microbial contaminants in milk and cheese (O'Reilly et al., 2000; Arqués et al., 2006). They offer the advantage of application after cheese manufacture, when the contamination of the cheese interior is no longer possible, with the only exception being blue-veined cheeses, which are pierced some days after manufacture. The effect of HP treatments on the characteristics of cheeses made from bovine milk (O'Reilly et al., 2003; Evert-Arriagada et al., 2012), ovine milk (Arqués et al., 2007; Juan et al., 2007), and caprine milk (Saldo et al., 2002; Delgado et al., 2012) has been investigated. Pressurization may also be useful to impede BA formation during cheese ripening. To our knowledge, HP treatments with this objective have been assayed only on a pasteurized goat milk cheese, but BA contents of control cheese in the experiment were so low that it was not possible to establish differences with respect to the pressurized cheese (Novella-Rodríguez et al., 2002).

The effect of HP treatments on the chemical characteristics of blue-veined cheeses has been studied only on an Irish cow milk blue cheese, which was pressurized on d 42 of ripening and stored afterward at 4°C for 28 d (Voigt et al., 2010). However, no information is available on the effect of HP treatments on BA formation in blue-veined cheeses. Moreover, the effect of pressurization on the characteristics of ovine milk blue-veined cheeses, which include reputed varieties such as Roquefort, has not been studied. The objective of the present work was to investigate the effect of HP treatments at 400 or 600 MPa, applied after 3, 6 or 9 wk of ripening, on the primary and secondary proteolysis, the formation of BA, and the sensory characteristics of ovine milk blue-veined cheese.

MATERIALS AND METHODS

Cheese-Making and HP Treatments

Pasteurized (73°C for 16 s) ovine milk was used for the manufacture of blue-veined cheese in duplicate trials carried out on consecutive days. Lactic cultures (Flora Danica, 120 g; Chr. Hansen, Tres Cantos, Spain), *P. roqueforti* (Choozit PA, 1 dose; Danisco, Sassenage, France), and $CaCl_2$ (120 g, in aqueous solution) were added to milk. Milk (1,200 L) was coagulated at 32°C for 35 min with animal rennet (Naturen Premium, 200 mL; Chr. Hansen). Curd was cut into 1.5-cm cubes and held at 32°C for 20 min. Whey was drained out and the nonpressed curd was distributed into cylindrical molds. Cheeses, 18 cm in diameter and 10 cm high, were turned over 5 times while held at 21°C for 24 h, followed by another 48 h at 15°C. Afterward, they were salted by rubbing dry salt onto all the surfaces and pierced from the 2 flat surfaces. Ripening took place at 10°C and 93% relative humidity until d 30 and at 5°C from d 30 to d 90. Refrigerated storage at 3°C lasted until d 360.

Cheeses from each trial were pressurized for 5 min at 400 or 600 MPa after ripening for 3, 6 or 9 wk and coded as 400W3, 600W3, 400W6, 600W6, 400W9, and 600W9, respectively. Before HP treatments, cheeses were vacuum-packaged in CN300 bags (Cryovac Grace S.A., Barcelona, Spain). A 120-L capacity isostatic press (NC Hyperbaric, Burgos, Spain) was used for HP treatments. Come-up times to reach 400 and 600 MPa were 1.82 and 2.65 min and depressurization times were 6 and 8 s, respectively. Temperature of the water used as transmitting fluid remained under 13°C during the process. After HP treatments cheeses were unpackaged, ripening and storage proceeded under the same conditions as for control cheese. A total of 44 cheeses per trial (1 per treatment and sampling date) were used for analytical determinations.

Microbiological Analyses

Representative cheese samples (10 g) were homogenized with 90 mL of a sterile 2% (wt/vol) sodium citrate solution at 45°C in a Colworth Stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions of samples were prepared in sterile 0.1% peptone

solution. Total viable counts, LAB, enterococci, and lactobacilli were determined in duplicate on plates of PCA (Biolife, Milano, Italy) incubated for 48 h at 30°C, M17 agar (acidified at pH 5.7 with acetic acid; Biolife) incubated for 48 h at 30°C, KF Streptococcus agar (Oxoid, Basingstoke, UK) incubated for 48 h at 37°C, and Rogosa agar (acidified at pH 5.4 with acetic acid; Biolife) incubated anaerobically for 48 h at 37°C, respectively. *Micrococcaceae* were determined in duplicate on plates of mannitol salt agar (Oxoid) incubated for 72 h at 30°C, coagulase-positive staphylococci were determined on Baird-Parker agar (Oxoid) with RPF Supplement II (Biolife) incubated at 37°C for 48 h, gram-negative bacteria were determined on McConkey agar (Biolife) incubated for 24 h at 30°C, and coliforms were determined on VRBA agar (Oxoid) incubated for 24 h at 37°C. Molds and yeasts were determined in duplicate on plates of Chloramphenicol Glucose Agar (Scharlab, Barcelona, Spain) incubated for 72 h at 25°C. Microbial counts were expressed in log cfu per gram of cheese.

Physicochemical and Enzymatic Determinations

Proteins were analyzed by capillary gel electrophoresis according to a previously described method (Garde et al., 2002), with some modifications, on an automated P/ACE MDQ capillary electrophoresis apparatus controlled by a 32 Karat Software (Beckman Instruments España S.A., Madrid, Spain). Briefly, 5 g of cheese were mixed with 25 mL of 2% sodium citrate solution and homogenized for 1 min in an Ultra-Turrax T-10 blender (IKA, Staufen, Germany) at high speed on ice. Twenty microliters of cheese homogenate was mixed with 170 μL of 100 m*M* Tris-HCl buffer (pH 9.0) containing 1% SDS, 10 μL of 2-mercaptoethanol, and 4 μL of a 10 kDa internal standard (Beckman Instruments España S.A.), and heated at 95°C for 10 min before injection at 5 kV. Electrophoretic separation was performed at 15 kV for 30 min after a 4-min ramp in a bare-fused silica capillary column (Beckman Instruments España S.A.) of 50 μm internal diameter and 30 cm total length, in SDS-buffer gel (Beckman Instruments España S.A.). To calculate the molecular weight of peaks monitored at 214 nm, the coefficient of relative time mobility to the internal standard was compared with those of a mixture of 10, 20, 35, 50, 100, 150, and 225 kDa protein standards (SDS-molecular weight protein size standard; Beckman Instruments España S.A.). Commercial standards (Sigma-Aldrich, Alcobendas, Spain) of bovine α-CN, β-CN, κ-CN, α-LA, β-LG, serum albumin, and lactoferrin were used for the identification of proteins. Protein peaks were quantified with respect to the internal standard area and expressed as milligrams of protein per grams of cheese DM.

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by reverse-phase HPLC according to a previously described method (Lau et al., 1991), using a Beckman System Gold chromatograph (Beckman Instruments España S.A.) equipped with a diode array detector module 168 with detection wavelength at 280 nm. Peaks with retention times from 5.5 to 14.6 min were considered to correspond to hydrophilic peptides and those with retention times from 14.6 to 20.5 min to hydrophobic peptides. Results were expressed in arbitrary units (**AU**), calculated as units of chromatogram area per milligram of cheese DM.

Free AA and BA were simultaneously extracted from duplicate samples according to a previously described method (Krause et al., 1995). Analysis of individual free AA was carried out by reverse-phase HPLC after derivatization with Waters AccQ Fluor Reagent, using a Waters AccQ Tag (Waters, Milford, MA) column. Quantitative analysis of individual BA after derivatization with dabsyl chloride was carried out by reverse-phase HPLC using a System Gold HPLC apparatus (Beckman Coulter, Palo Alto, CA) equipped with a Nova-pack C18 column (Waters). A standard mixture of BA (Sigma-Aldrich) was used for their identification and quantification.

Aminopeptidase activity released into the cheese was measured on duplicate samples of an extract obtained by homogenizing 10 g of cheese with 20 mL of 100 m*M* sodium phosphate buffer, pH 7, at 20°C for 2 min in an Ultra-Turrax T-10 blender, followed by centrifuging (10,000 × *g*, 20 min, 4°C) and filtering through Whatman No. 2 paper (GE Healthcare UK Ltd., Buckinghamshire, UK). Lysine *p*-nitroanilide (Lys-*p*-NA) and leucine *p*-nitroanilide (Leu-*p*-NA) were used as substrates. One activity unit corresponds to the activity of enzyme (s) producing 1 nmol of *p*-nitroaniline per minute per gram of cheese.

Overall proteolysis determined by the *o*-phthaldialdehyde test was analyzed in duplicate, as previously described (Church et al., 1983), and expressed as the absorbance at 340 nm. Cheese DM was determined in triplicate after cheese grinding with sand by drying to a constant weight in an oven at 102°C. Cheese pH was measured in triplicate directly by means of a Crison penetration electrode (model 52–3,2; Crison Instruments S.A., Barcelona, Spain) coupled to a Crison GPL 22 pH-meter. A cheese sag index was defined and calculated as the percentage of sagging in the central point of the flat surface with respect to the height at the edge of the cheese.

Sensory Evaluation

Fifteen trained panelists evaluated the cheeses on d 90, 180, 240, and 360 for flavor quality (overall acceptance), flavor intensity (overall intensity), and the flavor attributes acid, bitter, salty, sweet, and umami on a 0- to 10-point scale, using a horizontal line anchored in the middle and at both ends, as previously described (Garde et al., 2006). Cheeses were cut in representative triangular slices (15–20 g), which were held for 2 h at 20 to 22°C before sensory evaluation. Four cheeses per session (1 control and 3 experimental cheeses, manufactured on the same day), coded with random 3-digit numbers, were randomly presented to panelists. Bread and water were used as rinsing agents between cheeses.

Statistical Treatment

An ANOVA with HP treatment (6 treatments and control) and ripening time as main effects was performed on the analytical variables by means of SPSS Win 14.0 program (SPSS Inc., Chicago, IL). Calculation of correlations and comparison of means by Tukey's test, with the significance assigned at $P < 0.05$, were carried out using the same program.

RESULTS AND DISCUSSION

Microbial Groups

Total viable counts and LAB were significantly ($P < 0.05$) affected by HP treatments. Both the pressure level and, at a lesser degree, the ripening time at pressurization influenced mortality rates, which were almost coincident for total viable counts and LAB, an expected result given that LAB were the predominant microbial group. Counts of LAB, which reached a maximum of 9.54 log cfu/g on d 1, decreased by 1.06, 4.69, 0.43, 3.98, 0.12, and 3.99 log units in 400W3, 600W3, 400W6, 600W6, 400W9, and 600W9 cheeses, respectively, immediately after pressurization with respect to control cheese (data not shown). Counts of LAB recovered by approximately 0.5 log units after treatment at 400 MPa, but no recovery was observed in 600 MPa cheeses during the rest of the ripening period. In pressurized Irish blue-veined cheese, counts of lactococci declined by 1.6 log units at 400 MPa and recovered by up to 1.9 log units during storage, whereas at 600 MPa they declined by 1.9 log units and did not recover during storage (Voigt et al., 2010). In the present work, LAB counts declined during refrigerated storage, from d 90 to 360, in control, 400W6, and 400W9 cheeses, and remained fairly constant in the rest of the cheeses (Table 1), in which the mortality caused by HP treatments had been more marked.

Penicillium roqueforti counts reached 7.73, 7.92, and 7.74 log cfu/g on d 21, 42, and 63, respectively. Its population declined by 5.33, 0.74, and 0.71 log units following HP treatment at 400 MPa after 3, 6, and 9 wk of ripening, respectively, and fell below detection level immediately after HP treatment in all 600 MPa cheeses, independently of the day of pressurization (data not shown). Decreases reported for *P. roqueforti* in Irish blue-veined cheese pressurized on d 42 at 400 and 600 MPa were 2.16 and 2.68 log units, respectively (Voigt et al., 2010). The high *P. roqueforti* mortality observed in the present work for HP treatment on d 21 may be ascribed to the physiological status of the strain at that time, with profuse mycelium growth but still with a low level of spores, presumably more baroresistant forms. Underestimation of survivors, in particular of sublethally injured cells when plated on a selective agar, can be also responsible for the low *P. roqueforti* count in 400W3 cheese immediately after treatment on d 21. The differences in *P. roqueforti* mortality caused by HP treatments on d 42 in both works can be associated to the use of different strains in cheese manufacture. From d 90 onwards, *P. roqueforti* declined gradually in control and 400 MPa cheeses and was not detected in any of the 600 MPa cheeses (Table 1). The population of non-*Penicillium* fungi, mostly *Geotrichum* and yeasts, reached a maximum of 7.07 log cfu/g on d 42, and was lowered by 2.91, 1.73, and 0.32 log units in 400W3, 400W6, and 400W9 cheeses, respectively, immediately after pressurization with respect to control cheese (data not shown). Similarly to *P. roqueforti*, these microorganisms were not detected on d 90 in 600 MPa cheeses. From d 90 onwards, non-*Penicillium* fungi declined gradually in control cheese and were occasionally detected in pressurized cheeses (Table 1). Yeast counts were not lowered by pressurization in Irish blue-veined cheese treated at 400 MPa, and declined by 2.10 log units in cheese treated at 600 MPa (Voigt et al., 2010).

Lactobacilli and enterococci counts were below 5 log cfu/g in all cheeses from d 1 to 90. They remained below 5 log cfu/g in control cheese and in cheeses treated at 400 MPa from d 90 to 360, and were only occasionally detected in 600 MPa cheeses, at levels below 4 log cfu/g (data not shown). In Irish blue-veined cheese, counts of nonstarter LAB and enterococci were below 6 log cfu/g in control and 400 MPa cheeses, and below 4 log cfu/g in 600 MPa cheeses (Voigt et al., 2010). In the present study, *Micrococcaceae*, staphylococci, gram-negative bacteria, and coliforms were detected occasionally, at counts below 3 log cfu/g (data not shown).

Table 1. Counts[1] of the main microbial groups in control ovine milk blue cheese and pressurized cheeses

Microbial group	Days	Control cheese	400W3[2]	600W3[2]	400W6[2]	600W6[2]	400W9[2]	600W9[2]
Lactic acid bacteria	90	8.24 ± 0.02^{c}	6.34 ± 0.59^{b}	3.58 ± 0.39^{a}	7.98 ± 0.23^{c}	4.07 ± 0.14^{a}	7.74 ± 0.03^{c}	4.08 ± 0.27^{a}
	180	8.02 ± 0.03^{d}	6.70 ± 0.14^{b}	3.75 ± 0.04^{a}	7.46 ± 0.10^{c}	3.49 ± 0.04^{a}	7.07 ± 0.05^{bc}	3.31 ± 0.31^{a}
	270	7.64 ± 0.06^{d}	6.71 ± 0.22^{c}	4.04 ± 0.12^{b}	6.31 ± 0.30^{c}	3.15 ± 0.13^{a}	6.60 ± 0.04^{c}	3.86 ± 0.16^{ab}
	360	6.57 ± 0.13^{c}	6.41 ± 0.32^{c}	3.21 ± 0.70^{a}	5.63 ± 0.02^{b}	3.33 ± 0.32^{a}	5.45 ± 0.17^{b}	3.67 ± 0.06^{a}
Penicillium roqueforti	90	7.53 ± 0.10^{c}	5.96 ± 0.06^{b}	ND[3a]	5.80 ± 0.67^{b}	ND^{a}	5.93 ± 0.04^{b}	ND^{a}
	180	7.10 ± 0.04^{d}	4.36 ± 0.78^{b}	ND^{a}	5.94 ± 0.33^{c}	ND^{a}	5.77 ± 0.07^{c}	ND^{a}
	270	6.56 ± 0.23^{d}	3.24 ± 0.72^{b}	ND^{a}	5.61 ± 0.22^{c}	ND^{a}	5.07 ± 0.47^{c}	ND^{a}
	360	5.66 ± 0.08^{c}	2.79 ± 0.46^{b}	ND^{a}	3.38 ± 0.31^{b}	ND^{a}	2.61 ± 0.36^{b}	ND^{a}
Non-*Penicillium* fungi	90	5.19 ± 0.76^{c}	4.11 ± 0.47^{c}	ND^{a}	4.71 ± 0.22^{c}	ND^{a}	2.73 ± 0.42^{b}	ND^{a}
	180	4.02 ± 0.45^{b}	3.65 ± 0.38^{b}	3.14 ± 0.36^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	270	3.49 ± 0.60^{bc}	4.05 ± 1.19^{c}	2.48 ± 0.28^{b}	ND^{a}	ND^{a}	3.13 ± 0.66^{bc}	ND^{a}
	360	ND^{a}	3.64 ± 0.46^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	2.73 ± 0.65^{b}

$^{a–d}$Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Microbial counts are expressed as log colony-forming units per gram of cheese.

[2]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[3]ND = not detected.

Table 2. Values of pH, DM, and sag index in control ovine milk blue cheese and pressurized cheeses

Item	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Cheese pH[2]	90	5.86 ± 0.06^{b}	5.29 ± 0.04^{a}	5.24 ± 0.06^{a}	5.62 ± 0.05^{b}	5.80 ± 0.06^{b}	5.67 ± 0.04^{b}	5.70 ± 0.04^{b}
	180	5.47 ± 0.02^{c}	5.09 ± 0.02^{a}	5.01 ± 0.01^{a}	5.38 ± 0.02^{bc}	5.43 ± 0.02^{bc}	5.35 ± 0.02^{b}	5.35 ± 0.03^{b}
	270	5.26 ± 0.01^{cd}	5.11 ± 0.04^{b}	4.94 ± 0.01^{a}	5.23 ± 0.02^{c}	5.19 ± 0.03^{bc}	5.34 ± 0.03^{d}	5.27 ± 0.02^{cd}
	360	5.34 ± 0.01^{c}	5.15 ± 0.04^{b}	5.06 ± 0.02^{a}	5.36 ± 0.02^{c}	5.38 ± 0.02^{c}	5.34 ± 0.01^{c}	5.40 ± 0.01^{c}
DM (%)[2]	90	53.95 ± 0.54^{a}	53.27 ± 0.75^{a}	53.86 ± 0.50^{a}	52.06 ± 0.74^{a}	54.46 ± 0.42^{a}	53.67 ± 0.31^{a}	52.75 ± 1.13^{a}
	180	54.43 ± 0.36^{ab}	52.95 ± 0.47^{a}	52.98 ± 0.30^{a}	53.39 ± 1.03^{ab}	54.61 ± 0.70^{ab}	54.85 ± 0.78^{ab}	55.05 ± 0.43^{b}
	270	53.16 ± 0.69^{a}	54.40 ± 0.61^{a}	55.08 ± 0.19^{a}	54.10 ± 0.76^{a}	53.59 ± 0.84^{a}	55.23 ± 0.44^{a}	53.99 ± 0.80^{a}
	360	53.35 ± 0.27^{ab}	53.60 ± 0.79^{ab}	55.16 ± 0.57^{b}	54.49 ± 1.16^{ab}	52.96 ± 0.28^{a}	54.54 ± 0.73^{ab}	54.46 ± 0.46^{ab}
Sag index[2,3]	90	15.04 ± 1.08^{a}	36.24 ± 1.43^{c}	34.21 ± 0.35^{c}	27.16 ± 1.63^{b}	25.49 ± 0.74^{b}	30.25 ± 1.99^{bc}	29.22 ± 0.47^{bc}
	180	13.51 ± 0.49^{a}	32.15 ± 0.52^{d}	29.45 ± 1.27^{cd}	26.10 ± 0.78^{bc}	21.62 ± 1.72^{b}	23.92 ± 1.16^{bc}	30.09 ± 0.66^{cd}
	270	14.84 ± 0.80^{a}	31.94 ± 0.73^{c}	28.30 ± 1.07^{bc}	23.28 ± 0.78^{b}	22.92 ± 1.49^{b}	28.03 ± 1.35^{bc}	27.96 ± 1.07^{bc}
	360	15.14 ± 0.56^{a}	31.78 ± 1.75^{d}	30.49 ± 1.54^{cd}	24.06 ± 1.46^{b}	21.68 ± 2.94^{b}	24.61 ± 1.06^{bc}	23.23 ± 1.55^{b}

$^{a–d}$Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE (n = 6) of triplicate determinations in 2 cheese-making experiments.

[3]Cheese sag index was calculated as 100 − (100 height at the central point/height at the edge).

Cheese pH, DM, and Sag Index

Control cheese pH, which had declined to 4.76 on d 1, fell further to 4.63 by d 7, and rose afterward gradually to values of 5.65 on d 21, 6.10 on d 42, and 6.24 on d 63 (data not shown), most likely because of lactic acid consumption by molds and yeasts. This pH pattern is similar to that recorded for Stilton cheese (Madkor et al., 1987). In pressurized cheeses, pH values immediately after HP treatments were similar to those recorded for the respective control cheeses, with differences always under 0.2 pH units. However, the pH evolved differently during ripening of 400W3 and 600W3 cheeses than in the rest, with significantly ($P < 0.05$) lower values on d 90 in cheeses pressurized after 3 wk of ripening, most probably because of impaired lactic acid consumption by damaged *P. roqueforti* cells (Table 2). Dry matter of control cheese, which was 46.63% on d 1, increased to 50.73 and 52.97% on d 21 and 63 (data not shown), respectively, and to 53.95% on d 90 (Table 2). During refrigerated storage, DM remained fairly constant, with differences between cheeses under 3% at all sampling times.

The sag index, measuring the subsiding at the central point of the flat surface with respect to height at the edge, was significantly ($P < 0.05$) higher in all the pressurized cheeses, independently of the pressure level or the ripening time at pressurization, than in control cheese (Table 2). The sag index of control cheese did not vary during refrigerated storage, whereas gradual shape recovery took place in all the pressurized cheeses from d 90 to 360, with increases in the sag index ranging from 3.1 to 6.0%.

Proteolysis

Changes in the concentration of proteins from milk to 1-d-old cheese can be explained by losses during whey drainage and by enzymatic hydrolysis. Losses in whey were responsible for the decline observed in the contents of the water-soluble proteins α-LA, β-LG, and serum albumin from milk to 1-d-old cheese (Table 3). In the absence of proteolysis, contents of α-CN, β-CN, and κ-CN should have increased from milk to 1-d-old cheese, due to the retention and concentration of CN micelles in the curd. However, because of the activity of rennet and starter proteinases the contents of α-CN, β-CN, and κ-CN referred to DM declined by 65, 11, and 71% (Table 3), respectively, from milk to 1-d-old cheese. On d 90, α-CN and κ-CN were no longer detectable in control cheese, whereas β-CN was 99% lower and para-κ-CN 79% lower than on d 1. A more rapid hydrolysis of α-CN than of β-CN was also recorded for Stilton cheese (Madkor et al., 1987). The only whey protein detected on d 90 in control cheese was β-LG, at a level 99% lower than on d 1 (Table 3). Considerably higher levels of residual α-CN and β-CN are found in non-*Penicillium* ripened cheeses (Gomez et al., 1999). In addition to the rennet and starter LAB proteinases acting in most cheese varieties, *P. roqueforti* proteinases (Modler et al., 1974; Trieu-Cuot et al., 1982) were most likely responsible for the intense proteolysis observed during ripening of blue-veined cheese in the present work. During the refrigerated storage period proteolysis still progressed. Thus, β-CN and para-κ-CN declined from d 90 to 360 in control cheese by 96 and 80%, respectively, and γ-CN, the degradation products resulting from primary proteolysis, by 81% (Table 4). From d 90 onwards, the highest levels of β-CN, para-κ-CN, and γ-CN were generally found in the 600W3 cheese. Protein concentrations in all cheeses during refrigerated storage were at such low levels, with the exception of γ-CN, that a significant contribution of residual proteins to the formation of peptides and free AA at this stage is to be precluded. A less pronounced proteolysis, as shown by the lower pH 4.6- and TCA-soluble N contents, was recorded for Irish blue-veined cheese pressurized at 600 MPa than for control cheese or for 400 MPa cheese (Voigt et al., 2010). The lower proteolysis in cheeses treated at 600 MPa can be ascribed to a lower activity of chymosin and, probably, of *P. roqueforti* proteinases. Chymosin is partially inactivated by pressurization at 400 MPa or higher pressures, but plasmin, a more baroresistant enzyme, maintains full activity after pressurization at 500 or 600 MPa (Malone et al., 2003; Juan et al., 2007). To our knowledge, no information is available on the barotolerance of *P. roqueforti* proteinases.

Hydrophilic peptides increased gradually during the ripening of control cheese, with levels attaining 6.79, 24.51, 25.78, and 27.10 AU respectively on d 1, 21, 42, and 63, whereas the levels of hydrophobic peptides reached a maximum of 7.48 AU on d 21 (data not shown) and declined thereafter to 5.66 AU on d 90. Both hydrophilic peptides and hydrophobic peptides suffered minor variations from d 90 to 360 (Table 5). In the case of hydrophilic peptides, the differences between control and pressurized cheeses at the same sampling time were generally not significant. However, hydrophobic peptides were generally found at higher levels in pressurized cheeses than in the respective control cheese. Consequently, the hydrophobic peptides-to-hydrophilic peptides ratio, which has been reported to correlate well with cheese bitterness (Gomez et al., 1997), was lower in control cheese than in most of the pressurized cheeses (Table 5). The highest levels of hydrophobic peptides and of the peptide ratio from d 90 to 360 were generally found for the 600W3 cheese. Contrary to

Table 3. Main proteins[1] in milk and control ovine milk blue cheese during ripening

Days	α-CN	β-CN	κ-CN	para-κ-CN	γ-CN	α-LA	β-LG	Serum albumin
Milk	59.52 ± 2.95	205.47 ± 13.21	15.23 ± 1.34	ND[2]	7.97 ± 1.26	2.19 ± 0.10	49.15 ± 2.65	5.54 ± 0.67
1	20.76 ± 3.69^{c}	183.67 ± 19.42^{c}	4.36 ± 0.36^{c}	28.35 ± 2.34^{c}	7.95 ± 1.02^{a}	0.87 ± 0.08^{b}	7.37 ± 0.79^{b}	0.80 ± 0.15^{c}
21	1.49 ± 0.48^{b}	25.05 ± 2.44^{b}	0.95 ± 0.25^{b}	15.25 ± 1.46^{b}	44.87 ± 2.80^{c}	ND^{a}	0.21 ± 0.03^{a}	0.13 ± 0.08^{b}
42	1.12 ± 0.21^{b}	13.38 ± 1.72^{ab}	0.43 ± 0.13^{b}	9.64 ± 1.14^{ab}	32.05 ± 2.40^{bc}	ND^{a}	0.15 ± 0.06^{a}	0.05 ± 0.03^{b}
63	0.54 ± 0.19^{b}	6.20 ± 1.38^{a}	0.02 ± 0.01^{b}	8.22 ± 0.96^{ab}	30.05 ± 3.06^{b}	ND^{a}	0.18 ± 0.07^{a}	ND^{a}
90	ND^{a}	1.22 ± 0.35^{a}	ND^{a}	5.94 ± 0.82^{a}	19.60 ± 4.21^{ab}	ND^{a}	0.07 ± 0.03^{a}	ND^{a}

$^{a–c}$Means for cheese samples on the same column followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Proteins are expressed in milligrams per gram of milk or cheese DM.

[2]ND = not detected.

Table 4. Main caseins[1] in control ovine milk blue cheese and pressurized cheeses

Casein	Days	Control cheese	400W3[2]	600W3[2]	400W6[2]	600W6[2]	400W9[2]	600W9[2]
β-CN	90	1.22 ± 0.35^{a}	3.58 ± 0.84^{b}	3.22 ± 0.76^{ab}	0.63 ± 0.24^{a}	2.40 ± 0.12^{ab}	0.59 ± 0.21^{a}	0.71 ± 0.14^{a}
	180	0.80 ± 0.22^{ab}	1.20 ± 0.20^{ab}	1.98 ± 0.30^{b}	0.62 ± 0.17^{ab}	0.66 ± 0.19^{ab}	0.33 ± 0.09^{a}	0.39 ± 0.17^{a}
	270	0.07 ± 0.05^{a}	0.30 ± 0.11^{ab}	0.85 ± 0.26^{b}	0.16 ± 0.02^{a}	0.35 ± 0.09^{ab}	0.09 ± 0.04^{a}	0.15 ± 0.05^{a}
	360	0.05 ± 0.03^{a}	0.13 ± 0.06^{ab}	0.17 ± 0.04^{ab}	0.16 ± 0.07^{ab}	0.32 ± 0.09^{b}	0.07 ± 0.03^{ab}	0.09 ± 0.02^{a}
para-κ-CN	90	5.94 ± 0.82^{a}	5.71 ± 1.12^{a}	6.41 ± 0.77^{a}	3.15 ± 0.62^{a}	6.00 ± 0.68^{a}	3.68 ± 0.83^{a}	4.84 ± 0.30^{a}
	180	3.50 ± 0.95^{ab}	2.94 ± 0.31^{ab}	4.54 ± 0.65^{b}	2.56 ± 0.29^{ab}	3.97 ± 0.47^{ab}	1.65 ± 0.49^{a}	2.60 ± 0.40^{ab}
	270	1.28 ± 0.22^{ab}	1.43 ± 0.18^{abc}	2.95 ± 0.62^{c}	0.60 ± 0.18^{a}	2.29 ± 0.29^{bc}	0.91 ± 0.28^{ab}	1.71 ± 0.61^{abc}
	360	1.16 ± 0.24^{ab}	1.31 ± 0.29^{ab}	2.01 ± 0.35^{b}	0.75 ± 0.16^{a}	1.21 ± 0.32^{ab}	0.60 ± 0.11^{a}	1.25 ± 0.08^{ab}
γ-CN	90	19.60 ± 3.21^{ab}	14.41 ± 2.81^{ab}	17.24 ± 2.46^{ab}	9.99 ± 1.93^{a}	22.08 ± 2.09^{b}	11.90 ± 2.18^{a}	12.31 ± 0.37^{ab}
	180	12.87 ± 2.92^{ab}	8.95 ± 0.53^{ab}	14.17 ± 1.97^{b}	10.10 ± 1.49^{ab}	13.14 ± 0.38^{ab}	5.92 ± 1.26^{a}	8.37 ± 1.13^{ab}
	270	4.66 ± 0.97^{ab}	5.50 ± 0.86^{ab}	10.42 ± 1.97^{c}	2.82 ± 0.67^{a}	7.54 ± 0.75^{bc}	3.82 ± 0.52^{ab}	4.80 ± 1.23^{ab}
	360	3.77 ± 0.48^{ab}	3.85 ± 1.09^{ab}	6.03 ± 0.98^{b}	2.56 ± 0.29^{a}	4.80 ± 0.70^{ab}	2.79 ± 0.25^{a}	5.18 ± 0.19^{ab}

$^{a–c}$Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Caseins are expressed in milligrams per gram of cheese DM. α-Casein and κ-CN were not detected on d 90 and afterward.

[2]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

our results, no significant differences in the late-eluting region, corresponding to hydrophobic peptides, were found between control and pressurized Irish blue-veined cheeses (Voigt et al., 2010). The shorter cheese ripening and storage periods in their work may explain the different finding.

Aminopeptidase activity in control cheese reached maximum levels of 24.83 nmol *p*-nitroaniline per minute per gram on d 42 (with Leu-*p*-NA as substrate) and 24.58 nmol *p*-nitroaniline per minute per gram on d 63 (with Lys-*p*-NA as substrate; data not shown), and then declined during the rest of the ripening period to 19.61 and 19.43 nmol *p*-nitroaniline per minute per gram, respectively, on d 90 (Table 6). Aminopeptidase activity, which originates mostly from starter LAB but also from other added or contaminating microorganisms, was not determined in Irish blue-veined cheese (Voigt et al., 2010). In Gorgonzola cheese, not only aminopeptidase activity but also carboxypeptidase and iminopeptidase activities, attributed to *P. roqueforti* by the authors, increased until d 86, the last sampling date (Gobbetti et al., 1997). High-pressure treatments show differences in the inactivation of bacterial peptidases, depending not only on process parameters, but also on bacterial species and assay substrates, and the effect may vary for different peptidolytic enzymes within a bacterial strain (Malone et al., 2003). In the present work, pressurization lowered aminopeptidase activity by 20 to 30% at 400 MPa and by 40 to 50% at 600 MPa immediately after treatment (data not shown), in agreement with previous results for a nonmold-ripened ovine milk cheese (Garde et al., 2007). On d 90, aminopeptidase activity levels were significantly ($P < 0.05$) lower in all the pressurized cheeses than in control cheese. Aminopeptidase activity declined further during refrigerated storage, to levels under 20% of the maximum values, and the differences between cheeses persisted until d 360 (Table 6).

Overall proteolysis, as determined by the *o*-phthaldialdehyde method, increased in control cheese from a value of 0.15 on d 1 to 0.89 on d 21, 2.93 on d 42, 5.45 on d 63 (data not shown), and 6.89 on d 90 (Table 7). Proteolysis values during ripening were considerably higher than those reported for cheese varieties not mold-ripened (Garde et al., 2002, 2007). Proteolysis values in pressurized cheeses immediately after treatment were similar to those of the respective control cheese (data not shown). However, significant ($P < 0.05$) differences in the overall proteolysis values of cheeses were recorded on d 90, the lowest value being that of 600W3 cheese (Table 7). During refrigerated storage, overall proteolysis increased by 51% in control cheese, and by 37 to 68% in pressurized cheeses. On d 360, control cheese and cheeses treated at 400 MPa on wk 6 or 9 showed the highest overall proteolysis values, and 600W3 cheese the lowest value.

Total free AA concentration increased in control cheese from 1.49 mg/g of DM on d 1 to 61.20 mg/g of DM on d 90, a considerably higher concentration than those reported for Gorgonzola cheese and other strongly proteolyzed cheeses of approximately the same ripening time (Gobbetti et al., 1997; Garde et al., 2002, 2007), but lower than the concentration of free AA in Stilton cheese (Madkor et al., 1987). In the present work, free AA concentrations in pressurized cheeses immediately after treatment were close to those of the respective control cheese (data not shown). From d 90 to 360, total free AA increased by 91% in control cheese and by 59 to 116% in pressurized cheeses. Similar to the pattern observed for overall proteolysis, the concentration of total free AA during refrigerated storage tended to reach its maximum levels in control cheese and in 400W6 and 400W9 cheeses, probably favored by conformational changes in the CN of these cheeses that facilitate the access of enzymes to their substrates (O'Reilly et al., 2003), whereas the lowest values were those of 600W3 cheese (Table 7). Early pressurization (after 3 wk of ripening) at a high pressure level (600 MPa) was the most effective treatment in retarding secondary proteolysis, even though the lowest aminopeptidase activity values were not those of 600W3 cheese.

Aminopeptidase activities on Leu-*p*-NA and Lys-*p*-NA as substrates correlated well with each other (r^2 values = 0.922 and 0.982 on d 90 and 360, respectively), and this was also true for the correlation of overall proteolysis with total free AA (r^2 values = 0.782 and 0.942 on d 90 and 360). However, a significant correlation of aminopeptidase activities with overall proteolysis or free AA concentration in cheeses was not observed (r^2 values = <0.10 and <0.40 on d 90 and 360). It must be taken into account that aminopeptidases were at levels far from their maximum values during refrigerated storage, and also that aminopeptidases present in intact microbial cells not lysed by treatment at 400 MPa, which most likely are not recovered in the cheese extracts used for aminopeptidase assays, contribute to cheese secondary proteolysis too. The low correlation of aminopeptidase activity to overall proteolysis and total free AA can be also attributed to the fact that carboxypeptidase and iminopeptidase activities coming from *P. roqueforti*, which enhance overall proteolysis and participate in free AA formation, were not evaluated when using Leu-*p*-NA and Lys-*p*-NA as substrates.

BA

Histamine, cadaverine, and spermine were not detected at any of the sampling times in any of the

Table 5. Hydrophilic peptides, hydrophobic peptides, and the hydrophobic-to-hydrophilic peptide ratio in control ovine milk blue cheese and pressurized cheeses

Peptide	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Hydrophilic peptides[2]	90	28.16 ± 0.82^{a}	36.64 ± 0.41^{d}	27.42 ± 0.63^{a}	32.58 ± 0.96^{bc}	28.91 ± 1.22^{ab}	30.70 ± 0.51^{abc}	33.00 ± 0.08^{cd}
	180	28.08 ± 1.49^{a}	31.76 ± 0.28^{a}	29.40 ± 1.55^{a}	26.68 ± 1.14^{a}	29.20 ± 0.14^{a}	27.93 ± 0.19^{a}	27.13 ± 1.66^{a}
	270	29.39 ± 0.55^{ab}	27.80 ± 0.35^{ab}	25.86 ± 1.23^{a}	30.19 ± 1.11^{b}	27.90 ± 0.23^{ab}	30.72 ± 0.86^{b}	31.12 ± 1.05^{b}
	360	29.81 ± 0.87^{ab}	30.10 ± 2.73^{ab}	26.86 ± 0.11^{a}	33.11 ± 0.53^{bc}	27.55 ± 0.74^{a}	36.05 ± 0.49^{c}	31.93 ± 0.12^{ab}
Hydrophobic peptides[2]	90	5.66 ± 0.14^{a}	9.40 ± 0.36^{d}	8.85 ± 0.36^{cd}	7.16 ± 0.20^{b}	7.72 ± 0.68^{bc}	6.73 ± 0.06^{ab}	6.70 ± 0.13^{ab}
	180	4.80 ± 0.19^{a}	7.61 ± 0.30^{c}	9.08 ± 0.55^{d}	6.11 ± 0.13^{ab}	7.12 ± 0.30^{bc}	5.88 ± 0.40^{ab}	5.24 ± 0.33^{a}
	270	4.44 ± 0.32^{a}	4.14 ± 0.12^{a}	6.53 ± 0.26^{bc}	4.78 ± 0.96^{ab}	5.35 ± 0.49^{ab}	6.82 ± 0.35^{bc}	7.33 ± 0.22^{c}
	360	4.54 ± 0.18^{a}	6.54 ± 0.01^{b}	8.33 ± 0.32^{d}	6.81 ± 0.49^{bc}	6.61 ± 0.16^{b}	6.93 ± 0.07^{bc}	7.85 ± 0.11^{cd}
Ratio[2]	90	0.20 ± 0.01^{a}	0.26 ± 0.01^{bc}	0.32 ± 0.01^{d}	0.22 ± 0.01^{ab}	0.27 ± 0.01^{c}	0.22 ± 0.01^{ab}	0.20 ± 0.01^{a}
	180	0.17 ± 0.00^{a}	0.24 ± 0.01^{b}	0.31 ± 0.03^{c}	0.23 ± 0.01^{ab}	0.24 ± 0.01^{b}	0.21 ± 0.02^{ab}	0.19 ± 0.01^{ab}
	270	0.15 ± 0.01^{a}	0.15 ± 0.00^{a}	0.25 ± 0.00^{b}	0.16 ± 0.03^{a}	0.19 ± 0.02^{ab}	0.22 ± 0.01^{b}	0.24 ± 0.02^{b}
	360	0.15 ± 0.01^{a}	0.22 ± 0.02^{c}	0.31 ± 0.01^{d}	0.21 ± 0.01^{bc}	0.24 ± 0.01^{c}	0.19 ± 0.00^{ab}	0.25 ± 0.00^{c}

[a–d]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Peptides, determined at 280 nm, are expressed in arbitrary units (AU), calculated as units of chromatogram area per milligram of cheese DM.

Table 6. Aminopeptidase activity on Leu-*p*-NA and Lys-*p*-NA in control ovine milk blue cheese and pressurized cheeses

Aminopeptidase activity	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Activity on Leu-*p*-NA[2]	90	19.61 ± 0.99^{d}	12.34 ± 0.31^{c}	11.34 ± 0.27^{bc}	7.58 ± 0.12^{a}	11.55 ± 0.70^{bc}	11.39 ± 0.72^{bc}	8.19 ± 0.32^{ab}
	180	6.66 ± 0.48^{d}	4.38 ± 0.21^{bc}	4.77 ± 0.52^{c}	3.25 ± 0.52^{ab}	2.63 ± 0.31^{a}	2.86 ± 0.07^{ab}	2.65 ± 0.05^{a}
	270	5.30 ± 0.83^{c}	2.31 ± 0.07^{b}	2.34 ± 0.15^{b}	1.44 ± 0.07^{a}	1.42 ± 0.01^{a}	2.13 ± 0.10^{b}	1.21 ± 0.01^{a}
	360	3.26 ± 0.27^{c}	1.11 ± 0.18^{a}	1.39 ± 0.05^{ab}	1.85 ± 0.08^{b}	0.92 ± 0.06^{a}	1.77 ± 0.04^{b}	0.89 ± 0.12^{a}
Activity on Lys-*p*-NA[2]	90	19.43 ± 0.48^{e}	10.92 ± 0.61^{cd}	9.48 ± 0.34^{abc}	8.16 ± 0.27^{ab}	12.87 ± 0.59^{d}	10.34 ± 0.99^{bcd}	7.23 ± 0.09^{a}
	180	6.26 ± 0.42^{b}	3.65 ± 0.08^{a}	3.24 ± 0.35^{a}	3.32 ± 0.36^{a}	2.38 ± 0.23^{a}	2.94 ± 0.16^{a}	2.22 ± 0.08^{a}
	270	4.33 ± 0.67^{b}	1.46 ± 0.02^{a}	1.06 ± 0.03^{a}	1.30 ± 0.10^{a}	0.97 ± 0.02^{a}	1.70 ± 0.13^{a}	1.23 ± 0.07^{a}
	360	3.21 ± 0.27^{c}	1.00 ± 0.19^{ab}	1.02 ± 0.03^{ab}	1.71 ± 0.13^{b}	0.91 ± 0.09^{ab}	1.66 ± 0.05^{ab}	0.75 ± 0.06^{a}

[a–e]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Activity is expressed in nanomole *p*-nitroaniline per minute per gram.

Table 7. Overall proteolysis and free AA in control ovine milk blue cheese and pressurized cheeses

Item	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Overall proteolysis[2]	90	6.89 ± 0.17^{bc}	5.25 ± 0.62^{ab}	4.03 ± 0.37^{a}	6.83 ± 0.04^{bc}	5.23 ± 0.05^{ab}	7.06 ± 0.08^{c}	6.55 ± 0.83^{bc}
	180	8.28 ± 0.54^{d}	6.51 ± 0.04^{b}	4.49 ± 0.12^{a}	8.62 ± 0.87^{d}	7.32 ± 0.09^{c}	8.85 ± 0.40^{d}	7.00 ± 0.32^{bc}
	270	8.65 ± 0.37^{c}	6.88 ± 0.24^{b}	4.81 ± 0.45^{a}	8.83 ± 0.25^{c}	7.85 ± 0.21^{bc}	9.01 ± 0.12^{c}	8.52 ± 0.37^{c}
	360	10.42 ± 0.29^{c}	8.10 ± 0.14^{b}	5.53 ± 0.42^{a}	11.38 ± 0.51^{c}	8.78 ± 0.16^{b}	10.40 ± 0.17^{c}	8.96 ± 0.63^{b}
Total free AA[2]	90	61.20 ± 3.67^{c}	42.09 ± 2.89^{b}	25.21 ± 1.31^{a}	64.68 ± 6.64^{c}	59.64 ± 2.51^{c}	63.04 ± 3.70^{c}	59.66 ± 2.91^{c}
	180	88.61 ± 2.10^{c}	60.10 ± 3.28^{b}	36.46 ± 0.72^{a}	90.96 ± 1.08^{cd}	80.98 ± 0.55^{c}	92.91 ± 1.58^{d}	78.97 ± 5.74^{c}
	270	101.13 ± 3.89^{cd}	73.55 ± 5.61^{b}	38.77 ± 2.62^{a}	108.28 ± 0.24^{d}	84.73 ± 1.35^{b}	101.86 ± 3.83^{cd}	88.27 ± 10.03^{bc}
	360	116.76 ± 1.42^{d}	81.45 ± 3.46^{b}	54.33 ± 5.70^{a}	116.68 ± 0.77^{d}	95.80 ± 1.42^{c}	123.48 ± 1.08^{d}	94.81 ± 1.15^{c}

[a–d]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Overall proteolysis estimated by the *o*-phthaldialdehyde method is expressed as the absorbance at 340 nm. Total free AA are expressed in milligrams per gram cheese DM.

Table 8. Main biogenic amines in control ovine milk blue cheese and pressurized cheeses

Biogenic amine	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Tyramine[2]	180	7.15 ± 2.66^{ab}	7.80 ± 1.09^{b}	6.57 ± 1.70^{ab}	4.91 ± 0.66^{a}	5.78 ± 1.36^{a}	6.24 ± 0.99^{ab}	4.62 ± 1.21^{a}
	270	26.70 ± 2.92^{a}	25.78 ± 6.30^{a}	24.78 ± 5.85^{a}	18.63 ± 2.83^{a}	24.46 ± 3.84^{a}	23.15 ± 2.45^{a}	23.84 ± 3.37^{a}
	360	52.20 ± 9.56^{c}	32.01 ± 2.55^{b}	33.43 ± 2.43^{b}	20.90 ± 1.68^{a}	32.39 ± 1.92^{b}	30.62 ± 5.86^{b}	27.22 ± 1.79^{ab}
Tryptamine[2]	180	61.88 ± 7.30^{ab}	67.07 ± 9.71^{b}	46.33 ± 5.06^{a}	54.29 ± 6.01^{ab}	42.85 ± 5.63^{a}	69.40 ± 6.72^{b}	57.91 ± 8.02^{ab}
	270	70.59 ± 5.17^{ab}	86.28 ± 2.85^{b}	65.49 ± 12.44^{a}	70.89 ± 3.72^{ab}	73.59 ± 2.61^{ab}	81.57 ± 3.68^{ab}	74.76 ± 5.10^{ab}
	360	71.11 ± 3.82^{a}	102.90 ± 7.91^{bc}	76.77 ± 4.93^{a}	74.69 ± 4.32^{a}	104.40 ± 3.10^{bc}	93.95 ± 2.61^{ab}	120.09 ± 3.94^{c}
Phenylethylamine[2]	180	13.25 ± 0.94^{c}	8.13 ± 2.53^{ab}	11.77 ± 3.38^{bc}	5.07 ± 0.80^{a}	8.18 ± 0.43^{ab}	4.11 ± 0.56^{a}	7.21 ± 2.07^{a}
	270	57.04 ± 6.51^{a}	64.49 ± 2.55^{ab}	61.05 ± 5.55^{ab}	66.31 ± 1.60^{ab}	54.50 ± 4.15^{a}	80.68 ± 9.81^{b}	75.31 ± 9.22^{b}
	360	61.44 ± 2.79^{a}	74.22 ± 5.47^{b}	77.71 ± 9.95^{bc}	57.49 ± 4.49^{a}	63.11 ± 5.58^{ab}	70.70 ± 6.19^{ab}	92.05 ± 5.31^{c}
Putrescine[2]	180	17.28 ± 1.33^{bc}	14.51 ± 1.68^{a}	15.90 ± 2.01^{ab}	12.67 ± 0.87^{a}	19.52 ± 3.15^{c}	13.03 ± 1.40^{a}	18.70 ± 2.46^{bc}
	270	33.46 ± 2.09^{a}	33.96 ± 2.85^{a}	36.27 ± 2.58^{a}	33.22 ± 1.30^{a}	46.79 ± 3.96^{b}	38.34 ± 1.45^{a}	42.79 ± 2.81^{ab}
	360	32.97 ± 2.05^{ab}	34.48 ± 3.11^{ab}	39.50 ± 3.55^{bc}	30.38 ± 0.54^{a}	44.05 ± 2.81^{cd}	31.00 ± 2.81^{a}	49.70 ± 2.79^{d}

[a–d]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Biogenic amines are expressed in milligrams per kilogram of cheese DM.

cheeses. Spermidine was found on d 270 and 360 at low concentrations, ranging from 5.06 to 14.03 mg/kg of DM, in control cheese and in some of the HP-treated cheeses (data not shown). Spermine and spermidine may be formed by starter LAB. These polyamines were detected at low levels, 0.20 mg/L of spermine and 0.75 mg/L of spermidine, in sterilized nonfat milk, and increased during the fermentation of milk with added rennet by *Lactococcus lactis* to levels as high as 1.7 and 10 mg/L, respectively (Santos et al., 2003). According to those authors, spermine reached a maximum after 12 h at 20°C and had practically disappeared 12 h later. Concentrations of spermidine and spermine in the present study are in agreement with those found for pressurized and control goat milk cheeses, which contained higher concentrations of spermidine, ranging from 14.7 to 26.4 mg/kg of DM, than of spermine, which ranged from 0.9 to 3.5 mg/kg of DM (Novella-Rodríguez et al., 2002).

Tyramine, which was not detected on d 90 in any of the cheeses, attained significantly ($P < 0.05$) lower levels on d 360 in all the pressurized cheeses than in control cheese (Table 8). The low tyramine concentrations found in the present work and the absence of histamine can be related to the low counts of enterococci and lactobacilli, potential tyrosine and histidine decarboxylase-positive bacteria, but also to the low cheese-ripening and storage temperatures used, 5°C from d 30 to 90, and 3°C from d 90 onwards, which impair BA formation (Stratton et al., 1991). Tyrosine and histidine concentrations in cheeses on d 360, which ranged from 4.11 to 9.29 and from 3.80 to 9.86 mg/g of DM (data not shown), respectively, did not appear as limiting factors for tyramine and histamine formation. Tryptamine, which was below detection level in all cheeses on d 90, reached higher concentrations in 400W3, 600W6, and 600W9 cheeses by d 360 than in control cheese. Phenylethylamine was found in all cheeses on d 90 at low concentrations, ranging from 4.96 to 13.28 mg/kg of DM (data not shown), and attained higher levels in 400W3, 600W3, and 600W9 by d 360 cheeses than in control cheese (Table 8). Putrescine was also detected in all cheeses at low concentrations on d 90, ranging from 5.38 to 8.65 mg/kg of DM (data not shown), and reached higher concentrations in 600W6 and 600W9 cheeses by d 360 than in control cheese (Table 8). During the fermentation of sterilized nonfat milk with added rennet by *L. lactis*, up to 0.82 mg/L of tyramine and 0.15 mg/L of putrescine were formed, but phenylethylamine and tryptamine were not detected (Santos et al., 2003). In the present work, the concentrations of phenylalanine, tryptophan, and arginine in control and pressurized cheeses did not seem to limit the formation of the respective biogenic amines, similarly to tyrosine and histidine.

Biogenic amines formation through free AA decarboxylation may constitute an alternative energy source for cheese microbiota in the absence of fermentable carbohydrates (Fernandez-García et al., 2000). Conversely, some strains of *Lactobacillus*, *Pediococcus*, and *Micrococcus* are capable of degrading BA, such as tyramine and histamine, by means of monoamine oxidases, preferably under aerobic conditions (Leuschner et al., 1998). Accumulation of BA is thus the result of BA formation and degradation by cheese microbiota.

Concentrations of BA reported for blue-veined cheeses show a considerable variability, and do not seem to be related to the use of raw or pasteurized milk. Roquefort cheese contained, on average, 19, 40, 65, and 158 mg/kg of histamine, tyramine, putrescine, and cadaverine, respectively (De Vuyst et al., 1976), and Stilton cheese contained 39, 121, 11, and 126 mg/kg of histamine, tyramine, phenyletylamine, and putrescine, respectively (Baker et al., 1987). Roquefort cheese has been always made from raw milk, and Stilton cheese was made from unpasteurized milk at the time of the cited work (Gkatzionis et al., 2009). High concentrations of BA, 490 mg/kg of histamine and 625 mg/kg of tyramine, were found for Danish blue cheese (Ingles et al., 1985) and for Spanish blue cheese made from raw milk, which contained 1,042 mg/kg of histamine and 1,052 mg/kg of tyramine (Fernández et al., 2007). For Egyptian raw milk blue cheese, up to 36, 2,220, 27, 12, and 16 mg/kg of DM of histamine, tyramine, putrescine, and cadaverine, respectively, were found (Rabie et al., 2011). Those authors achieved significant decreases in histamine and tyramine contents by cheese irradiation at 4 kGy, and in putrescine and cadaverine contents by irradiation at 6 kGy.

Sensory Characteristics

Flavor intensity scores of pressurized cheeses did not differ from those of control cheese, with the only exception of 600W3 cheese scores, which were significantly ($P < 0.05$) lower than those of control cheese (Table 9). The scores obtained for flavor quality were also generally lower for 600W3 cheeses than for the rest (Table 9). The low flavor scores of 600W3 cheese can be associated to its higher levels of hydrophobic peptides and peptide ratio, and its lower levels of overall proteolysis and total free AA. In fact, significant ($P < 0.05$) r^2 values of 0.758 and 0.808 were obtained for the regressions of flavor intensity scores on free AA contents in all cheeses on d 270 and 360, respectively, and r^2 values of 0.830 and 0.934 for the regressions of flavor quality scores on

Table 9. Sensory characteristics of control ovine milk blue cheese and pressurized cheeses

Sensory characteristic	Days	Control cheese	400W3[1]	600W3[1]	400W6[1]	600W6[1]	400W9[1]	600W9[1]
Flavor intensity[2]	90	6.77 ± 0.16^{ab}	7.01 ± 0.26^{ab}	6.48 ± 0.29^{a}	7.25 ± 0.24^{b}	6.86 ± 0.26^{ab}	7.17 ± 0.31^{b}	6.88 ± 0.34^{ab}
	180	7.30 ± 0.17^{b}	7.28 ± 0.23^{b}	6.41 ± 0.34^{a}	7.02 ± 0.21^{ab}	7.41 ± 0.29^{b}	7.39 ± 0.27^{b}	6.80 ± 0.31^{ab}
	270	7.23 ± 0.18^{b}	7.09 ± 0.32^{b}	6.27 ± 0.36^{a}	7.18 ± 0.28^{b}	7.11 ± 0.27^{b}	7.36 ± 0.25^{b}	7.45 ± 0.30^{b}
	360	7.55 ± 0.19^{b}	7.09 ± 0.40^{ab}	6.50 ± 0.28^{a}	7.90 ± 0.34^{b}	7.31 ± 0.29^{ab}	7.39 ± 0.49^{ab}	7.13 ± 0.37^{ab}
Flavor quality[2]	90	7.12 ± 0.17^{b}	6.39 ± 0.36^{a}	6.11 ± 0.35^{a}	7.07 ± 0.29^{b}	6.74 ± 0.38^{ab}	6.79 ± 0.44^{ab}	6.86 ± 0.31^{ab}
	180	7.19 ± 0.19^{bc}	6.45 ± 0.40^{b}	5.33 ± 0.42^{a}	7.28 ± 0.21^{c}	6.51 ± 0.43^{b}	6.63 ± 0.38^{b}	7.45 ± 0.29^{c}
	270	6.49 ± 0.22^{bc}	6.19 ± 0.35^{b}	5.18 ± 0.46^{a}	7.06 ± 0.26^{bc}	6.55 ± 0.33^{bc}	7.16 ± 0.28^{c}	7.09 ± 0.26^{c}
	360	7.03 ± 0.21^{b}	6.50 ± 0.40^{ab}	5.53 ± 0.46^{a}	7.30 ± 0.32^{b}	6.63 ± 0.34^{ab}	7.09 ± 0.35^{b}	6.57 ± 0.28^{ab}

[a-c]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Treatments were pressurized at 400 MPa after 3 wk of ripening (400W3), 600 MPa after 3 wk (600W3), 400 MPa after 6 wk (400W6), 600 MPa after 6 wk (600W6), 400 MPa after 9 wk (400W9), and 600 MPa after 9 wk (600W9).

[2]Mean ± SE of determinations in 2 cheese-making experiments by 15 trained panelists on a 0- to 10-point scale.

free AA contents on d 270 and 360. Cheese treatment at a high pressure level (600 MPa) at an early ripening stage (3 wk) negatively affected biochemical changes and sensory characteristics. The flavor attributes acid, bitter, salty, sweet, and umami attained similar scores in control and pressurized cheeses and, with the exception of umami scores, which increased significantly ($P < 0.05$) with time, did not vary during the refrigerated storage period (data not shown).

No sensory evaluation was carried out on Irish blue-veined cheese (Voigt et al., 2010). In the case of Gorgonzola blue cheese, treatments at 600 and 700 MPa did not reduce the acceptance by untrained panelists, and no changes in the bitter, salty, and piquant attributes were observed (Carminati et al., 2004). Conversely, a negative effect of irradiation on the odor and taste of Egyptian raw milk blue cheese was recorded when this procedure was assayed to reduce the formation of BA (Rabie et al., 2011).

CONCLUSIONS

Pressurization of blue-veined cheese lowered microbial counts, in particular treatments at 600 MPa, which reduced LAB counts by more than 4 log units and *P. roqueforti* counts by more than 6 log units. The levels of residual caseins were generally higher in 600 MPa cheeses than in the rest of the cheeses. Hydrophilic peptides reached similar levels in pressurized and control cheeses from d 90 onwards, but the level of hydrophobic peptides tended to be higher in pressurized cheeses. Aminopeptidase activity, overall proteolysis, and free AA contents were generally higher in control cheese than in pressurized cheeses. Tyramine concentration was lower in pressurized cheeses than in control cheese, but higher tryptamine, phenylethylamine, and putrescine contents were found in some of the pressurized cheeses, in particular in cheese pressurized at 600 MPa after 9 wk of ripening, than in control cheese. Differences in sensory characteristics between pressurized and control cheeses were generally negligible, with the exception of treatment at a high pressure level (600 MPa) at an early ripening stage (3 wk), which negatively affected biochemical changes and sensory characteristics.

ACKNOWLEDGMENTS

This work was supported by project AGL2009-07801 from the Ministry of Science and Innovation (MICINN; Madrid, Spain). J. Calzada was the recipient of a MICINN fellowship. The authors are grateful for the valuable help of V. Carbonero (Lácteas Toledo, Guadamur, Spain) with cheese supply and of F. Purroy (NC Hyperbaric, Burgos, Spain) with high pressure treatments.

REFERENCES

Arqués, J. L., S. Garde, E. Fernández-García, P. Gaya, and M. Nuñez. 2007. Volatile compounds, odor, and aroma of La Serena cheese high-pressure treated at two different stages of ripening. J. Dairy Sci. 90:3627–3639.

Arqués, J. L., S. Garde, P. Gaya, M. Medina, and M. Nuñez. 2006. Inactivation of microbial contaminants in raw milk La Serena cheese by high pressure treatments. J. Dairy Sci. 89:888–891.

Baker, G. B., J. T. F. Wong, R. T. Coutts, and F. M. Pasutto. 1987. Simultaneous extraction and quantitation of several bioactive amines in cheese and chocolate. J. Chromatogr. 392:317–331.

Carminati, D., M. Gatti, B. Bonvini, E. Neviani, and G. Mucchetti. 2004. High-pressure processing of Gorgonzola cheese: Influence on *Listeria monocytogenes* inactivation and on sensory characteristics. J. Food Prot. 67:1671–1675.

Church, F. C., H. E. Swaisgood, D. H. Porter, and G. L. Catignani. 1983. Spectrophotometric assay using *o*-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66:1219–1227.

De Vuyst, A., W. Vervack, and M. Foulon. 1976. Détection d'amines non volatiles dans quelques fromages. Lait 56:414–422.

Delgado, F. J., J. Gonzalez-Crespo, R. Cava, and R. Ramirez. 2012. Changes in microbiology, proteolysis, texture and sensory characteristics of raw goat milk cheeses treated by high-pressure at different stages of maturation. LWT-Food Sci. Technol. 48:268–275.

Edwards, S. T., and W. E. Sandine. 1981. Public health significance of amines in cheese. J. Dairy Sci. 64:2431–2438.

Evert-Arriagada, K., M. M. Hernández-Herrero, B. Juan, B. Guamis, and A. J. Trujillo. 2012. Effect of high-pressure on fresh cheese shelf-life. J. Food Eng. 110:248–253.

Fernández, M., D. M. Linares, B. del Río, V. Ladero, and M. A. Alvarez. 2007. HPLC quantification of biogenic amines in cheeses: Correlation with PCR-detection of tyramine-producing microorganisms. J. Dairy Res. 74:276–282.

Fernandez-García, E., J. Tomillo, and M. Nuñez. 2000. Formation of biogenic amines in raw milk Hispánico cheese manufactured with proteinases and different levels of starter culture. J. Food Prot. 63:1551–1555.

Garde, S., J. L. Arqués, P. Gaya, M. Medina, and M. Nuñez. 2007. Effect of high pressure treatments on the proteolysis and texture of ewes' raw milk La Serena cheese. Int. Dairy J. 17:1424–1433.

Garde, S., M. Ávila, P. Gaya, M. Medina, and M. Nuñez. 2006. Proteolysis of Hispánico cheese manufactured using lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639. J. Dairy Sci. 89:840–849.

Garde, S., J. Tomillo, P. Gaya, M. Medina, and M. Nuñez. 2002. Proteolysis in Hispánico cheese manufactured using a mesophilic starter, a thermophilic starter and bacteriocin-producing *Lactococcus lactis* ssp. *lactis* INIA 415 adjunct culture. J. Agric. Food Chem. 50:3479–3485.

Gkatzionis, K., R. S. T. Linforth, and C. E. R. Dodd. 2009. Volatile profile of Stilton cheeses: Differences between zones within a cheese and dairies. Food Chem. 113:506–512.

Gobbetti, M., R. Burzigotti, E. Smacchi, A. Corsetti, and M. De Angelis. 1997. Microbiology and biochemistry of Gorgonzola cheese during ripening. Int. Dairy J. 7:519–529.

Gomez, M. J., S. Garde, P. Gaya, M. Nuñez, and M. Medina. 1997. Relationship between level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk. J. Dairy Res. 64:289–297.

Gomez, M. J., E. Rodríguez, P. Gaya, M. Nuñez, and M. Medina. 1999. Characteristics of Manchego cheese manufactured from raw and pasteurized ovine milk and with defined-strain and or commercial mixed-strain starter cultures. J. Dairy Sci. 82:2300–2307.

Gripon, J.-C., M. J. Desmazeaud, D. Le Bars, and J. L. Bergère. 1977. Role of proteolytic enzymes of *Streptococcus lactis*, *Penicillium roqueforti*, and *Penicillium caseicolum* during cheese ripening. J. Dairy Sci. 60:1532–1538.

Ingles, D. L., J. F. Black, D. Gallimore, R. Tindale, and K. J. Shaw. 1985. Estimation of biogenic-amines in foods. J. Sci. Food Agric. 36:402–406.

Joosten, H. M. L. J., and M. D. Northolt. 1987. Conditions allowing the formation of biogenic-amines in cheese. 2. Decarboxylase properties of some nonstarter bacteria. Neth. Milk Dairy J. 41:259–280.

Juan, B., V. Ferragut, M. Buffa, B. Guamis, and A. J. Trujillo. 2007. Effects of high pressure on proteolytic enzymes in cheese: Relationship with the proteolysis of ewe milk cheese. J. Dairy Sci. 90:2113–2125.

Krause, I., A. Bockhardt, H. Neckermann, T. Henle, and H. Klostermeyer. 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. J. Chromatogr. A 715:67–79.

Lau, K. Y., D. M. Barbano, and R. R. Rasmussen. 1991. Influence of pasteurization of milk on protein breakdown in Cheddar cheese during aging. J. Dairy Sci. 74:727–740.

Leuschner, R. G., M. Heidel, and W. P. Hammes. 1998. Histamine and tyramine degradation by food fermenting microorganisms. Int. J. Food Microbiol. 39:1–10.

Madkor, S., P. F. Fox, S. I. Shalabi, and N. H. Metwalli. 1987. Studies on the ripening of Stilton cheese: Proteolysis. Food Chem. 25:13–29.

Malone, A. S., C. Wick, T. H. Shellhammer, and P. D. Courtney. 2003. High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. J. Dairy Sci. 86:1139–1146.

Modler, H. W., J. R. Brunner, and C. M. Stine. 1974. Extracellular protease of *Penicillium roqueforti*. I. Production and characteristics of crude enzyme preparation. J. Dairy Sci. 57:523–527.

Novella-Rodríguez, S., M. T. Veciana-Nogués, J. Saldo, and M. C. Vidal-Carou. 2002. Effects of high hydrostatic pressure treatments on biogenic amine contents in goat cheeses during ripening. J. Agric. Food Chem. 50:7288–7292.

O'Reilly, C. E., A. L. Kelly, J. C. Oliveira, P. M. Murphy, M. A. E. Auty, and T. P. Beresford. 2003. Effect of varying high-pressure treatment conditions on acceleration of ripening of Cheddar cheese. Innov. Food Sci. Emerg. Technol. 4:277–284.

O'Reilly, C. E., P. M. O'Connor, A. L. Kelly, T. P. Beresford, and P. M. Murphy. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl. Environ. Microbiol. 66:4890–4896.

Pircher, A., F. Bauer, and P. Paulsen. 2007. Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. Eur. Food Res. Technol. 226:225–231.

Rabie, M. A., H. I. Siliha, S. M. El-Saidy, A. A. El-Badawy, and F. X. Malcata. 2011. Effect of γ-irradiation upon biogenic amine formation in blue cheese during storage. Int. Dairy J. 21:373–376.

Saldo, J., P. L. H. McSweeney, E. Sendra, A. L. Kelly, and B. Guamis. 2002. Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening. Int. Dairy J. 12:35–44.

Santos, W. C., M. R. Souza, M. M. O. P. Cerqueira, and M. B. A. Gloria. 2003. Bioactive amines formation in milk by *Lactococcus* in the presence or not of rennet and NaCl at 20 and 32 °C. Food Chem. 90:219–230.

Silla Santos, M. H. 1996. Biogenic amines: Their importance in foods. Int. J. Food Microbiol. 29:213–231.

Stratton, J. E., R. W. Hutkins, and S. L. Taylor. 1991. Biogenic amines in cheese and other fermented foods. J. Food Prot. 54:460–470.

Taylor, S. L. 1986. Histamine food poisoning: Toxicology and clinical aspects. Crit. Rev. Toxicol. 17:91–128.

ten Brink, B., C. Damink, H. M. L. J. Joosten, and J. H. Huis in 't Veld. 1990. Occurrence and formation of biologically-active amines in foods. Int. J. Food Microbiol. 11:73–84.

Til, H. P., H. E. Falke, M. K. Prinsen, and M. I. Willems. 1997. Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. Food Chem. Toxicol. 35:337–348.

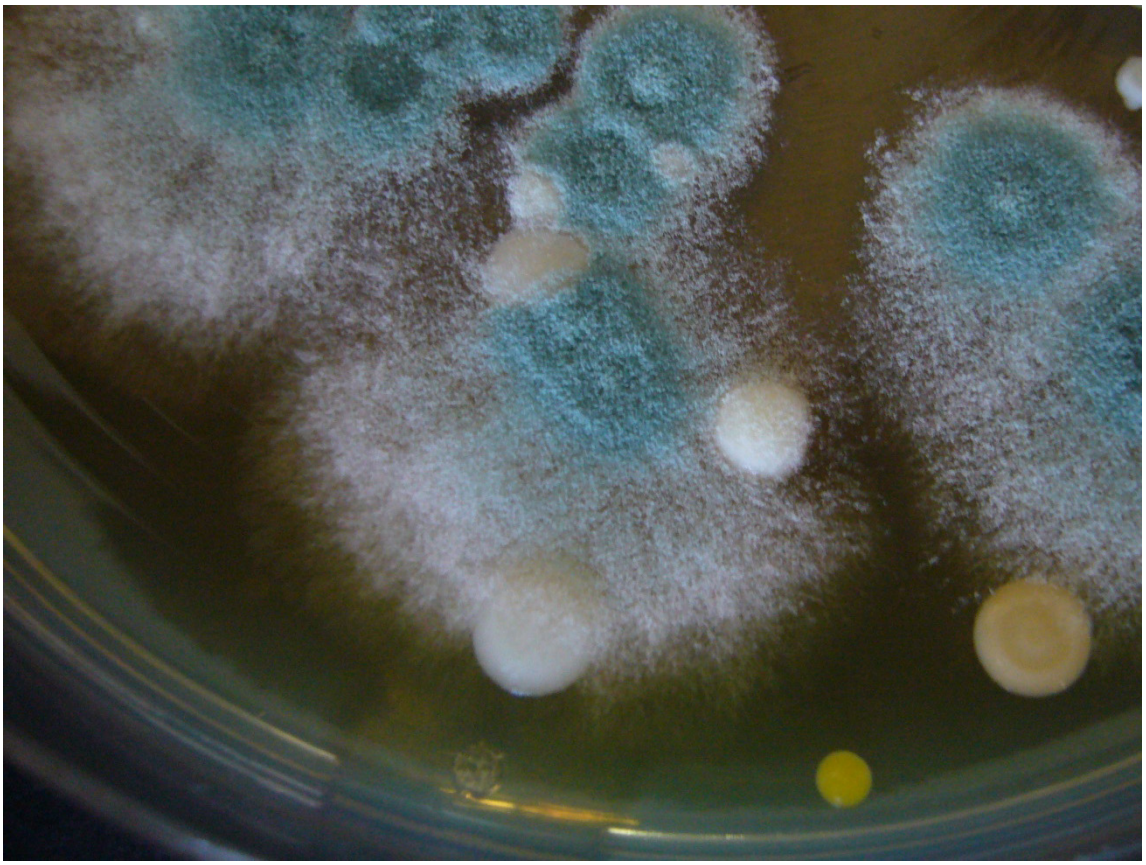
Trieu-Cuot, P., M.-J. Archieri-Haze, and J.-C. Gripon. 1982. Effect of aspartyl proteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on caseins. J. Dairy Res. 49:487–500.

Visser, S. 1993. Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. J. Dairy Sci. 76:329–350.

Voigt, D. D., F. Chevalier, M. C. Qian, and A. L. Kelly. 2010. Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavor compounds in mature blue-veined cheese. Innov. Food Sci. Emerg. Technol. 11:68–77.

Wick, C., U. Nienaber, O. Anggraeni, T. H. Shellhammer, and P. D. Courtney. 2004. Texture, proteolysis and viable lactic acid bacteria in commercial Cheddar cheeses treated with high pressure. J. Dairy Res. 71:107–115.

Yvon, M., and L. Rijnen. 2001. Cheese flavor formation by amino acid catabolism. Int. Dairy J. 11:185–201.



Capítulo 3.

High-pressure processing decelerates lipolysis and formation of volatile compounds in ovine milk blue-veined cheese.

Fotografía: colonias de *Penicillium roqueforti* y bacterias lácticas en agar M17.

J. Dairy Sci. 96:7500–7510
http://dx.doi.org/10.3168/jds.2013-7221

High-pressure processing decelerates lipolysis and formation of volatile compounds in ovine milk blue-veined cheese

J. Calzada, A. Del Olmo, A. Picon, P. Gaya, and M. Nuñez[1]
Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28040 Madrid, Spain

ABSTRACT

Enzyme-rich cheeses are prone to over-ripening during refrigerated storage. Blue-veined cheeses fall within this category because of the profuse growth of *Penicillium roqueforti* in their interior, which results in the production of highly active proteinases, lipases, and other enzymes responsible for the formation of a great number of flavor compounds. To control the excessive formation of free fatty acids (FFA) and volatile compounds, blue-veined cheeses were submitted to high-pressure processing (HPP) at 400 or 600 MPa on d 21, 42, or 63 after manufacture. Cheeses were ripened for 30 d at 10°C and 93% relative humidity, followed by 60 d at 5°C, and then held at 3°C until d 360. High-pressure processing influenced the concentrations of acetic acid and short-chain, medium-chain, and long-chain FFA. The effect was dependent on treatment conditions (pressure level and cheese age at the time of treatment). The lowest concentrations of acetic acid and FFA were recorded for cheeses treated at 600 MPa on d 21; these cheeses showed the lowest esterase activity values. Acetic acid and all FFA groups increased during ripening and refrigerated storage. The 102 volatile compounds detected in cheese belonged to 10 chemical groups (5 aldehydes, 12 ketones, 17 alcohols, 12 acids, 35 esters, 9 hydrocarbons, 5 aromatic compounds, 3 nitrogen compounds, 3 terpenes, and 1 sulfur compound). High-pressure processing influenced the levels of 97 individual compounds, whereas 68 individual compounds varied during refrigerated storage. Total concentrations of all groups of volatile compounds were influenced by HPP, but only ketones, acids, esters, and sulfur compounds varied during refrigerated storage. The lowest total concentrations for most groups of volatile compounds were recorded for the cheese pressurized at 600 MPa on d 21. A principal component analysis combining total concentrations of groups of FFA and volatile compounds discriminated cheeses by age and by the pressure level applied to HPP cheeses.

Key words: high-pressure processing, lipolysis, volatile compound, blue-veined cheese

INTRODUCTION

Flavor, rheological properties, and visual appearance determine cheese quality (Fox and Wallace, 1997). Cheese flavor, probably the main trait influencing its quality, is caused by the interaction of many compounds responsible for taste and aroma. These compounds are produced during manufacture and ripening through the metabolism of lactose, lactate, and citrate, the liberation of FFA, and the degradation of caseins to peptides and free amino acids (McSweeney and Sousa, 2000; Collins et al., 2003). Primary degradation is followed by the secondary catabolism of the resulting products to compounds that, in many cases, have higher flavor impact than their respective precursors. More than 600 volatile compounds have been identified in cheese, most of which have been associated with particular odor and aroma notes (Molimard and Spinnler, 1996; Curioni and Bosset, 2002).

Coagulant enzymes, together with lactic starter cultures and their enzymes, are responsible for the biochemical changes occurring during the manufacture and ripening of semihard and hard cheeses made from pasteurized milk, because most of the microorganisms and enzymes present in raw milk have been inactivated by the thermal treatment. In the case of blue-veined cheeses, *Penicillium roqueforti* is an additional major ripening agent responsible for their unique flavor. *Penicillium roqueforti* consumes lactic acid, causing an increase in cheese pH value favorable for many chemical reactions, produces extracellular proteinases and lipases (Gripon et al., 1977; Lamberet and Menassa, 1983), and has the ability to form methyl ketones through the β-oxidation of FFA followed by a decarboxylation reaction (Kinsella and Hwang, 1976).

The activity of enzymes and microorganisms persists during the refrigerated storage of ripe cheese at distribution and retail, which can cause over-ripening if levels of flavor compounds above the desired balanced concentrations for a particular cheese variety are attained. Thus, the cheese purchased by the con-

Received July 2, 2013.
Accepted September 5, 2013.
[1]Corresponding author: nunez@inia.es

sumer may have a stronger or different flavor than the manufacturer intended (Wick et al., 2004). Blue-veined cheeses, because of their richness in enzymatic activities, seem particularly prone to over-ripening defects during the refrigerated storage of ripe cheese. An approach to prevent over-ripening and prolong the shelf life of ripe cheese is frozen storage. Although cheese flavor remains unchanged at thawing, both texture and visual appearance are negatively affected by freezing (Tejada et al., 2000; Van Hekken et al., 2005).

High-pressure processing (**HPP**), with a negligible effect on flavor characteristics, meets the increasing consumer demand for fresh-tasting, minimally processed foods. It has been successfully applied to milk and cheese for the inactivation of pathogenic and spoilage microorganisms (O'Reilly et al., 2000; Arqués et al., 2006). In addition, HPP may be a useful tool for the inactivation of enzymes present in cheese such as proteinases (García-Risco et al., 2003; Huppertz et al., 2004), peptidases (Malone et al., 2003; Juan et al., 2007), and esterases (Ávila et al., 2007). The formation of volatile compounds in cheese is also influenced by HPP, at a variable degree that depends on the pressure level applied and the age of cheese at the time of treatment (Ávila et al., 2006; Arqués et al., 2007). Consequently, HPP seems a feasible procedure to prevent over-ripening during the refrigerated storage of blue-veined cheese.

In a previous study, we reported the effect of HPP on the proteolysis and formation of biogenic amines in blue-veined cheese made from ovine milk (Calzada et al., 2013). However, the effects of HPP on the lipolysis and formation of volatile compounds in blue-veined cheese are not well known. In the only work published on the subject (Voigt et al., 2010), the authors did not find significant differences in the concentrations of FFA and methyl ketones when comparing pressurized and control blue-veined cheeses, a result that could be ascribed to the short refrigerated storage period of cheeses after HPP (only 28 d). In the present work, we investigated the influence of HPP applied to ovine milk blue-veined cheese at 400 or 600 MPa on d 21, 42, or 63 after manufacture on the lipolysis and formation of volatile compounds during a 90-d ripening period and a further 270-d refrigerated storage period.

MATERIALS AND METHODS

Cheese Manufacture and HPP

The manufacturing procedure of blue-veined cheese from pasteurized ovine milk was described in a previous work (Calzada et al., 2013). Two batches of blue-veined cheese were made on consecutive days, each from 1,200 L of milk inoculated with lactic cultures and *P. roqueforti*. Cheeses, 18 cm in diameter and 10 cm high, were ripened at 10°C and 93% relative humidity until d 30 and then at 5°C from d 30 to d 90. After 90 d, they were held at 3°C until d 360.

Cheeses were pressurized at 400 or 600 MPa for 5 min, after 21, 42, or 63 d of ripening, as described by Calzada et al. (2013). Treatments were coded as **400W3**, **600W3**, **400W6**, **600W6**, **400W9**, and **600W9** according to the pressure level applied (400 or 600 MPa) and the age of cheese (3, 6, or 9 wk) at pressurization. Cheeses were unpackaged after HPP, and ripening and storage proceeded under the same conditions as for control cheese.

A different cheese per treatment (1 control and 6 HPP) was sampled at each of the times of analysis. Two 100-g pieces per cheese were wrapped in aluminum foil, vacuum-packaged, and frozen at −40°C for chemical analyses.

FFA Determination

Acetic acid, propionic acid, and FFA from butyric ($C_{4:0}$) to linolenic acid ($C_{18:3}$) in cheese were determined by gas chromatography with flame-ionization detection, as described by Fernández-García et al. (2006), with elution in 8 mL of diethyl ether containing 2% formic acid. Frozen cheese pieces were thawed overnight at 4°C before analysis. At all sampling times, acids were extracted from cheeses using a solid-phase extraction technique, with pentanoic, nonanoic, and heptadecanoic acids added as internal standards. A Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, Las Rozas, Spain) equipped with an automatic sampler (HP 7683), a split/splitless injector, a FFAP column (Agilent Technologies, 30 m × 0.32 mm i.d. × 0.25 μm film thickness) and a flame-ionization detector was used for the analysis. Injection (1 μL of sample) was performed in split mode at 1:20 split ratio, at 260°C. Helium was the carrier gas, with the flow set for maintaining a constant pressure of 0.80 kg/cm^2. For chromatographic separation, the temperature was increased from 65 to 240°C at a rate of 10°C/min, and held at 240°C for 12.5 min. Fifteen standard solutions of FA were used for the calculation of calibration curves. Individual FFA were separated, identified, and quantified, and their concentrations expressed in milligrams per gram of cheese DM.

Determination of Esterase Activity

Esterase activity was determined in duplicate on cheese extracts according to the method described by Ávila et al. (2007) with some modifications. Ten grams

of cheese was homogenized with 20 mL of phosphate buffer (0.1 *M*, pH 7.0) in an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany), followed by centrifugation at 10,000 × *g* for 20 min at 4°C and filtering through Whatman No. 2 paper (Whatman International Ltd., Maidstone, UK). The chromogenic substrate was α-naphthylbutyrate (Sigma-Aldrich, Steinhem, Germany). The assay mixture contained 30 μL of chromogenic substrate, 600 μL of distilled water, and different volumes (100, 200, or 400 μL) of cheese homogenate and phosphate buffer (0.1 *M*, pH 7.5) to a final volume of 1,230 μL. The assay mixture was incubated for 1 h at 37°C in a water bath and centrifuged at 12,000 × *g* for 5 min at room temperature. Finally, 900 μL of supernatant was mixed with 150 μL of Fast Red TR salt (Sigma-Aldrich) aqueous solution (2.7 mg/mL). After 5 min at room temperature, the absorbance was measured at 537 nm using a DU650 spectrophotometer (Beckman Coulter Inc., Brea, CA). Esterase activity was calculated from absorbance values in the range of 0.1 to 0.9 by means of a α-naphthol standard curve. One unit of enzymatic activity was defined as the amount of enzyme that liberated 1 pmol of α-naphthol per minute and gram of cheese at 37°C and pH 7.5.

Determination of Volatile Compounds

Volatile compounds were extracted from cheese using a solid-phase microextraction method (Mallia et al., 2005). Five grams of cheese was homogenized in a mechanical grinder with 35 g of Na_2SO_4 and 100 μL of an aqueous solution of 1,058 mg/L cyclohexanone as internal standard. Two grams of the mixture was weighed in a 15-mL headspace glass vial sealed with a polytetrafluoroethylene (PTFE)-faced silicone septum (Supelco, Bellefonte, PA). Vials were submerged in a thermostat-controlled bath at 30°C (D3 model, Haake, Berlin, Germany) for both equilibration (20 min) and extraction (30 min) phases. A solid-phase microextraction manual holder equipped with a 75-μm StableFlex carboxen/polydimethylsiloxane (CAR/PDMS) coated fiber (Supelco) was inserted through the PTFE septum for headspace extraction, after which it was inserted into the GC injection port for desorption (270°C/10 min in splitless mode). Before use, the fiber was conditioned in the injection port of the GC (300°C/ 1 h) as recommended by manufacturer. After each run, the fiber was cleaned up to avoid carryover problems and, periodically, fiber sensitivity was tested with an aqueous solution of the internal standard. All analyses were run using the same fiber unit.

Chromatography (GC-MS) was carried out in a capillary column (60 m long, 0.25 mm i.d., 0.5 μm film thickness; Zebron-WAX plus, Phenomenex, Torrance, CA), with helium flow at 1.4 mL/min for 1 min followed by 1 mL/min, and the following temperature program: 7 min at 40°C, first ramp 2°C/min to 90°C, second ramp 3°C/min to 150°C, final ramp 9°C/min to 240°C, and 8 min at 240°C. Detection was performed with electron impact ionization, with 70 eV ionization energy operating in the full-scan mode at 1.74 scans/s. Source and quadrupole temperatures were 230 and 150°C, respectively. Compound identification was carried out by injection of commercial standards and by spectra comparison using the Wiley7Nist05 Library (Wiley and Sons Inc., Weinheim, Germany). The sum of abundances of characteristic ions was used for semiquantitation of compounds. The relative abundances of volatile compounds were calculated by multiplying the respective peak areas by 10^3 and dividing by the cyclohexanone peak area..

Statistical Treatment

Data obtained were analyzed by a 2-way ANOVA, with treatment (6 HPP treatments and control) and cheese age as the main effects. Means were compared using Tukey's test, with significance declared at $P \leq 0.05$. Principal component analysis was carried out on the total concentrations of groups of FFA and volatile compounds of 180-d and 360-d cheeses for the discrimination of samples according to treatment and cheese age. The SPSS Win 14.0 software (SPSS Inc., Chicago, IL) was used for the statistical analysis of data.

RESULTS AND DISCUSSION

Acetic and Propionic Acids

Acetic acid derives mostly from metabolism of lactose, lactate, and citrate by lactic acid bacteria and other microorganisms (McSweeney and Sousa, 2000). Both treatment and cheese age significantly influenced ($P < 0.001$) the concentration of acetic acid in blue-veined ovine milk cheese (Table 1). In control cheese, it increased during ripening from 1.11 mg/g of DM on d 1 to 1.62 mg/g of DM on d 90, and afterward declined slightly during refrigerated storage, to 1.39 mg/g of DM on d 360 (Table 2). High-pressure processing of cheeses on d 21 arrested the production of acetic acid, independently of the pressure level applied, more markedly than in cheeses pressurized on d 42 or d 63. At the end of ripening, the 400W3 and 600W3 cheeses showed the lowest concentrations of acetic acid, differences that persisted until d 360. Acetic acid, a major odorant of Cheddar, Gruyère, and Emmental cheeses (Curioni and Bosset, 2002), plays an important role in cheese flavor and aroma by itself and as a substrate for

Table 1. Levels of significance of the ANOVA main effects high-pressure processing (HPP) treatment and cheese age (T), and their interaction (HPP × T), on the total concentrations of the main groups of carboxylic acids and volatile compounds in ovine milk blue-veined cheeses

Chemical compound or group	Factor		
	HPP	T	HPP × T
Acetic acid	***	***	***
Short-chain FFA	***	***	**
Medium-chain FFA	***	***	***
Long-chain FFA	***	***	***
Aldehydes	***	NS	***
Ketones	***	***	***
Alcohols	***	NS	NS
Acids	***	***	*
Esters	***	**	**
Hydrocarbons	***	NS	NS
Aromatic compounds	***	NS	NS
Nitrogen compounds	***	NS	*
Terpenes	***	NS	NS
Sulfur compounds	*	**	NS

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

ester formation through esterification reactions. In the present work, the decline in acetic acid concentration generally observed during refrigerated storage may be ascribed to ester formation.

Propionic acid was found at low concentrations, ranging from 0.01 to 0.03 mg/g of cheese DM (data not shown), during ripening and refrigerated storage of control and HPP cheeses, without a clear influence of treatment or cheese age. Propionic acid, mainly formed by lactate-metabolizing microorganisms, is characteristic of Swiss-type cheeses and has also been detected in Cheddar and Camembert cheeses (Curioni and Bosset, 2002). Within blue-veined varieties, its presence has been reported only in Gorgonzola cheese, at low concentrations that accounted for 0.4 to 0.6% of total carboxylic acids (Moio et al., 2000).

Branched-chain carboxylic acids, deriving from the metabolism of leucine, isoleucine, and valine (Yvon and Rijnen, 2001), were not detected in control or HPP cheeses. These compounds have been found in many cheese varieties (Curioni and Bosset, 2002), including Gorgonzola cheese, in which they represented up to 4.4% of total carboxylic acids (Moio et al., 2000). Branched-chain carboxylic acids may be formed by certain strains of lactic acid bacteria and gram-negative bacteria such as *Pseudomonas* spp. (Morales et al., 2005). The high microbiological quality of the cheeses studied in the present work, with contaminating bacteria at very low levels (Calzada et al., 2013), probably impeded the formation of these compounds.

Free Fatty Acids

Short-chain (**SC**, $C_{4:0}$ to $C_{8:0}$) FFA originate from esterase- or lipase-mediated hydrolysis of triacylglycerides, but also from the fermentation of lactose and lactate, from the degradation of amino acids, and from the oxidation of some ketones, esters, and aldehydes (Molimard and Spinnler, 1996; Collins et al., 2003). In the present work, the formation of SC FFA was significantly ($P < 0.001$) influenced by treatment and cheese age (Table 1). Total SC FFA concentration increased 51.7-fold during ripening of control cheese from d 1 to 90, and 2.9-fold during refrigerated storage until d 360 (Table 2). The respective levels of individual SC FFA $C_{4:0}$, $C_{6:0}$, and $C_{8:0}$ were 1.32, 0.85, and 0.93 mg/g of DM on d 90, and reached 2.94, 2.39, and 3.56 mg/g of DM on d 360.

Table 2. Concentrations of acetic acid and short-chain free fatty acids (SC FFA) during ripening and refrigerated storage of ovine milk blue-veined control and high-pressure processed (HPP) cheeses pressurized at 400 or 600 MPa after 3, 6, or 9 wk (W3, W6, W9) of ripening[1]

Chemical compound or group	Day	Control	400W3	600W3	400W6	600W6	400W9	600W9
Acetic acid	1	1.11 ± 0.03						
	21	0.63 ± 0.10^{b}	0.45 ± 0.08^{b}	0.19 ± 0.02^{a}				
	42	1.37 ± 0.09^{c}	0.52 ± 0.05^{ab}	0.24 ± 0.02^{a}	0.83 ± 0.09^{b}	0.62 ± 0.09^{b}		
	63	1.72 ± 0.21^{d}	0.61 ± 0.07^{ab}	0.39 ± 0.02^{a}	1.08 ± 0.13^{bc}	0.75 ± 0.08^{abc}	1.21 ± 0.11^{bcd}	1.35 ± 0.10^{cd}
	90	1.62 ± 0.15^{c}	0.50 ± 0.06^{a}	0.44 ± 0.07^{a}	1.72 ± 0.18^{c}	1.20 ± 0.14^{bc}	1.51 ± 0.23^{c}	0.79 ± 0.12^{ab}
	180	1.67 ± 0.12^{d}	0.46 ± 0.01^{ab}	0.39 ± 0.02^{a}	1.33 ± 0.03^{cd}	0.87 ± 0.12^{bc}	0.92 ± 0.10^{bc}	0.68 ± 0.09^{ab}
	270	1.49 ± 0.09^{d}	0.55 ± 0.09^{ab}	0.36 ± 0.02^{a}	1.11 ± 0.09^{cd}	0.70 ± 0.04^{abc}	1.42 ± 0.09^{d}	0.92 ± 0.08^{bc}
	360	1.39 ± 0.11^{d}	0.55 ± 0.02^{ab}	0.28 ± 0.01^{a}	1.42 ± 0.07^{d}	0.72 ± 0.06^{bc}	1.07 ± 0.06^{cd}	0.58 ± 0.03^{ab}
SC FFA ($C_{4:0}$-$C_{8:0}$)	1	0.06 ± 0.01						
	21	0.72 ± 0.15^{b}	0.74 ± 0.11^{b}	0.19 ± 0.08^{a}				
	42	2.82 ± 0.38^{bc}	0.82 ± 0.16^{a}	0.34 ± 0.10^{a}	3.25 ± 0.22^{c}	2.47 ± 0.39^{b}		
	63	2.37 ± 0.20^{b}	0.91 ± 0.19^{a}	0.88 ± 0.21^{a}	3.95 ± 0.36^{c}	3.50 ± 0.42^{bc}	3.81 ± 0.16^{c}	2.25 ± 0.25^{ab}
	90	3.10 ± 0.33^{ab}	1.44 ± 0.10^{a}	1.20 ± 0.41^{a}	5.78 ± 0.58^{c}	5.35 ± 0.90^{c}	4.50 ± 0.91^{bc}	5.66 ± 0.69^{c}
	180	5.06 ± 0.49^{c}	1.92 ± 0.08^{ab}	1.70 ± 0.80^{a}	6.38 ± 0.78^{c}	4.40 ± 0.85^{abc}	5.95 ± 0.83^{c}	4.56 ± 0.53^{bc}
	270	6.32 ± 0.68^{b}	2.83 ± 0.43^{a}	1.58 ± 0.54^{a}	7.94 ± 0.93^{b}	6.67 ± 1.08^{b}	7.20 ± 1.16^{b}	7.74 ± 0.91^{b}
	360	8.88 ± 0.78^{cd}	5.21 ± 0.84^{ab}	2.21 ± 0.42^{a}	11.81 ± 1.29^{d}	7.17 ± 0.16^{bc}	10.02 ± 1.02^{cd}	7.55 ± 0.55^{bc}

[a–d]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Results are expressed in milligrams per gram of cheese DM.

Table 3. Concentrations of medium-chain (MC) and long-chain (LC) FFA during ripening and refrigerated storage of ovine milk blue-veined control and high-pressure processed (HPP) cheeses pressurized at 400 or 600 MPa after 3, 6, or 9 wk (W3, W6, W9) of ripening[1]

Chemical group	Day	Control	400W3	600W3	400W6	600W6	400W9	600W9
MC FFA ($C_{10:0}$-$C_{14:0}$)	1	0.27 ± 0.07						
	21	0.92 ± 0.28^{b}	0.92 ± 0.17^{b}	0.32 ± 0.11^{a}				
	42	3.86 ± 0.83^{b}	1.10 ± 0.14^{a}	0.78 ± 0.26^{a}	5.67 ± 0.78^{b}	3.61 ± 0.63^{b}		
	63	3.29 ± 0.39^{abc}	1.38 ± 0.23^{a}	2.03 ± 0.55^{ab}	6.30 ± 1.14^{c}	5.22 ± 0.80^{bc}	5.89 ± 0.32^{c}	3.01 ± 0.41^{abc}
	90	5.47 ± 0.54^{abc}	1.79 ± 0.15^{a}	3.60 ± 1.22^{ab}	10.84 ± 1.73^{bc}	11.57 ± 1.61^{c}	7.93 ± 1.47^{abc}	11.53 ± 1.96^{c}
	180	12.17 ± 1.40^{b}	2.70 ± 0.51^{a}	4.25 ± 1.01^{a}	14.26 ± 2.26^{b}	9.31 ± 1.66^{ab}	12.27 ± 2.67^{b}	7.96 ± 1.82^{ab}
	270	14.26 ± 1.79^{b}	4.33 ± 0.88^{a}	3.84 ± 1.27^{a}	14.64 ± 2.01^{b}	10.95 ± 1.80^{b}	16.67 ± 2.36^{b}	14.40 ± 2.45^{b}
	360	18.31 ± 1.72^{bc}	11.84 ± 2.59^{ab}	4.50 ± 0.19^{a}	25.74 ± 3.32^{c}	13.55 ± 0.87^{ab}	18.60 ± 2.07^{bc}	12.92 ± 1.96^{ab}
LC FFA ($C_{16:0}$-$C_{18:3}$)	1	1.06 ± 0.13						
	21	5.62 ± 1.21^{b}	5.44 ± 1.57^{b}	2.74 ± 0.88^{a}				
	42	16.94 ± 2.67^{bc}	5.52 ± 1.19^{a}	3.85 ± 0.70^{a}	24.44 ± 2.43^{c}	13.21 ± 2.73^{ab}		
	63	15.16 ± 1.51^{abc}	5.98 ± 1.40^{a}	6.72 ± 1.12^{a}	28.06 ± 3.15^{c}	23.49 ± 3.04^{bc}	19.98 ± 1.32^{bc}	10.38 ± 1.08^{ab}
	90	19.21 ± 1.83^{abc}	7.13 ± 0.94^{a}	11.50 ± 2.79^{ab}	34.69 ± 4.02^{bc}	38.77 ± 4.86^{c}	26.70 ± 5.02^{abc}	37.85 ± 6.69^{c}
	180	36.14 ± 4.00^{c}	10.55 ± 1.23^{a}	12.19 ± 3.82^{ab}	41.09 ± 6.06^{c}	31.19 ± 7.06^{abc}	39.84 ± 6.82^{c}	32.89 ± 5.02^{bc}
	270	44.12 ± 5.62^{b}	12.09 ± 2.29^{a}	13.01 ± 2.55^{a}	45.20 ± 7.50^{b}	32.00 ± 8.50^{ab}	50.28 ± 8.95^{b}	45.01 ± 7.39^{b}
	360	56.63 ± 4.54^{bc}	38.30 ± 7.96^{ab}	15.11 ± 1.07^{a}	66.85 ± 9.08^{c}	44.17 ± 3.40^{bc}	54.66 ± 5.87^{bc}	40.23 ± 6.81^{b}

[a–c]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Results are expressed in milligrams per gram of cheese DM.

Medium-chain (**MC**, $C_{10:0}$ to $C_{14:0}$) FFA are formed through the lipase-mediated hydrolysis of triacylglycerides. *Penicillium roqueforti* produces 2 extracellular lipases, an acidic lipase and an alkaline lipase that retains activity at pH 4.5 (Lamberet and Menassa, 1983). The formation of MC FFA was significantly ($P < 0.001$) influenced by treatment and cheese age (Table 1). Total MC FFA concentration increased 20.3-fold during ripening of control cheese and 3.3-fold during refrigerated storage (Table 3). Individual MC FFA $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ levels were 2.32, 1.17, and 1.99 mg/g of DM, respectively, on d 90 and attained 7.89, 3.80, and 6.62 mg/g of DM on d 360.

Long-chain (**LC**, $C_{16:0}$ to $C_{18:3}$) FFA in blue-veined cheeses are mostly derived from triacylglycerides, by the action of *P. roqueforti* lipases. The accumulation of LC FFA in cheese was significantly ($P < 0.001$) influenced by treatment and cheese age (Table 1). Total LC FFA concentration increased 18.1-fold during ripening of control cheese and 2.9-fold during refrigerated storage (Table 3). The major LC FFA in control cheese, $C_{16:0}$ and $C_{18:1}$, reached levels of 4.31 and 11.14 mg/g of DM, respectively, on d 90, and 19.43 and 27.03 mg/g of DM on d 360. As previously recorded for Stilton cheese by Madkor et al. (1987), LC FFA were present at higher concentrations than SC and MC FFA.

Pressurization of cheeses on d 21 limited the formation of SC, MC, and LC FFA (Tables 2 and 3). In contrast, Voigt et al. (2010) did not observe significant differences between the FFA content of pressurized and control blue-veined cheeses. In the present work, the effect was more marked for 600W3 cheese than for 400W3 cheese. On d 360, the concentrations of total SC, MC, and LC FFA in 600W3 cheese were less than half those in 400W3 cheese. Milk lipoprotein lipase was presumably inactivated by milk pasteurization in the present work.

Lactic acid bacteria esterases may withstand cheese pressurization, according to Ávila et al. (2007). To our knowledge, no information on the barotolerance of *P. roqueforti* lipolytic enzymes has been reported. The esterase activity values found in the present work, up to 38.53 pmol of α-naphthol per min per gram for control cheese on d 42 (Table 4), were markedly higher than those reported for a non-mold-ripened variety, which ranged from 0.49 to 1.39 pmol of α-naphthol per min per gram of cheese (Ávila et al., 2007). The high esterase activity values of blue-veined cheese must be attributed to *P. roqueforti* lipolytic enzymes. These enzymes exhibited a certain baroresistance, according to the data presented in Table 4. Even in the 600W3 cheese, which showed the lowest ($P < 0.05$) esterase activity values throughout ripening and refrigerated storage, esterase activity reached 9.12 pmol of α-naphthol per min per gram on d 90 of ripening. Total FFA concentrations in 90-d cheeses did not correlate significantly with the respective esterase activity values. However, total FFA concentrations in 360-d cheeses correlated significantly ($P < 0.05$) with the respective esterase activity values, and even more strongly ($P < 0.01$) with the esterase activity values obtained for the respective cheeses on d 90. Levels of significance for the correlations between SC, MC, and LC FFA and esterase activity values equaled those found for total FFA and esterase activity.

Butanoic acid plays an important role in the flavor of many cheese varieties, although at high concentrations (usually in cheeses with the late blowing defect caused by the butyric acid fermentation of lactate by

Table 4. Esterase activity during ripening and refrigerated storage of ovine milk blue-veined control cheese and high-pressure processed (HPP) cheeses pressurized at 400 or 600 MPa after 3, 6, or 9 wk (W3, W6, W9) of ripening[1]

Day	Control	400W3	600W3	400W6	600W6	400W9	600W9
21	16.72 ± 0.40^{c}	12.62 ± 0.59^{b}	4.66 ± 0.67^{a}				
42	38.53 ± 1.35^{d}	13.71 ± 0.80^{b}	4.98 ± 0.43^{a}	28.85 ± 0.12^{c}	17.74 ± 2.21^{b}		
63	23.78 ± 0.67^{b}	15.19 ± 1.05^{ab}	6.57 ± 1.05^{a}	26.80 ± 2.41^{b}	15.96 ± 1.47^{ab}	13.96 ± 0.61^{ab}	10.17 ± 1.90^{a}
90	22.01 ± 2.47^{bc}	17.82 ± 4.02^{abc}	9.12 ± 2.30^{a}	27.36 ± 3.00^{c}	12.46 ± 0.96^{ab}	19.53 ± 0.91^{abc}	16.17 ± 2.60^{abc}
180	33.56 ± 2.25^{bc}	12.49 ± 0.46^{a}	7.65 ± 0.36^{a}	34.47 ± 4.03^{c}	28.37 ± 5.96^{bc}	19.64 ± 3.13^{ab}	10.71 ± 2.67^{a}
270	31.74 ± 0.33^{d}	10.89 ± 1.43^{ab}	5.52 ± 0.80^{a}	32.12 ± 3.76^{d}	17.02 ± 0.72^{bc}	32.01 ± 1.48^{d}	19.75 ± 0.49^{c}
360	28.16 ± 2.06^{c}	8.41 ± 0.43^{ab}	4.31 ± 0.22^{a}	26.18 ± 0.88^{c}	5.95 ± 0.23^{ab}	11.63 ± 0.10^{b}	8.47 ± 0.71^{ab}

$^{a–d}$Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Results are expressed in picomoles of α-naphthol per minute and gram of cheese.

clostridia), it becomes undesirable. Hexanoic and octanoic acids are characteristic flavor compounds of aged Grana Padano and Roncal cheeses (Curioni and Bosset, 2002). Medium-chain FFA such as $C_{10:0}$ and $C_{12:0}$ are key aroma compounds in varieties such as Cheddar, Roncal, and probably others, because of their relatively low perception thresholds. In contrast, LC FFA have high perception thresholds, which limit their contribution to cheese flavor, in spite of the high concentrations commonly reached in many cheese types (Curioni and Bosset, 2002).

Volatile Compounds

One hundred two compounds were identified in the volatile fraction of ovine milk blue-veined cheese by solid-phase microextraction followed by GC-MS. Lower numbers of volatile compounds have generally been reported for other blue-veined cheeses made exclusively from bovine milk, such as Gorgonzola or Stilton (Moio et al., 2000; Gkatzionis et al., 2009), or including ovine milk in their composition, such as Cabrales and Gamonedo (González de Llano et al., 1990; De Frutos et al., 1991), although 108 volatile compounds were identified or tentatively identified in ovine milk Roquefort cheese (Gallois and Langlois, 1990). The 102 volatile compounds identified in the present work included 5 aldehydes, 12 ketones, 17 alcohols, 12 acids, 35 esters, 9 hydrocarbons, 5 aromatic compounds, 3 nitrogen compounds, 3 terpenes, and 1 sulfur compound. Total concentrations of all groups of volatile compounds were significantly influenced by HPP, but only ketones, acids, esters, and sulfur compounds varied significantly with cheese age during refrigerated storage (Table 1). Out of the 102 individual volatile compounds, 97 were significantly influenced by HPP (86 compounds at $P < 0.001$), whereas 68 varied significantly with cheese age (52 compounds at $P < 0.001$).

Total aldehydes reached higher levels in control cheese and 400 MPa cheeses than in 600 MPa cheeses on d 180 (Table 5), with acetaldehyde being the main aldehyde at that time. During cheese manufacture and the first days of ripening, acetaldehyde may be formed through the metabolism of lactose, but later it mostly derives from the catabolism of threonine (McSweeney and Sousa, 2000). The population of lactic acid bacteria, which declined on average 0.5 log cfu/g in 400 MPa cheeses and 4.2 log cfu/g in 600 MPa cheeses immediately after HPP, attained respective mean counts of 8.0, 7.1, and 3.5 log cfu/g in control, 400 MPa, and 600 MPa cheeses on d 180 (Calzada et al., 2013). The lower acetaldehyde content of 600 MPa cheeses on d 180 may be explained by their low lactic acid bacteria counts and total aerobic counts, which preclude a metabolic activity capable of influencing cheese chemical parameters. Total aldehydes increased in 600 MPa cheeses from d 180 to 360 due to the formation of 3-methylbutanal (the main aldehyde on d 360), 2-methylbutanal, and 2-methylpropanal from leucine, isoleucine, and valine, respectively, during the last stages of refrigerated storage. The increase in total aldehydes recorded from d 180 to 360 in 600 MPa cheeses cannot be attributed to microbial metabolism, because of the low counts of lactic acid bacteria, *P. roqueforti*, and other microbial groups in those cheeses. Therefore, abiotic chemical reactions seem the most plausible origin of the branched-chain aldehydes produced from d 180 to 360 in 600 MPa cheeses, which doubled their contents during this period. Aldehydes are key odorants in cheese, with green, sweet, pungent notes for acetaldehyde; green, malty, acrid, pungent notes that turn into pleasant fruity at low concentrations for 3-methylbutanal; malty, nutty notes for 2-methylbutanal; and malty, floral notes for 2-methylpropanal (Curioni and Bosset, 2002).

Total ketones reached their maximum concentration on d 180 in control cheese (Table 5), with 2-pentanone, 2-heptanone, and 2-propanone as the major ketones. *Penicillium roqueforti* is a main producer of methyl ketones via the β-oxidation and decarboxylation of FFA, with enhanced production by germinating spores

Table 5. Levels of total volatile aldehydes, ketones, alcohols, acids, and esters during refrigerated storage of ovine milk blue-veined control and high-pressure processed (HPP) cheeses pressurized at 400 or 600 MPa after 3, 6, or 9 wk (W3, W6, W9) of ripening[1]

Volatile group	Day	Control cheese	400W3	600W3	400W6	600W6	400W9	600W9
Aldehydes	180	15.26 ± 1.43^{b}	14.51 ± 1.39^{b}	5.32 ± 0.26^{a}	15.34 ± 1.65^{b}	6.63 ± 0.38^{a}	10.40 ± 1.46^{ab}	5.40 ± 0.66^{a}
	360	10.64 ± 0.88^{a}	10.58 ± 0.66^{a}	8.88 ± 1.14^{a}	7.06 ± 0.78^{a}	11.74 ± 1.74^{a}	9.57 ± 0.72^{a}	11.35 ± 0.79^{a}
Ketones	180	$1{,}285 \pm 178^{b}$	809 ± 107^{ab}	397 ± 25^{a}	355 ± 39^{a}	847 ± 126^{ab}	649 ± 106^{a}	726 ± 149^{ab}
	360	$1{,}364 \pm 152^{cd}$	$1{,}717 \pm 201^{d}$	670 ± 18^{ab}	$1{,}837 \pm 133^{d}$	$1{,}005 \pm 91^{abc}$	$1{,}280 \pm 112^{bcd}$	534 ± 53^{a}
Alcohols	180	$1{,}452 \pm 105^{d}$	806 ± 21^{bc}	209 ± 9^{a}	$1{,}070 \pm 21^{c}$	512 ± 74^{ab}	881 ± 95^{bc}	332 ± 28^{a}
	360	$1{,}496 \pm 83^{c}$	744 ± 54^{b}	239 ± 7^{a}	$1{,}281 \pm 115^{c}$	481 ± 26^{ab}	775 ± 46^{b}	242 ± 13^{a}
Acids	180	$2{,}465 \pm 107^{b}$	$1{,}517 \pm 144^{a}$	$1{,}441 \pm 211^{a}$	$2{,}631 \pm 55^{b}$	$2{,}506 \pm 88^{b}$	$2{,}804 \pm 123^{b}$	$2{,}844 \pm 88^{b}$
	360	$4{,}211 \pm 166^{c}$	$2{,}955 \pm 529^{ab}$	$2{,}090 \pm 212^{a}$	$4{,}455 \pm 270^{c}$	$3{,}611 \pm 77^{bc}$	$4{,}390 \pm 112^{c}$	$3{,}971 \pm 64^{bc}$
Esters	180	868 ± 95^{d}	200 ± 15^{ab}	47.2 ± 6.1^{a}	698 ± 52^{cd}	284 ± 23^{ab}	506 ± 66^{bc}	190 ± 14^{ab}
	360	$1{,}493 \pm 167^{c}$	420 ± 36^{ab}	61.2 ± 4.8^{a}	760 ± 55^{b}	311 ± 30^{ab}	841 ± 24^{b}	173 ± 15^{a}

[a–d]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Results are expressed as relative abundances with respect to the internal standard.

(Kinsella and Hwang, 1976). On d 180, *P. roqueforti* counts reached 7.1 log cfu/g in control cheese and 5.4 log cfu/g on average in 400 MPa cheeses, whereas *P. roqueforti* counts were below the detection level in 600 MPa cheeses (Calzada et al., 2013). These differences in *P. roqueforti* counts may help to explain the higher ketone content in control cheese than in HPP cheeses on d 180. Reduced formation of ketones during the refrigerated storage of pressurized blue-veined cheeses compared with control cheese has been reported by Voigt et al. (2010). In the present work, the formation of methyl ketones in 600 MPa cheeses, with *P. roqueforti* counts below detection level following HPP treatments, needs further explanation. Either sublethally injured *P. roqueforti* cells unable to grow on selective media carried out the chemical reactions responsible for methyl ketone formation or these reactions took place abiotically in the absence of viable *P. roqueforti* cells. From d 180 to 360, we observed marked increases in the ketone content of 400 MPa cheeses, whereas ketones remained stable in control cheese. Methyl ketones are the characteristic flavor compounds of blue-veined cheeses, in particular, 2-heptanone, which has a blue cheese note and a low perception threshold, but also 2-pentanone, with a sweet fruity odor note, and 2-propanone, with hay and wood pulp odor notes (Molimard and Spinnler, 1996).

Total alcohols were at higher levels in control cheese and 400 MPa cheeses than in 600 MPa cheeses on d 180 (Table 5). The main alcohols at d 180 were 2-pentanol, 2-propanol, and ethanol, followed by 1-butanol, 2-heptanol, and 3-methyl-1-butanol. The formation of 2-alkanols through the reduction of the corresponding 2-alkanones by both spores and mycelium of *P. roqueforti* has been suggested as a cellular detoxifying mechanism (Kinsella and Hwang, 1976). The lowest content of 2-alkanols was found in 600W3 cheese, in which *P. roqueforti* had the shortest period to exert its metabolic activity before being inactivated by HPP. The formation of other alcohols is independent from the metabolic activity of *P. roqueforti*. Branched-chain 3-methyl-1-butanol is produced through the reduction of the respective aldehyde, derived from leucine (Yvon and Rijnen, 2001). Ethanol is considered to be mostly produced in cheese through the fermentation of lactose by lactic acid bacteria (Fox and Wallace, 1997). However, its content doubled in control cheese from d 180 to 360, a stage at which lactose, glucose, and galactose were certainly exhausted, whereas ethanol content did not vary in HPP cheeses. Reduction of acetaldehyde seems a plausible source of ethanol during the refrigerated storage of control cheese. Reduction of 1-butanal may be also the origin of 1-butanol, which increased from d 180 to 360 in control cheese but not in HPP cheeses. Primary alcohols show green, alcoholic notes, and secondary alcohols have fruity, herbaceous notes, whereas 3-methyl-1-butanol has pleasant, fresh cheese notes (Curioni and Bosset, 2002). They all contribute to cheese flavor, directly and as substrates for ester formation.

Total volatile acids attained lower levels on d 180 in 400W3 and 600W3 cheeses (Table 5). The main acids on d 180 as determined by GC-MS were butanoic, hexanoic, and acetic acids, which accounted for 54, 29, and 12%, respectively, of total acids in cheeses, on average. Total acids increased in all cheeses from d 180 to 360, more so in control cheese and 400 MPa cheeses than in 600 MPa cheeses, in spite of their contribution to ester formation, with significant increases of all the individual acids. Because acids are formed through microbial metabolism and lipolysis, these results indicate that both phenomena probably remained active even after HPP of cheese at the higher pressure level.

Total esters were negatively affected by HPP, in particular at 600 MPa, with 600W3 cheese showing the lowest ester contents on d 180 and 360, and control

cheese the highest contents (Table 5). Ethyl butanoate, methyl hexanoate, and ethyl hexanoate were the major esters on d 180, followed by methyl butanoate, ethyl octanoate, and methyl octanoate. On d 360, ethyl butanoate, ethyl hexanoate, and methyl hexanoate were predominant, followed by ethyl octanoate, methyl butyrate, and ethyl acetate. Esters may be formed in cheese by esterification, from alcohols and carboxylic acids, or by alcoholysis, a transferase reaction in which fatty acyl groups from acylglycerols and acyl-CoA derivatives are directly transferred to alcohols, the second mechanism being the major one in lactic acid bacteria and yeasts (Liu et al., 2004). To our knowledge, the existence of alcohol acyltransferases in *Penicillium* spp. has not been reported. It may be hypothesized that ester formation in control and 400 MPa cheeses took place by both mechanisms, whereas in 600 MPa cheeses, with a negligible microbial population, the first mechanism would predominate. Esters generally show sweet, fruity, and floral notes, have a low perception threshold, and are key odorants in many cheeses. At low concentrations, esters contribute positively to the overall flavor balance in cheese, by themselves and by masking the effect of unclean off-flavors with pungent, sharp, cowy, and barny notes. At higher concentrations, a fruity flavor defect may arise (Curioni and Bosset, 2002; Liu et al., 2004). Two cyclic esters, γ-butyrolactone and γ-caprolactone, were detected at low levels and increased moderately from d 180 to 360. Their highest contents were found in control and 400 MPa cheeses. Lactones are considered to be generated by the hydrolysis of hydroxy-fatty acid triglycerides followed by lactonization (Jolly and Kosikowski, 1975). They generally have pronounced fruity flavor notes and a low perception threshold, and may be of importance to the aroma of some cheese varieties such as Bleu d'Auvergne (Gallois and Langlois, 1990). However, pungent, fetid, buttery notes have been assigned to γ-butyrolactone (Molimard and Spinnler, 1996).

Total hydrocarbons tended to reach higher levels in control cheese than in HPP cheeses, although the differences were significant ($P < 0.05$) only for 600W3, 400W9, and 600W9 cheeses on d 180 and for 600W9 cheeses on d 360 (Table 6). We observed no significant increase in the hydrocarbon content during refrigerated storage of cheeses from d 180 to 360. The main hydrocarbons found—octane, hexane, and pentamethylheptane—have solvent, gasoline-like odor notes. The presence of hydrocarbons, generally derived from lipid oxidation (Carbonell et al., 2002; Collins et al., 2003), has been reported in ovine milk cheeses such as La Serena and Manchego (Carbonell et al., 2002; Fernández-García et al., 2002) but not in Roquefort, Gorgonzola, or Stilton blue-veined cheeses (Gallois and Langlois, 1990; Moio et al., 2000; Gkatzionis et al., 2009).

Total aromatic compounds were generally at lower levels in 400W3 and 600W3 cheeses than in the others (Table 6). Early HPP treatment, on d 21, which severely impaired growth and metabolism of *P. roqueforti* (Calzada et al., 2013), was more crucial than the pressure level applied. The main aromatic compounds found were toluene and 1-methoxy-4-methylbenzene, which did not vary significantly from d 180 to 360; phenol, which declined; and 4-methyl-phenol, which increased. Aromatic compounds may be formed in cheese through the microbial catabolism of aromatic amino acids (Yvon and Rijnen, 2001). Toluene and phenol have been detected in the volatile fraction of cheeses made from ovine raw milk (Carbonell et al., 2002; Fernández-García et al., 2004), but not in blue-veined cheeses (Gallois and Langlois, 1990; Lawlor et al., 2003).

Total nitrogen compounds were significantly influenced by HPP and did not vary with cheese age from

Table 6. Levels of total volatile hydrocarbons, aromatic compounds, terpenes, nitrogen compounds, and sulfur compounds during refrigerated storage of ovine milk blue-veined control and high-pressure processed (HPP) cheeses pressurized at 400 or 600 MPa after 3, 6, or 9 wk (W3, W6, W9) of ripening[1]

Volatile group	Day	Control	400W3	600W3	400W6	600W6	400W9	600W9
Hydrocarbons	180	10.29 ± 0.85^{b}	7.84 ± 1.00^{ab}	6.31 ± 0.90^{a}	6.61 ± 0.62^{ab}	7.27 ± 1.12^{ab}	6.30 ± 0.59^{a}	5.76 ± 0.48^{a}
	360	9.86 ± 1.27^{b}	8.14 ± 0.73^{ab}	6.27 ± 0.57^{ab}	6.93 ± 0.70^{ab}	6.87 ± 0.79^{ab}	6.00 ± 0.45^{ab}	4.56 ± 0.28^{a}
Aromatic compounds	180	17.37 ± 1.61^{c}	8.86 ± 0.29^{ab}	5.71 ± 0.63^{a}	14.44 ± 0.91^{bc}	13.67 ± 0.88^{bc}	10.42 ± 0.13^{ab}	14.03 ± 0.68^{bc}
	360	15.71 ± 1.43^{c}	8.33 ± 0.38^{ab}	5.42 ± 0.09^{a}	11.94 ± 0.45^{bc}	13.05 ± 0.52^{bc}	11.05 ± 0.45^{b}	10.79 ± 0.42^{b}
Nitrogen compounds	180	3.37 ± 0.17^{ab}	2.26 ± 0.28^{a}	3.14 ± 0.38^{ab}	3.91 ± 0.27^{b}	3.67 ± 0.28^{b}	3.78 ± 0.31^{b}	3.46 ± 0.41^{ab}
	360	5.40 ± 0.60^{b}	2.89 ± 0.45^{a}	2.41 ± 0.20^{a}	4.06 ± 0.40^{ab}	3.34 ± 0.40^{ab}	4.38 ± 0.69^{ab}	3.29 ± 0.34^{ab}
Terpenes	180	1.50 ± 0.10^{b}	1.12 ± 0.08^{ab}	0.89 ± 0.02^{a}	1.72 ± 0.22^{b}	1.64 ± 0.13^{b}	1.43 ± 0.16^{ab}	1.47 ± 0.13^{ab}
	360	1.90 ± 0.27^{b}	1.05 ± 0.07^{ab}	0.56 ± 0.09^{a}	1.36 ± 0.22^{ab}	1.66 ± 0.10^{b}	1.53 ± 0.06^{ab}	1.43 ± 0.12^{ab}
Sulfur compounds	180	0.95 ± 0.07^{a}	0.80 ± 0.07^{a}	0.72 ± 0.06^{a}	1.00 ± 0.10^{a}	1.01 ± 0.05^{a}	1.04 ± 0.05^{a}	0.90 ± 0.04^{a}
	360	1.18 ± 0.12^{a}	0.94 ± 0.10^{a}	0.92 ± 0.07^{a}	1.22 ± 0.13^{a}	1.04 ± 0.12^{a}	1.18 ± 0.10^{a}	1.05 ± 0.10^{a}

[a–c]Means in the same row followed by different superscripts differ ($P < 0.05$).

[1]Mean ± SE (n = 4) of duplicate determinations in 2 cheese-making experiments. Results are expressed as relative abundances with respect to the internal standard.

d 180 to 360 (Tables 1 and 6). Methanamide, butyramide, and 2,6-dimethylpyrazine, the nitrogen compounds detected in the present work, were at low levels in all cheeses (data not shown). The concentrations of butyramide and 2,6-dimethylpyrazine more than doubled in control cheese from d 180 to 360, whereas they remained largely constant in HPP cheeses during this period. The increases of these compounds in control cheese might be associated with the metabolism of cheese microbiota, but their origin could not be traced to a particular microbial group.

Total terpenes attained lower levels in 400W3 and 600W3 cheeses than in the other cheeses (Table 6). Terpenes are secondary plant metabolites, whose presence in an ovine milk cheese not ripened by molds has been traced to the feed (Fernández-García et al., 2008). Additionally, *P. roqueforti* strains cultivated on synthetic media have been reported to produce terpenes (Chalier and Crouzet, 1993). Pressurization of cheeses on d 21, which impeded normal growth of *P. roqueforti* (Calzada et al., 2013), likely hindered terpene synthesis during ripening and refrigerated storage of 400W3 and 600W3 cheeses. Terpenes found in the present work were endoborneol and 2 unidentified sesquiterpenes, all common volatile compounds in ovine milk cheeses (Carbonell et al., 2002) but not in blue-veined cheeses (Lawlor et al., 2003). In contrast to our results, the latter authors reported the presence of α-pinene and limonene in the 6 blue-veined varieties they investigated. Terpenes confer herbaceous, floral, and fruity odor notes, and may be useful as geographical markers of cheese origin (Bugaud et al., 2001).

Dimethylsulfone, the only sulfur compound detected in blue-veined ovine milk cheese, varied with HPP and cheese age (Tables 1 and 6). Dimethylsulfone has been detected in Cheddar cheese at levels that doubled from medium-age to extra sharp cheese (Burbank and Qian, 2005). It can be formed through the oxidation of dimethylsulfide (Al-Attabi et al., 2009) or may derive directly from the pasture, because it is present in cruciferous and bulbaceous plants. More common sulfur compounds such as dimethylsulfide or dimethyldisulfide (formed through the degradation of methionine), which have been reported in other blue-veined cheese varieties (Gallois and Langlois, 1990; Lawlor et al., 2003), were not found in HPP or control cheeses in the current study.

The sensory characteristics of control cheeses and HPP cheeses except the 600W3 cheese showed negligible variations during the refrigerated storage period, according to the results obtained in a previous work (Calzada et al., 2013). In contrast, the flavor quality of the 600W3 cheese declined during refrigerated storage, which can be attributed to the lower contents of some groups of flavor compounds, which might be unsatisfactorily balanced from a sensory point of view.

Principal Component Analysis

Principal component analysis was carried out on the total concentrations of the 4 groups of carboxylic acids (acetic + propionic, SC FFA, MC FFA, and LC FFA) determined by GC and the total levels of the 10 groups of volatile compounds (aldehydes, ketones, alcohols, acids, esters, hydrocarbons, aromatic compounds, nitrogen compounds, terpenes, and sulfur compounds) determined by GC-MS. Two functions explained each over 10% of the variance. Function 1, formed by the 4 groups of carboxylic acids and 8 groups of volatile compounds (excluding aldehydes and hydrocarbons), explained 50.6% of the variance, whereas function 2, formed by aldehydes and hydrocarbons, explained 15.7% of the variance. Function 1 was associated with cheese age, and function 2 with absence of (control) or milder (400 MPa) HPP treatment. When cheeses were plotted against functions 1 and 2 (Figure 1), function 1 correctly separated all the 360-d cheeses, located in the positive semi-plane, from all the 180-d cheeses, located in the negative semi-plane, with the only exception being the 360-d 600W3 cheese, which was located in the negative semi-plane. The decelerating effect of early HPP (d 21) at the higher pressure level (600 MPa) on the biochemical changes occurring during cheese ripening and refrigerated storage was thus highlighted. The contents of FFA and volatile compounds in the 360-d 600W3 cheese were closer to those of 180-d cheeses than to the contents of 360-d cheeses.

CONCLUSIONS

High-pressure processing influenced the total concentrations of all groups of carboxylic acids and volatile compounds found in ovine milk blue-veined cheese. The greatest reduction in the concentrations of carboxylic acids during ripening and refrigerated storage was recorded for the cheese pressurized at 600 MPa on d 21, which showed the lowest esterase activity values. Control cheese was richest in alcohols, esters, and aromatic compounds, whereas cheese pressurized at 600 MPa on d 21 showed the lowest contents of aldehydes, ketones, alcohols, acids, esters, aromatic compounds, and terpenes. The reduced levels of flavor compounds in the 600W3 cheese, which were associated with low counts of *P. roqueforti* and other microbial groups, resulted in lower flavor quality scores during refrigerated storage. The high concentrations of carboxylic acids and volatile compounds in control cheese and the other HPP cheeses did not impair their sensory characteristics, probably

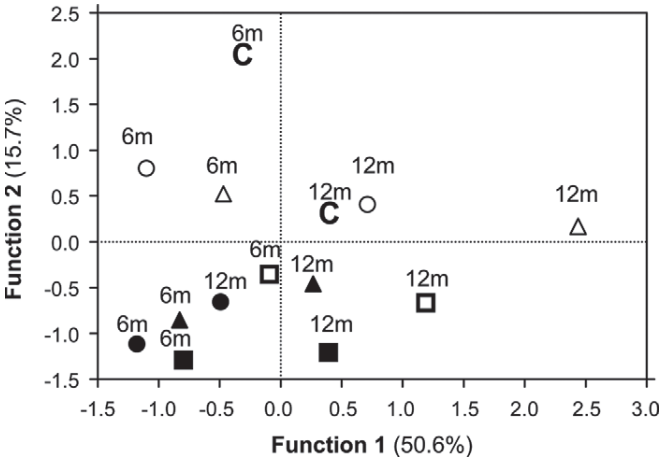


Figure 1. Distribution of control and high-pressure processed (HPP) blue-veined cheeses pressurized at 400 or 600 MPa on d 21, 42, or 63 after manufacture (W3, W6, W9) on the plane defined by functions 1 and 2 of the principal component analysis. Each symbol represents the averaged value of the 2 batches of cheese. Treatments are as follows: C = control; ○ = 400W3; ● = 600W3; Δ = 400W6; ▲ = 600W6; □ = 400W9; ■ = 600W9. Cheese age is as follows: 180 d = 6 mo; 360 d = 12 mo.

because an adequate balance of flavor compounds was maintained during refrigerated storage.

ACKNOWLEDGMENTS

This research was funded by AGL 2009-07801 project from the Ministry of Science and Innovation (MICINN, Spain). J. Calzada is the recipient of a FPI grant (MICINN, Spain). The authors thank the valuable help of V. Carbonero (Lácteas Toledo, Guadamur, Spain) with cheese supply and of F. Purroy (NC Hyperbaric, Burgos, Spain) with high pressure treatments.

REFERENCES

Al-Attabi, Z., B. R. D'Arcy, and H. C. Deeth. 2009. Volatile sulphur compounds in UHT milk. Crit. Rev. Food Sci. Nutr. 49:28–47.

Arqués, J. L., S. Garde, E. Fernández-García, P. Gaya, and M. Nuñez. 2007. Volatile compounds, odor, and aroma of La Serena cheese high-pressure treated at two different stages of ripening. J. Dairy Sci. 90:3627–3639.

Arqués, J. L., S. Garde, P. Gaya, M. Medina, and M. Nuñez. 2006. Inactivation of microbial contaminants in raw milk La Serena cheese by high-pressure treatments. J. Dairy Sci. 89:888–891.

Ávila, M., J. Calzada, S. Garde, and M. Nuñez. 2007. Effect of a bacteriocin-producing *Lactococcus lactis* strain and high-pressure treatment on the esterase activity and free fatty acids in Hispánico cheese. Int. Dairy J. 17:1415–1423.

Ávila, M., S. Garde, E. Fernández-García, M. Medina, and M. Nuñez. 2006. Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the odor and aroma of Hispánico cheese: Correlation of volatile compounds and sensory analysis. J. Agric. Food Chem. 54:382–389.

Bugaud, C., S. Buchin, J. B. Coulon, A. Hauwuy, and D. Dupont. 2001. Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk. Lait 81:401–414.

Burbank, H. M., and M. C. Qian. 2005. Volatile sulfur compounds in Cheddar cheese determined by headspace solid-phase microextraction and gas chromatograph-pulsed flame photometric detection. J. Chromatogr. A 1066:149–157.

Calzada, J., A. del Olmo, A. Picon, P. Gaya, and M. Nuñez. 2013. Proteolysis and biogenic amine buildup in high-pressure treated ovine milk blue-veined cheese. J. Dairy Sci. 96:4816–4829.

Carbonell, M., M. Nuñez, and E. Fernández-García. 2002. Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavor characteristics. Lait 82:683–698.

Chalier, P., and J. Crouzet. 1993. Production of volatile components by *Penicillium roqueforti* cultivated in the presence of soya bean oil. Flavour Frag. J. 8:43–49.

Collins, Y. F., P. L. H. McSweeney, and M. G. Wilkinson. 2003. Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. Int. Dairy J. 13:841–866.

Curioni, P. M. G., and J. O. Bosset. 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12:959–984.

De Frutos, M., J. Sanz, and I. Martinez-Castro. 1991. Characterization of artisanal cheeses by GC and GC/MS analysis of their medium volatility (SDE) fraction. J. Agric. Food Chem. 39:524–530.

Fernández-García, E., M. Carbonell, J. Calzada, and M. Nuñez. 2006. Seasonal variation of the free fatty acids contents of Spanish ovine milk cheeses protected by a designation of origin: A comparative study. Int. Dairy J. 16:252–261.

Fernández-García, E., M. Carbonell, P. Gaya, and M. Nuñez. 2004. Evolution of the volatile components of ewes raw milk Zamorano cheese. Seasonal variation. Int. Dairy J. 14:701–711.

Fernández-García, E., M. Carbonell, and M. Nuñez. 2002. Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. J. Dairy Res. 69:579–593.

Fernández-García, E., M. Imhof, H. Schlichtherle-Cerny, J. O. Bosset, and M. Nuñez. 2008. Terpenoids and benzenoids in La Serena cheese made at different seasons of the year with a *Cynara cardunculus* extract as coagulant. Int. Dairy J. 18:147–157.

Fox, P. F., and J. M. Wallace. 1997. Formation of flavor compounds in cheese. Adv. Appl. Microbiol. 45:17–85.

Gallois, A., and D. Langlois. 1990. New results in the volatile odorous compounds of French cheeses. Lait 70:89–106.

García-Risco, M. R., I. Recio, E. Molina, and R. López-Fandiño. 2003. Plasmin activity in pressurized milk. J. Dairy Sci. 86:728–734.

Gkatzionis, K., R. S. T. Linforth, and C. E. R. Dodd. 2009. Volatile profile of Stilton cheeses: Differences between zones within a cheese and dairies. Food Chem. 113:506–512.

González de Llano, D., M. Ramos, C. Polo, J. Sanz, and I. Martinez-Castro. 1990. Evolution of the volatile components of an artisanal blue cheese during ripening. J. Dairy Sci. 73:1676–1683.

Gripon, J.-C., M. J. Desmazeaud, D. Le Bars, and J. L. Bergère. 1977. Role of proteolytic enzymes of *Streptococcus lactis, Penicillium roqueforti*, and *Penicillium caseicolum* during cheese ripening. J. Dairy Sci. 60:1532–1538.

Huppertz, T., P. F. Fox, and A. L. Kelly. 2004. Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. J. Dairy Res. 71:496–499.

Jolly, R. C., and F. V. Kosikowski. 1975. Quantification of lactones in ripening pasteurized milk blue cheese containing added microbial lipases. J. Agric. Food Chem. 23:1175–1176.

Juan, B., V. Ferragut, M. Buffa, B. Guamis, and A. Trujillo. 2007. Effects of high pressure on proteolytic enzymes in cheese: Relationship with the proteolysis of ewe milk cheese. J. Dairy Sci. 90:2113–2125.

Kinsella, J. E., and D. Hwang. 1976. Biosynthesis of flavors by *Penicillium roqueforti*. Biotechnol. Bioeng. 18:927–938.

Lamberet, G., and A. Menassa. 1983. Purification and properties of an acid lipase from *Penicillium roqueforti*. J. Dairy Res. 50:459–468.

Lawlor, J. B., C. M. Delahunty, J. Sheehan, and M. G. Wilkinson. 2003. Relationships between sensory attributes and the volatile

compounds, non-volatile and gross compositional constituents of six blue-type cheese. Int. Dairy J. 13:481–494.
Liu, S.-Q., R. Holland, and V. L. Crow. 2004. Esters and their biosynthesis in fermented dairy products: A review. Int. Dairy J. 14:923–945.
Madkor, S., P. F. Fox, S. I. Shalabi, and N. H. Metwalli. 1987. Studies on the ripening of Stilton cheese: Lipolysis. Food Chem. 25:93–109.
Mallia, S., E. Fernández-García, and J. O. Bosset. 2005. Comparison of purge and trap and solid phase microextraction techniques for studying the volatile aroma compounds of three European PDO hard cheeses. Int. Dairy J. 15:741–758.
Malone, A. S., C. Wick, T. H. Shellhammer, and P. D. Courtney. 2003. High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. J. Dairy Sci. 86:1139–1146.
McSweeney, P. L. H., and M. J. Sousa. 2000. Biochemical pathways for the production of flavour compounds in cheese during ripening: A review. Lait 80:293–324.
Moio, L., L. Piombino, and F. Addeo. 2000. Odour-impact compounds of Gorgonzola cheese. J. Dairy Res. 67:273–285.
Molimard, P., and H. E. Spinnler. 1996. Compounds involved in the flavour of surface mold-ripened cheeses: Origins and properties. J. Dairy Sci. 79:169–184.
Morales, P., E. Fernández-García, and M. Nuñez. 2005. Volatile compounds produced in cheese by *Pseudomonas* strains of dairy origin belonging to six different species. J. Agric. Food Chem. 53:6835–6843.
O'Reilly, C. E., P. M. O'Connor, A. L. Kelly, T. P. Beresford, and P. M. Murphy. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl. Environ. Microbiol. 66:4890–4896.
Tejada, L., R. Gómez, M. Vioque, E. Sánchez, C. Mata, and J. Fernández-Salguero. 2000. Effect of freezing and frozen storage on the sensorial characteristics of Los Pedroches, a Spanish ewe milk cheese. J. Sens. Stud. 15:251–262.
Van Hekken, D. L., M. H. Tunick, and Y. W. Park. 2005. Effect of frozen storage on the proteolytic and rheological properties of soft caprine milk cheese. J. Dairy Sci. 88:1966–1972.
Voigt, D. D., F. Chevalier, M. C. Qian, and A. L. Kelly. 2010. Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavor compounds in mature blue-veined cheese. Innov. Food Sci. Emerg. Technol. 11:68–77.
Wick, C., U. Nienaber, O. Anggraeni, T. H. Shellhammer, and P. D. Courtney. 2004. Texture, proteolysis and viable lactic acid bacteria in commercial Cheddar cheeses treated with high pressure. J. Dairy Res. 71:107–115.
Yvon, M., and L. Rijnen. 2001. Cheese flavour formation by amino acid catabolism. Int. Dairy J. 11:185–201.

Capítulo 4.

Using high-pressure processing for reduction of proteolysis and prevention of over-ripening of raw milk cheese.

Fotografía: cortes de queso control (no presurizado) de Torta del Casar a día 60 (superior),120 (centro) y 240 (inferior).

Food Bioprocess Technol (2014) 7:1404–1413
DOI 10.1007/s11947-013-1141-5

ORIGINAL PAPER

Using High-Pressure Processing for Reduction of Proteolysis and Prevention of Over-ripening of Raw Milk Cheese

Javier Calzada · Ana del Olmo · Antonia Picon · Pilar Gaya · Manuel Nuñez

Received: 20 February 2013 / Accepted: 29 May 2013 / Published online: 13 June 2013

Abstract High-pressure-processing (HPP) at 400 or 600 MPa was applied to cheeses made from ewe raw milk, on days 21 or 35 after manufacturing, to reduce proteolysis and prevent over-ripening. The characteristics of HPP and non-pressurized (control) cheeses were compared during ripening at 8 °C until day 60 and further storage at 4 °C until day 240. HPP and control cheeses showed similar pH values throughout ripening, but on day 240 pH values remained 0.4–0.6 units lower for HPP cheeses than for the control cheeses. Casein degradation was significantly retarded in the 600 MPa cheeses. Their α-casein concentration was 48–52 % higher on day 60 and 30–33 % higher on day 240 than in the control cheeses while β-casein concentration was 25–26 % higher on day 60 and 100–103 % higher on day 240. No significant differences in para-κ-casein concentration between cheeses were found on day 60, but on day 240, it was 22–35 % higher in the 600 MPa cheeses than in the control cheese. Hydrophilic peptides, hydrophobic peptides and total free amino acids evolved similarly in HPP and control cheeses during the 60-day ripening period. However, on day 240 hydrophilic peptides were at 34–39 % lower levels in the 600 MPa cheeses than in the control cheeses, hydrophobic peptides at 7–16 % lower levels and total free amino acids at 25–29 % lower levels. Flavour intensity scores increased at a slower rate in HPP cheeses than in the control cheese. Flavour quality declined markedly in the control cheeses during refrigerated storage while it did not vary significantly in 600 MPa cheeses.

Keywords High-pressure processing · Proteolysis · Over-ripening · Cheese · Flavour

J. Calzada · A. del Olmo · A. Picon · P. Gaya · M. Nuñez (✉)
Departamento de Tecnología de Alimentos, INIA,
Carretera de La Coruña Km 7, Madrid 28040, Spain
e-mail: nunez@inia.es

Introduction

The breakdown of proteins and lipids, and the metabolism of lactose and citrate, are primary biochemical events which take place during cheese manufacturing and early ripening. Afterwards, secondary biochemical events such as the further hydrolysis of peptides and the catabolism of amino acids, fatty acids and lactate, give rise to the formation of the compounds responsible for the characteristic flavour and aroma of each particular cheese variety (McSweeney and Sousa 2000; Collins et al. 2003; Yvon and Rijnen 2001). Simultaneously, the typical texture and the microstructure of the product develop (O'Reilly et al. 2003; Picon et al. 2013b). Secondary biochemical events occurring during mid- and late ripening pursue during refrigerated storage of cheese at the dairy and during its shelf life at retailers and homes. Consequently, over-ripening defects may surge before consumption, particularly in strongly proteolyzed cheese varieties.

When exceeding a certain threshold, ammonia, amines, alcohols, aldehydes, carboxylic acids and thiol compounds formed in cheese through amino acid catabolism (Yvon and Rijnen 2001) are among the main causative agents for the flavor and aroma defects associated to over-ripening. Freezing of fully ripened cheeses has been assayed to prevent over-ripening and prolong their shelf life, by stopping or retarding enzyme activity and chemical reactions. Even though flavour characteristics of cheeses remained unchanged during frozen storage, texture defects at thawing were common (Tejada et al. 2000; Van Hekken et al. 2005).

High-pressure processing (HPP) is a technology that can achieve the food safety level of heat pasteurization whilst meeting consumer demand for fresher-tasting minimally processed foods (Norton and Sun 2008). The inactivation of pathogenic and spoilage microorganisms has been the main objective when applying HPP to milk and cheese (O'Reilly et al. 2000; Trujillo et al. 2000; Shao and Ramaswamy 2011).

In addition, HPP could be useful to stop or retard some of the chemical reactions taking place during cheese ripening, since cheese-related enzymes, including the proteinases and peptidases responsible for the formation of peptides and free amino acids (FAA), are affected by HPP (García-Risco et al. 2003; Malone et al. 2003; Juan et al. 2007). Pressure level is more crucial to enzyme inactivation than temperature or time of exposure, such as shown for chymosin which loses less than 5 % of its activity in Cheddar cheese at 400 MPa and 93–96 % at 600 MPa (Huppertz et al. 2004b). HPP has been reported to hinder the formation of FAA by some authors. Thus, lower FAA concentrations were found in 1-month-old Cheddar cheese pressurized at 400 MPa on day 1 than in the respective control cheese (O'Reilly et al. 2002) and in 7-month-old Cheddar cheeses pressurized on day 30 at 400, 500 or 800 MPa than in the respective control cheese (Wick et al. 2004). Within the current applications of emerging technologies as alternatives to milk pasteurization in cheese manufacture, pulsed electric fields have also been assayed, with satisfactory results (Yu et al. 2012).

In the present work, we applied HPP to Casar cheese, a variety made from ewe raw milk coagulated with an aqueous extract of *Cynara cardunculus* L. (cardoon) flowers. This extract contains the aspartic proteinases named cyprosins or cynarases, with optimum activity around pH 5.1 (Heimgartner et al.1990). The strong proteolytic activity of cyprosins, the microbial load of raw milk and the high pH value of the cheese thus produced make it prone to over-ripening. Because of the seasonality in ewe milk production, ripe cheeses made during the first half of the year may be held at refrigeration temperatures for several months before marketed. This is also valid for similar varieties such as La Serena and Los Pedroches cheeses in Spain, and Serra da Estrela and Azeitao cheeses in Portugal. With the objective of preventing the undesirable consequences of a prolonged storage period, we applied HPP to cheeses 3 or 5 weeks after manufacture. The breakdown of caseins, the formation of peptides and FAA, the texture and the sensory characteristics of HPP cheeses during a 60-day ripening period and a further 180-day refrigerated storage period were investigated and compared with the characteristics of control cheese.

Materials and Methods

Cheese Manufacture

Two batches of Casar cheese were made on consecutive days, each from 600 L of refrigerated ewe raw milk without added starter cultures, at a Protected Designation of Origin dairy. Milk was coagulated at 30 °C for 60 min in a semi-automated open vat with an aqueous extract obtained by macerating 600 g of dry cardoon flowers overnight in 6 L of tap water and filtering through a cheese cloth. Curds were cut into 10-mm cubes, held at 30 °C for 15 min and distributed into cylindrical moulds. Cheeses, 13 cm in diameter and 6-cm high, were pressed for 3 h in a horizontal press, salted by rubbing dry salt twice on all the surfaces, and ripened at 8 °C and 92 % relative humidity.

High-Pressure Processing

Cheeses coded as 400W3, 600W3, 400W5 and 600W5 were vacuum-packaged in CN300 bags (Cryovac Grace S.A., Barcelona, Spain) and pressurized at 400 or 600 MPa for 5 min, after 3 or 5 weeks of ripening, respectively. HPP was performed in a 120-L capacity isostatic press (Hiperbaric, Burgos, Spain). Times to reach 400 and 600 MPa were 1.85 and 2.83 min, respectively and depressurization times, 7 and 8 s. The temperature of the water used as transmitting fluid remained under 14 °C during the whole process. Pressurized cheeses were unpackaged after HPP. Control cheeses were vacuum-packaged after 3 weeks of ripening and unpackaged simultaneously with cheeses pressurized at that time. Cheeses were ripened at 8 °C and 92 % RH until day 60, and afterwards held at 4 °C until day 240.

Chemical Determinations

Caseins and whey proteins were determined on duplicate samples by capillary gel electrophoresis according to a previously described method (Garde et al. 2002) with some modifications, on an automated P/ACE^{TM} MDQ capillary electrophoresis apparatus controlled by the 32 Karat Software (Beckman Instruments España S.A., Madrid, Spain). Briefly, 5 g of cheese were mixed with 25 mL of 2 % trisodium citrate solution, homogenized for 1 min in an Ultra-Turrax T-10 blender (IKA, Staufen, Germany) at high speed on ice. Twenty microlitres of cheese homogenate was mixed with 170 μL of 100 mM Tris–HCl buffer (pH 9.0) containing 1 % SDS, 10 μL of 2-mercaptoethanol and 4 μL of a 10-kDa internal standard (Beckman), and heated at 95 °C for 10 min before injection at 5 kV. Electrophoretic separation was performed at 15 kV for 30 min after a 4-min ramp, in a bare-fused silica capillary column (Beckman) of 50 μm internal diameter and 30 cm total length, in SDS-buffer gel (Beckman). To calculate the MW of peaks monitored at 214 nm, the coefficient of relative time mobility to the internal standard was compared with those of a mixture of 10, 20, 35, 50, 100, 150 and 225 kDa protein standards (Beckman, SDS-MW protein size standard). Commercial standards (Sigma, Alcobendas, Spain) of bovine α-casein, β-casein, κ-casein, α-lactalbumin, β-lactalbumin, serum albumin and lactoferrin were used for the identification of proteins. Proteins were quantified with respect to the internal standard area and expressed as milligramme of protein per gramme of cheese dry matter (DM).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by RP-HPLC using a Beckman System Gold chromatograph (Beckman Instruments España) equipped with a diode array detector module 168, with detection wavelength at 280 nm, as previously described (Lau et al. 1991). Peaks with retention times from 5.5 to 14.6 min were considered to correspond to hydrophilic peptides and those with retention times from 14.6 to 20.5 min to hydrophobic peptides. Peptide levels were expressed in arbitrary units, calculated as units of chromatogram area per milligramme of cheese DM.

Free amino acids were extracted from duplicate samples (Krause et al. 1995) and individual FAA determined by RP-HPLC using a Beckman System Gold chromatograph, after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. They were expressed as milligrammes per gramme of cheese DM.

Cheese pH was measured in triplicate directly with a Crison penetration electrode (model 52–3,2; Crison Instruments, Barcelona, Spain) by means of a Crison GPL 22 pH-meter. Dry matter was determined on triplicate samples after drying to constant weight in an oven at 102 °C.

Cyprosin activity and overall peptidolytic activity in cheese were determined on duplicate samples. A homogenate of 5 g of cheese, 25 mL of 2 % trisodium citrate solution and 2 g of skim milk was acidified with 1 N HCl to pH 5.2, favourable for cyprosin activity, and incubated for 3 h at 37 °C. Then, the pH was adjusted with 1 N NaOH to 6.5, favourable for peptidase activity, and the incubation was prolonged for 3 h. The OPA test (Church et al. 1983), based on the reaction of released α-amino groups with this compound and with β-mercaptoethanol to form an adduct that absorbs strongly at 340 nm, was run on 0-h, 3-h and 6-h samples. Cyprosin activity was estimated as the increase in absorbance (OPA method) from 0 to 3 h during the incubation at pH 5.2 and 37 °C. Overall, peptidolytic activity was estimated as the increase in absorbance (OPA method) from 3 to 6 h during the incubation at pH 6.5, which reduces drastically cyprosin activity, and 37 °C. To discard the possible interference of milk plasmin with cyprosins or enzymes of bacterial origin present in cheese samples, the same assay was run on milk samples (taken from the vat, without added cardoon extract) at pH 6.7, the initial pH value of milk, and at pH values of 6.2, 5.7 and 5.2, after acidification with 1 N HCl.

Textural Determinations

Six cylinder-shaped (17-mm height, 17-mm diameter) samples from each cheese were compressed to 75 % of their original height after 10 min at room temperature (20–22 °C) using an Instron Compression Tester 4301 (Instron, High Wycombe, Bucks, UK), with crosshead and chart speeds of 50 and 500 mm/min, respectively. Fracturability (force at breaking point, expressed in Newtons), elasticity (apparent elastic modulus, expressed in Newtons per square millimetre) and firmness (work done on the cheese, expressed in Joules) were calculated from compression curves (Picon et al. 2013a).

Sensory Evaluation

A trained 15-member panel carried out the evaluation of flavour intensity and quality (preference), scoring on a 10-point scale as previously described (Nuñez et al. 1991). Five additional flavour attributes (acid, bitter, salty, sweet and umami) were also determined by panelists on a 10-point scale (Picon et al. 2013a).

Statistical Analysis

Data obtained were analyzed by a two-way analysis of variance, with HPP treatment and days of ripening as main effects. Means were compared using Tukey's test, with $p=0.05$. The SPSS Win 14.0 software (SPSS, Inc., Chicago, IL) was employed for the statistical analysis of data.

Results and Discussion

Protein Breakdown

Casein concentrations declined significantly ($p<0.001$) with ripening time in control cheese, according to the analysis of variance, from 38.36, 272.55 and 25.76 mg of α-, β- and para-κ-casein per gramme of cheese DM on day 1 (data not shown) to 5.80, 81.93 and 16.09 mg/g DM, respectively, on day 60. These marked decreases in casein levels agree with previous works on cheese varieties made from ewe milk coagulated with cardoon extract (Garde et al. 2007; Delgado et al. 2010). Regarding whey proteins, α-lactalbumin, at a concentration of 1.54 mg/g DM on day 1, could not be detected in control cheese from day 21 onwards (data not shown) while β-lactoglobulin concentration decreased significantly ($p<0.01$) in control cheese with ripening time, from 11.63 mg/g DM on day 1 to 5.13 mg/g DM on day 60.

HPP at 400 or 600 MPa did not significantly influence casein levels immediately after treatment. However, higher α-casein levels were found for the 600 MPa cheeses during ripening and refrigerated storage (Fig. 1), with a concentration of α-casein 48–52 % higher in the 600 MPa cheeses than in control cheeses on day 60 and 30–33 % higher on day 240. The concentration of β-casein in pressurized and control cheeses did not differ significantly during ripening, but it was 100–103 % higher in the 600 MPa cheeses than in the control cheese on day 240. No significant differences in

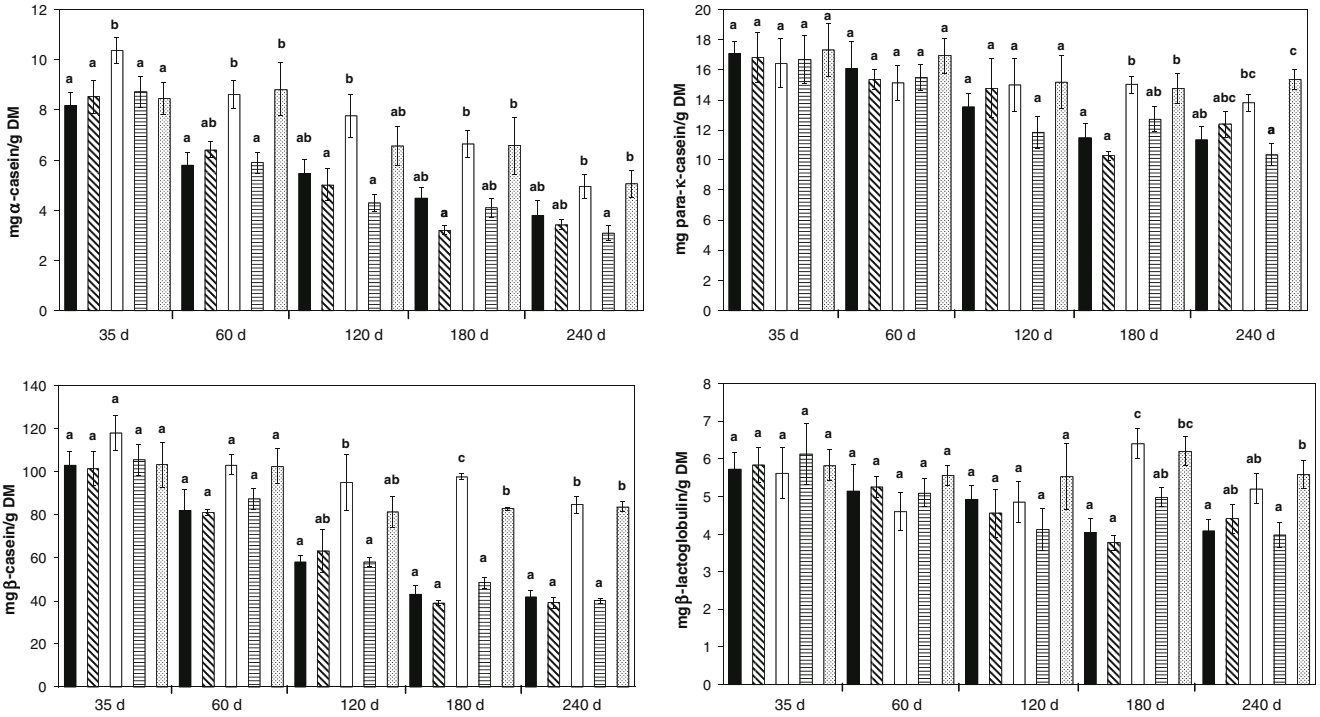


Fig. 1 Concentrations of caseins and β-lactoglobulin during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

para-κ-casein concentration between cheeses were found during ripening but on day 240 it was 22–35 % higher in the 600 MPa cheeses than in the control cheese. Similarly, there were no significant differences in β-lactoglobulin concentration between cheeses on day 60, whereas on day 240 it was 27–37 % higher in the 600 MPa cheeses than in the control cheese.

Protein degradation in the control cheese and in the pressurized cheeses until submitted to HPP can be associated to both plasmin and cyprosins. Cheese pressurization at 400 MPa has a negligible effect on plasmin activity, while treatment at 600 MPa reduces its activity by less than 10 % (Huppertz et al. 2004b). Therefore, considerable plasmin activity would remain in pressurized cheeses. The role of cyprosins in the proteolysis of HPP cheeses is difficult to establish since there is no information available on cyprosine barostability. In La Serena cheese, also made from ewe milk coagulated with cardoon extract, treatments at 300 and 400 MPa on day 2 resulted in higher levels of α_s- and β-caseins on days 30 and 60 of ripening than in the control cheese (Garde et al. 2007).

To ascertain the role of cyprosins in protein breakdown, cyprosin activity was estimated in HPP and control cheeses by determining the increase in absorbance by the OPA method after incubation for 3 h at 37 °C and pH 5.2, a pH value favourable for cyprosin activity (Heimgartner et al. 1990) but unfavourable for the activity of plasmin and most proteinases and peptidases from lactic acid bacteria. Apparently, there was a pressure-induced enhancement of cyprosin activity in 400 MPa cheeses, but only the cyprosin activity of 400W5 cheese on day 240 showed a significant ($p<0.05$) difference when compared with the respective control cheese (Table 1). Increases of activity after HPP have been reported for enzymes such as the cell envelope proteinase and the PepC aminopeptidase of *Lactococcus lactis* MG1363 (Malone et al. 2003). Cyprosin activity values in 600 MPa cheeses did not differ significantly from those of the respective control cheese. A high stability of cyprosins under cheese ripening conditions has been reported (Picon et al. 1999). In the present work, considerable cyprosin activity persisted, even in 600 MPa cheeses, until day 240. The increases in absorbance recorded for cheese samples during the 3 h of incubation at pH 5.2 cannot be attributed to the activity of milk plasmin. When the assay was run on milk without added cardoon extract, the increases of absorbance in the OPA test were as low as 0.022, 0.008, 0.004 and 0.003 for milk samples at pH values of 6.7, 6.2, 5.7 and 5.2, respectively. The overall peptidolytic activity was estimated by further incubation of cheese samples at pH 6.5 for 3 h, which resulted in additional increases in absorbance. The peptidolytic activity in 400 MPa cheeses and in control cheese did not differ (Table 1). However, the peptidolytic activity was 74 % lower in 600W3 cheese and 63 % lower in 600W5 cheese than in control cheese on day 60, while it was 90 % and 89 % lower,

Table 1 Cyprosin activity and peptidolytic activity in control and HPP cheeses at the end of ripening (day 60) and after refrigerated storage (day 240)

Days	Cheese	Cyprosin activity[a]	Peptidolytic activity[a]
60	Control	0.148±0.017ab	0.081±0.008b
	400W3	0.172±0.023ab	0.074±0.005b
	600W3	0.123±0.016a	0.021±0.016a
	400W5	0.207±0.025b	0.107±0.010b
	600W5	0.193±0.013ab	0.030±0.007a
240	Control	0.336±0.071a	0.119±0.038a
	400W3	0.439±0.052a	0.125±0.052a
	600W3	0.346±0.041a	0.012±0.015a
	400W5	0.596±0.045b	0.056±0.037a
	600W5	0.424±0.031a	0.013±0.022a

Results are expressed as mean±SEM of duplicate determinations on two cheese making trials. Means in the same column at the same sampling date with the same letters do not differ significantly

[a] Cyprosin and peptidolytic activities are expressed as increases in absorbance (OPA method)

respectively, on day 240 (Table 1). These results point to the inactivation of peptidolytic enzymes of bacterial origin at 600 MPa, in agreement with previous works (Malone et al. 2003; Avila et al. 2006), and may be of practical interest to control over-ripening at the cheese industry.

Cheese pH and DM increased significantly ($p<0.001$) with time, according to the analysis of variance. They modulate enzyme activity and may influence protein breakdown. Control cheese and HPP cheeses had similar pH values until day 60 (Fig. 2) but afterwards the pH rose more rapidly in the control cheese, which showed significantly ($p<0.05$) higher values than HPP cheeses from day 120 onwards. The higher pH values of the control cheese during refrigerated storage would have been less favourable for the activity of cyprosins, with optimum pH values around 5.1 (Heimgartner et al. 1990), but more favourable for plasmin, with maximal activity at slightly alkaline pH (Visser 1981). This fact may contribute to explain the lower concentrations of residual proteins in the control cheese than in the pressurized cheeses. Regarding cheese DM, no significant differences were found between HPP and control cheeses during ripening, until day 60. At the end of the refrigerated storage period, on day 240, higher ($p<0.05$) DM content was recorded for 600W3, 400W5 and 600W5 cheeses than for the 400W3 cheese and control cheese (Fig. 2).

Peptide and FAA Formation

The levels of hydrophilic and hydrophobic peptides and their ratio were significantly ($p<0.001$) influenced by time, according to the analysis of variance. There were no significant changes in peptide levels attributable to HPP immediately after treatment on days 21 (data not shown) and 35. On day 60, at the end of ripening, the 400W3 cheese had a significantly ($p<0.05$) higher level of hydrophilic peptides than the control cheese (Fig. 3) while the rest of the HPP cheeses did not differ from the control cheese. This result may be ascribed either to an enhancement of cyprosin activity by HPP at 400 MPa, a pressure level which apparently increased its activity, or to changes in the conformation of proteins caused by HPP (García-Risco et al. 2000; Huppertz et al. 2004a) which might have favoured the access of cyprosins to their substrates. The level of hydrophobic peptides on day 60 in control and HPP cheeses did not differ, with the only exception of 400W5 cheese which showed a lower level. In 60-day-old La Serena cheese pressurized at 400 MPa on day 2, hydrophilic peptides also attained a higher level than in the control cheese, while hydrophobic peptides were at a lower level (Garde et al. 2007). The hydrophobic peptides/hydrophilic peptides ratio reached on day 60 values of 1.55–1.68 in HPP cheeses and 1.87 in control cheese, markedly higher than the 0.54 mean value recorded for ewe raw milk Manchego cheese made using animal rennet (Gaya et al. 2005) but close to the 1.50 value found for La Serena cheese (Garde et al. 2007).

The level of hydrophilic peptides increased from day 60 to day 240 by factors of 3.19 in the control cheese and 1.78–2.56

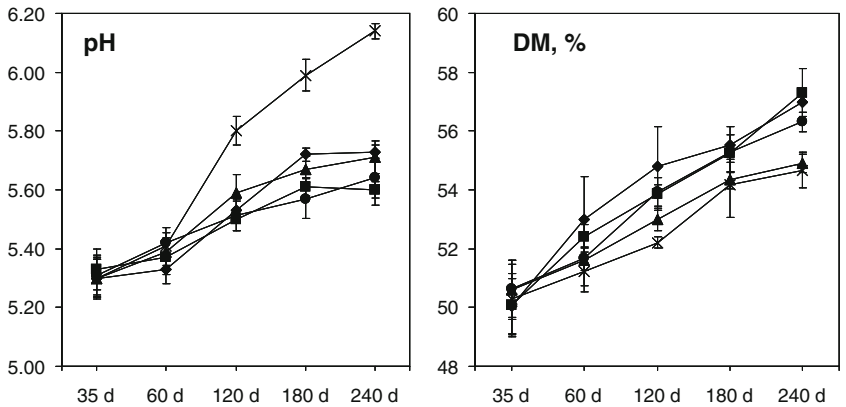


Fig. 2 Values (means with SEM) of pH and dry matter (DM) content during ripening and refrigerated storage of control and HPP cheeses. Control (*X*), 400W3 (*triangle*), 600W3 (*circle*), 400W5 (*diamond*), 600W5 (*square*)

in the HPP cheese. At the end of refrigerated storage, the level of hydrophilic peptides in the control cheese was significantly ($p<0.05$) higher than in the rest (Fig. 3), in agreement with its low concentrations of residual proteins. On the contrary, the levels of hydrophobic peptides hardly varied during refrigerated storage and on day 240 there were no significant differences in the contents of hydrophobic peptides between HPP and control cheeses. The hydrophobic peptides/hydrophilic peptides ratio fell sharply during refrigerated storage, to values of 0.64–0.92 on day 240, with no significant differences between HPP and control cheeses. To our knowledge, no information is available on the post-ripening evolution of peptides in the Casar or La Serena cheeses during the refrigerated storage period, which may span for several months because of the seasonality in milk and cheese production.

The concentration of FAA increased significantly ($p<0.001$) with time. It did not vary immediately after the HPP of cheeses on days 21 (data not shown) and 35. However, total FAA were on day 35 at significantly ($p<0.05$) higher levels in the 400W3 cheese than in the rest (Fig. 3), as already found for hydrophilic peptides, which served as substrates for the activity of peptidolytic enzymes. Pressurization of cheeses at 400 MPa most probably lyses a fraction of the bacterial cells without a negative effect on the activity of the enzymes which are released into the medium (Picon et al. 2013a), thus increasing the extracellular peptidolytic activity in cheese. Higher FAA concentrations on days 30 and 60 of ripening were also found for La Serena cheese pressurized at 400 MPa on day 2 (Garde et al. 2007). In the present work, the FAA concentration was 53 % higher in the 400W3 cheese than in the control cheese on day 60, but afterwards the differences in FAA between the 400W3 cheese and the control cheese were no longer statistically significant. The higher pH values of the control cheese during refrigerated storage most probably enhanced the activity of the peptidolytic enzymes of microbial origin, increasing the FAA concentration to levels close to those of the 400 MPa cheeses. At the end of the refrigerated storage period, on day 240, the cheeses pressurized at 600 MPa showed significantly ($p<0.05$) lower FAA concentrations than the rest (Fig. 3), a result which can be associated to the observed inactivation of peptidolytic enzymes at the higher pressure level (Table 1).

Texture

Firmness and elasticity declined significantly ($p<0.001$) with time. A marked softening of cheese texture was observed during the first weeks of ripening, resulting in the soft creamy texture desirable for this type of cheese. Firmness, as determined from the compression curves, reached low values in HPP and control cheeses (Fig. 4), consequently with the

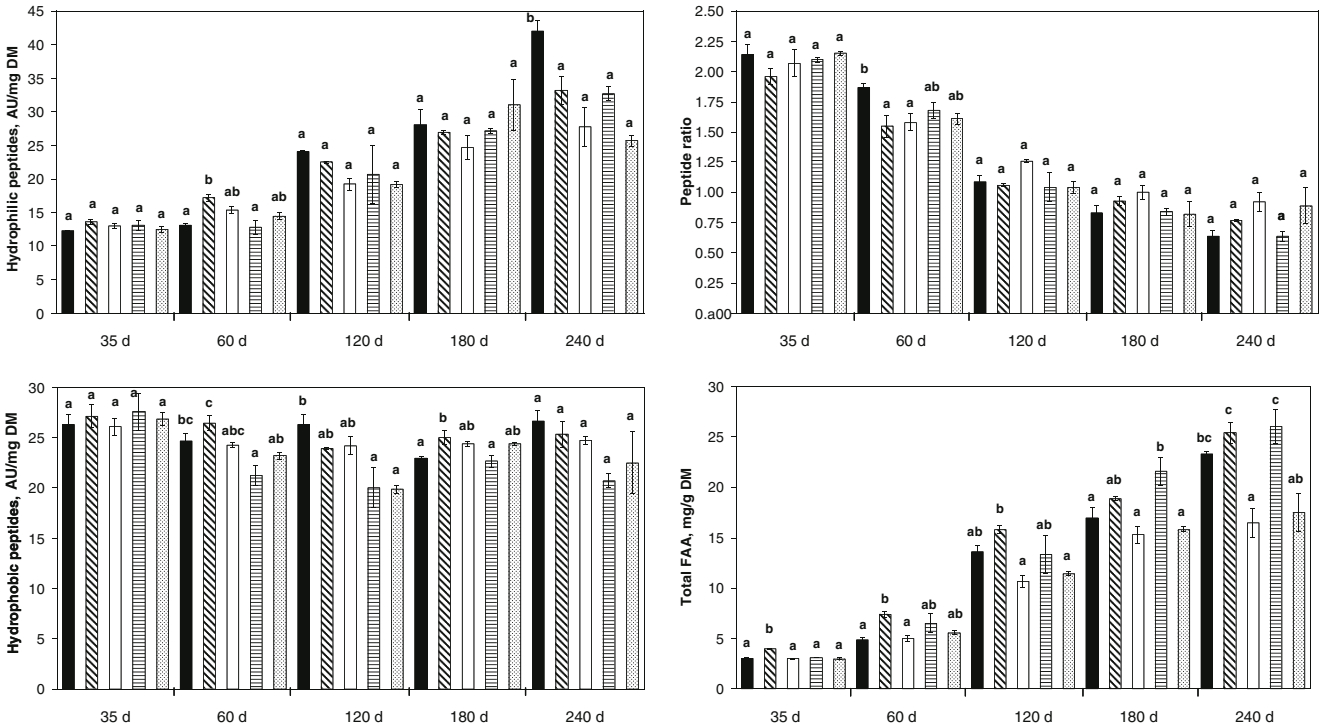


Fig. 3 Levels of hydrophilic peptides, hydrophobic peptides, hydrophobic/hydrophilic ratio and total free amino acids (FAA) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

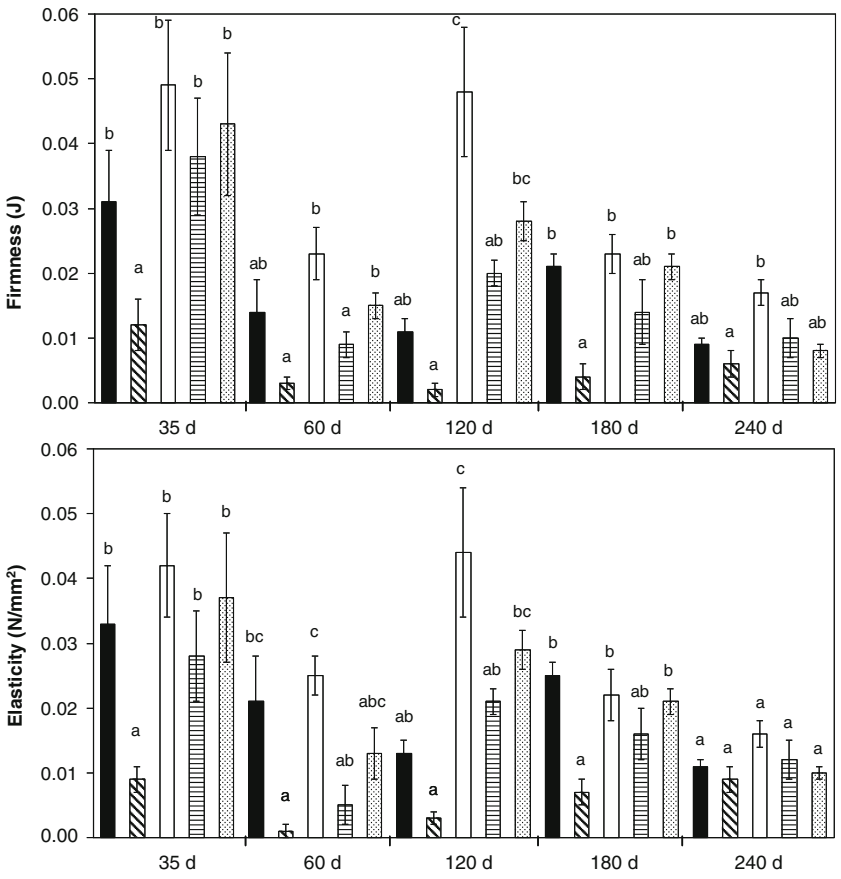


Fig. 4 Texture parameters (firmness and elasticity) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

extensive proteolysis due to the use of cardoon extract as milk coagulant. The level of intact caseins, in particular of α_{s1}-casein, influences the stability of the cheese protein network (Creamer and Olson 1982). In the present work, higher firmness values were generally found for the 600 MPa cheeses, which showed the highest levels of intact caseins. A strong correlation between residual caseins and firmness had been recorded for La Serena cheese (Fernández del Pozo et al. 1988). Also, HPP by itself strengthens the texture of cheeses made from milk coagulated with cardoon extract, with an increase in the values of texture parameters immediately after treatments which is more marked at higher pressure levels (Garde et al. 2007).

Two additional factors influencing cheese texture are pH value and DM content. At high pH values the casein molecules acquire a negative net charge and ionic interactions change from attraction to repulsion, weakening cheese texture (Creamer and Olson 1982). In the present work, the lower pH values of 600 MPa cheeses during refrigerated storage probably contributed to their relatively higher firmness. Negative correlations between pH values and firmness of La Serena cheese had been reported (Fernández del Pozo et al. 1988). A higher DM content is known to strengthen the matrix structure (Nuñez et al. 1991). However, the small differences in DM content between cheeses observed in the present work precluded a significant effect of DM on texture.

Elasticity exhibited a pattern similar to that of firmness, with higher values generally for the 600 MPa cheeses and lower values for the 400 MPa cheeses (Fig. 4). The compression curves showed no breaking point, impeding the determination of the fracturability parameter.

Sensory Evaluation

Both flavour intensity and flavour quality were significantly ($p<0.001$) influenced by time, according to the analysis of variance. HPP at 600 MPa applied on day 21 retarded flavour development during ripening, with lower ($p<0.05$) intensity scores on day 60 for the 600W3 cheese than for the respective control cheese (Fig. 5) and no significant differences between the control cheese and the rest of the HPP cheeses. Afterwards, flavour intensity increased in all cheeses, with significantly ($p<0.05$) lower scores for the 600 MPa cheeses than for the control cheese from day 180 onwards (Fig. 5) and no significant differences between the 400 MPa cheeses and control cheese. A significant ($p<0.01$) correlation was found between flavour intensity scores of cheeses during the whole refrigerated storage period and the respective levels of total FAA. Flavour intensity scores of 60-day-old La Serena cheeses pressurized at 300 or 400 MPa on days 2 and 50 after manufacturing did not differ significantly from that of control cheese (Garde et al. 2007). The results obtained in the present

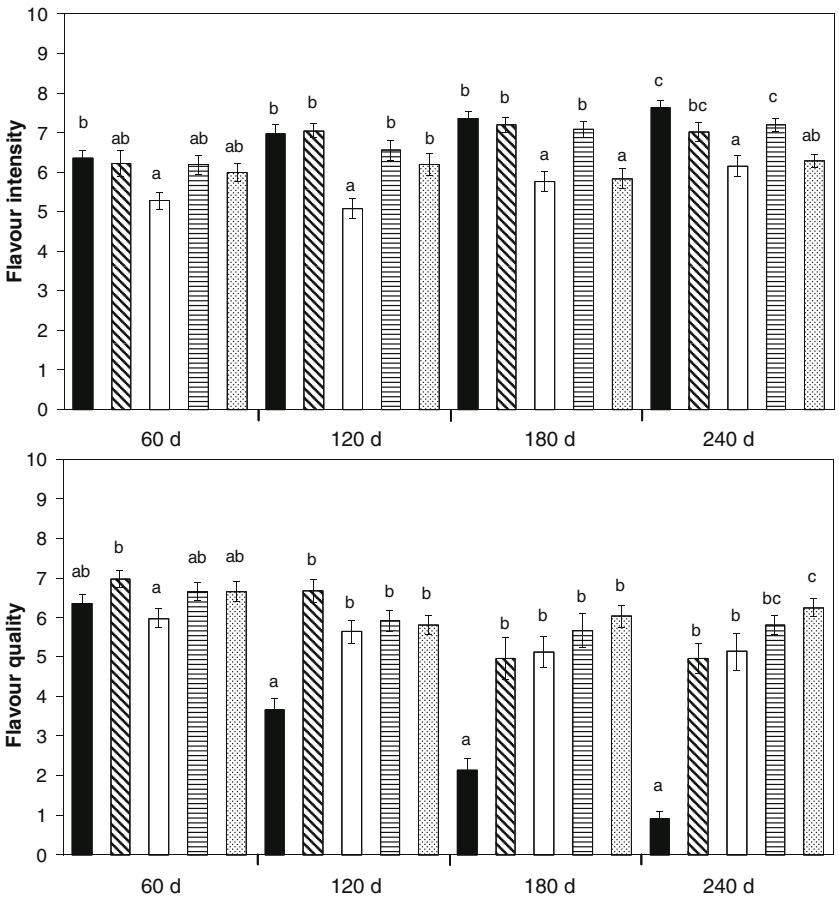


Fig. 5 Sensory characteristics (flavour intensity and flavour quality) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

work for 400 MPa cheeses are in agreement with those obtained for La Serena cheese treated at 400 MPa in spite of the different days of ripening at the time of pressurization.

Flavour quality scores at the end of ripening, on day 60, showed few significant differences between cheeses. However, the flavour quality of the control cheese declined dramatically throughout refrigerated storage reaching significantly ($p<0.05$) lower scores than all the HPP cheeses from day 120 onwards (Fig. 5). The flavour quality score of 400W3 cheese also declined with time, attaining significantly lower values on days 180 and 240 than on day 60. The highest flavour quality score on day 240 was that of 600W5 cheese, which did not vary during refrigerated storage. La Serena cheese pressurized at 400 MPa on day 2 after manufacture showed a significantly lower flavour quality score on day 60 than control cheese, but there was no difference in quality if the cheese was pressurized on day 50 (Garde et al. 2007). As the study on La Serena cheese ceased on day 60, the results of the present work during refrigerated storage cannot be compared.

Flavour descriptors "acid", "salty", "sweet" and "umami" were not influenced by HPP. Differences were occasionally found during refrigerated storage for "bitter" scores, which attained higher ($p<0.05$) values on day 120 for control cheese and 400W3 cheese in comparison with 600W3 cheese, on day 180 for control cheese in comparison with 600W3 and 600W5 cheeses, and on day 240 for control cheese in comparison with 600W3 cheese (data not shown). These differences could not be associated to the levels of hydrophobic peptides. An opposite effect of HPP was reported for La Serena cheese, which showed increased bitterness when pressurized on day 2 at 300 or 400 MPa and on day 50 at 300 MPa in comparison with the control cheese (Garde et al. 2007). The different ripening time of cheeses at HPP and the higher pressure level (600 MPa) applied in the present work may be responsible for the variation in results.

Conclusions

Research on HPP application to cheese has been mainly focused on its effects during the ripening period. The objective of the present work was to preserve cheese flavour quality during post-ripening refrigerated storage, mostly of cheese varieties with seasonal variations in production. According to the results obtained, HPP appears as a useful tool to prevent over-ripening of raw milk cheeses, in particular of those

varieties which suffer extensive proteolysis because of manufacturing procedures, e.g. if cardoon extract is used for milk coagulation. HPP at 600 MPa was particularly effective in retarding the breakdown of proteins and the formation of peptides and FAA during prolonged refrigerated storage. Flavour development during the 60-day ripening period was similar in pressurized and control cheeses. Afterwards, HPP prevented the dramatic decline in flavour quality recorded for the control cheese throughout the refrigerated storage. HPP cheeses, with the only exception of 400W3 cheese, retained until day 240 flavour quality scores not differing from those of the respective 60-day-old cheeses. The HPP of cheeses at 600 MPa may thus be recommended to prevent over-ripening and maintain flavour quality during prolonged refrigerated storage.

Acknowledgments This research was funded by the AGL 2009–07801 project (Ministry of Science and Innovation, Spain). The authors thank the Protected Designation of Origin dairy for providing the cheeses and Hiperbaric for the HPP treatments. J. Calzada is the recipient of an FPI grant (Ministry of Science and Innovation, Spain).

References

Avila, M., Garde, S., Gaya, P., Medina, M., & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy Science, 89*, 2882–2893.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*, 1219–1227.

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13*, 841–866.

Creamer, L. K., & Olson, N. F. (1982). Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science, 47*, 631–636.

Delgado, F.-J., Rodríguez-Pinilla, J., González-Crespo, J., Ramírez, R., & Roa, I. (2010). Proteolysis and texture changes of a Spanish soft cheese (Torta del Casar) manufactured with raw ewe milk and vegetable rennet during ripening. *International Journal of Food Science and Technology, 45*, 512–519.

Fernández del Pozo, B., Gaya, P., Medina, M., Rodríguez-Marín, M. A., & Nuñez, M. (1988). Changes in chemical and rheological characteristics of La Serena ewes' milk cheese during ripening. *Journal of Dairy Research, 55*, 457–464.

García-Risco, M. R., Olano, A., Ramos, M., & López-Fandiño, R. (2000). Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *Journal of Dairy Science, 83*, 2184–2189.

García-Risco, M. R., Recio, I., Molina, E., & López-Fandiño, R. (2003). Plasmin activity in pressurized milk. *Journal of Dairy Science, 86*, 728–734.

Garde, S., Tomillo, J., Gaya, P., Medina, M., & Nuñez, M. (2002). Proteolysis in Hispánico cheese manufactured using a mesophilic starter, a thermophilic starter and bacteriocin-producing *Lactococcus lactis* subsp. *lactis* INIA 415 adjunct culture. *Journal of Agricultural and Food Chemistry, 50*, 3479–3485.

Garde, S., Arqués, J. L., Gaya, P., Medina, M., & Nuñez, M. (2007). Effect of high-pressure treatments on proteolysis and texture of ewes' raw milk La Serena cheese. *International Dairy Journal, 17*, 1424–1433.

Gaya, P., Sánchez, C., Nuñez, M., & Fernández-García, E. (2005). Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation. *Journal of Dairy Research, 72*, 287–295.

Heimgartner, U., Pietrzak, M., Geertsen, R., Brodelius, P., Da Silva Figueiredo, A. C., & Pais, M. S. S. (1990). Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*. *Phytochemistry, 29*, 1405–1410.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). Dissociation of caseins in high pressure-treated bovine milk. *International Dairy Journal, 14*, 675–680.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71*, 496–499.

Juan, B., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. (2007). Effects of high pressure on proteolytic enzymes in cheese: relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science, 90*, 2113–2125.

Krause, I., Bockhardt, A., Neckermann, H., Henle, T., & Klostermeyer, H. (1995). Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. *Journal of Chromatography. A, 715*, 67–79.

Lau, K. Y., Barbano, D. M., & Rasmussen, R. R. (1991). Influence of pasteurization of milk on protein breakdown in Cheddar cheese during aging. *Journal of Dairy Science, 74*, 727–740.

Malone, A. S., Wick, C., Shellhammer, T. H., & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science, 86*, 1139–1146.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheese during ripening: A review. *Le Lait, 80*, 293–324.

Norton, T., & Sun, D.-W. (2008). Recent advances in the use of high pressure as an effective processing technique in the food industry. *Food and Bioprocess Technology, 1*, 2–34.

Nuñez, M., Guillén, A. M., Rodríguez-Marín, M. A., Marcilla, A. M., Gaya, P., & Medina, M. (1991). Accelerated ripening of ewes' milk Manchego cheese: the effect of neutral proteinases. *Journal of Dairy Science, 74*, 4108–4118.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology, 66*, 4890–4896.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L., & Beresford, T. P. (2002). Effects of high-pressure treatment on viability and autolysis of starter bacteria and proteolysis in Cheddar cheese. *International Dairy Journal, 12*, 915–922.

O'Reilly, C. E., Kelly, A. L., Oliveira, J. C., Murphy, P. M., Auty, M. A. E., & Beresford, T. P. (2003). Effect of varying high-pressure treatment conditions on acceleration of ripening of cheddar cheese. *Innovative Food Science and Emerging Technologies, 4*, 277–284.

Picon, A., Fernandez, J., Gaya, P., Medina, M., & Nuñez, M. (1999). Stability of chymosin and cyprosins under milk-coagulation and cheese-ripening conditions. *Journal of Dairy Science, 82*, 2331–2333.

Picon, A., Alonso, R., Gaya, P., & Nuñez, M. (2013a). High-pressure treatment and freezing of raw goat milk curd for cheese manufacture: effects on cheese characteristics. *Food and Bioprocess Technology.* doi:10.1007/s11947-012-0923-5.

Picon, A., Alonso, R., van Wely, K. H. M., & Nuñez, M. (2013b). Microstructural, textural and colour characteristics during ripening of

Hispánico cheese made using high-pressure-treated ovine milk curd. *Food and Bioprocess Technology*. doi:10.1007/s11947-012-0955-x.

Shao, Y. W., & Ramaswamy, H. S. (2011). *Clostridium sporogenes*-ATCC 7955 spore destruction kinetics in milk under high pressure and elevated temperature treatment conditions. *Food and Bioprocess Technology, 4*, 458–468.

Tejada, L., Gómez, R., Vioque, M., Sánchez, E., Mata, C., & Fernández-Salguero, J. (2000). Effect of freezing and frozen storage on the sensorial characteristics of Los Pedroches, a Spanish ewe milk cheese. *Journal of Sensory Studies, 15*, 251–262.

Trujillo, A. J., Capellas, M., Buffa, M., Royo, C., Gervilla, R., Felipe, X., Sendra, E., Saldo, J., Ferragut, V., & Guamis, B. (2000). Applications of high pressure treatment for cheese production. *Food Research International, 33*, 311–316.

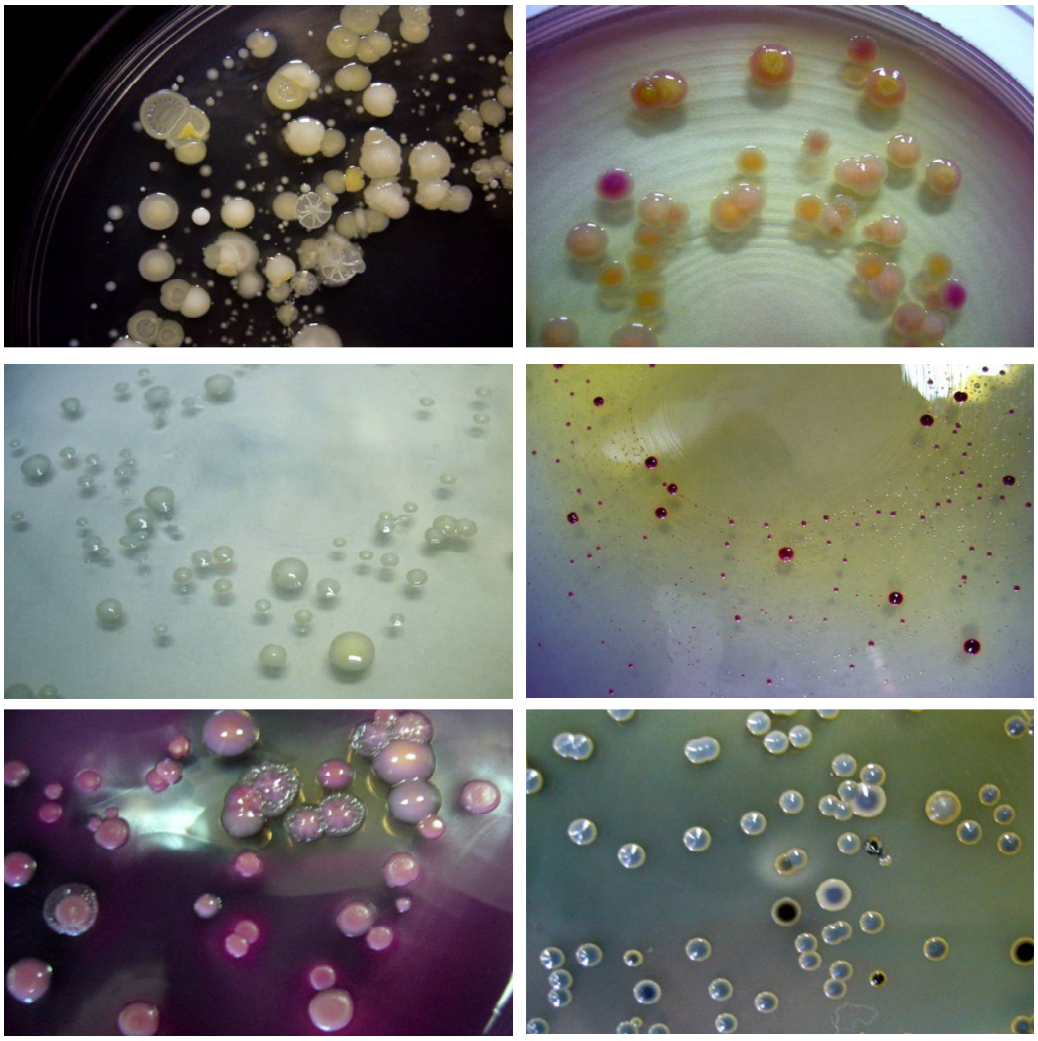
Van Hekken, D. L., Tunick, M. H., & Park, Y. W. (2005). Effect of frozen storage on the proteolytic and rheological properties of soft caprine milk cheese. *Journal of Dairy Science, 88*, 1966–1972.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. *Netherlands Milk and Dairy Journal, 35*, 65–88.

Wick, C., Nienaber, U., Anggraeni, O., Shellhammer, T. H., & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial Cheddar cheeses treated with high pressure. *Journal of Dairy Research, 71*, 107–115.

Yu, L. J., Ngadi, M., & Raghavan, V. (2012). Proteolysis of cheese slurry made from pulsed electric field-treated milk. *Food and Bioprocess Technology, 5*, 47–54.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11*, 185–201.



Capítulo 5.

Reducing biogenic-amine-producing bacteria, decarboxylase activity, and biogenic amines in raw milk cheese by high-pressure treatments.

Fotografía: colonias de bacterias aerobias totales en agar PC (superior, izquierda), bacterias Gram negativas en agar MacConkey (superior, derecha), lactobacilos en agar Rogosa (centro, izquierda), enterococos en agar KF (centro, derecha), coliformes y enterobacterias lactosa negativo en agar VRB (inferior, izquierda), y estafilococos coagulasa positivo y negativo en agar BP (inferior, derecha), obtenidas a partir de siembras de queso Torta del Casar.

AEM
Journals.ASM.org

Reducing Biogenic-Amine-Producing Bacteria, Decarboxylase Activity, and Biogenic Amines in Raw Milk Cheese by High-Pressure Treatments

Javier Calzada, Ana del Olmo, Antonia Picón, Pilar Gaya, Manuel Nuñez

Departamento de Tecnología de Alimentos, INIA, Madrid, Spain

Biogenic amines may reach concentrations of public health concern in some cheeses. To minimize biogenic amine buildup in raw milk cheese, high-pressure treatments of 400 or 600 MPa for 5 min were applied on days 21 and 35 of ripening. On day 60, counts of lactic acid bacteria, enterococci, and lactobacilli were 1 to 2 log units lower in cheeses treated at 400 MPa and 4 to 6 log units lower in cheeses treated at 600 MPa than in control cheese. At that time, aminopeptidase activity was 16 to 75% lower in cheeses treated at 400 MPa and 56 to 81% lower in cheeses treated at 600 MPa than in control cheese, while the total free amino acid concentration was 35 to 53% higher in cheeses treated at 400 MPa and 3 to 15% higher in cheeses treated at 600 MPa, and decarboxylase activity was 86 to 96% lower in cheeses treated at 400 MPa and 93 to 100% lower in cheeses treated at 600 MPa. Tyramine, putrescine, and cadaverine were the most abundant amines in control cheese. The total biogenic amine concentration on day 60, which reached a maximum of 1.089 mg/g dry matter in control cheese, was 27 to 33% lower in cheeses treated at 400 MPa and 40 to 65% lower in cheeses treated at 600 MPa. On day 240, total biogenic amines attained a concentration of 3.690 mg/g dry matter in control cheese and contents 11 to 45% lower in cheeses treated at 400 MPa and 73 to 76% lower in cheeses treated at 600 MPa. Over 80% of the histidine and 95% of the tyrosine had been converted into histamine and tyramine in control cheese by day 60. Substrate depletion played an important role in the rate of biogenic amine buildup, becoming a limiting factor in the case of some amino acids.

Biogenic amines (BA) are low-molecular-weight organic bases showing biological activity (1). Monoamines, diamines, and polyamines consist of an aliphatic, aromatic, or heterocyclic structure with one, two, or more attached reactive amino groups, respectively. The main monoamines are tyramine, a potent vasoconstrictor with an effect on healthy individuals usually limited to headache or migraine (2), and histamine, also vasoactive, which may cause urticaria, hypotension, headache, flushing, and abdominal cramps (3). The diamines putrescine and cadaverine can react with nitrite to form carcinogenic nitrosamines (4). Polyamines and diamines may be converted into stable carcinogenic *N*-nitroso compounds and enhance the growth of chemically induced aberrant crypt foci in the intestine (5). Accumulation of BA in cheese and other foods is therefore a matter of public health concern.

The sensitivity of individuals to BA varies considerably. Concentrations of histamine above 500 to 1,000 mg/kg of food are regarded as potentially dangerous for human health (6), but cheeses with lower histamine contents have been involved in outbreaks (7). Alcohol and other potentiating factors may increase the effect of biogenic amines. For this reason, acceptable BA levels for foods have not been established as a general rule. Only an upper limit for histamine, 100 mg/kg, has been set for certain fish and fish products. Threshold values of 100 to 800 mg/kg for tyramine and 30 mg/kg for phenylethylamine have been suggested as the toxic dose (6).

During cheese manufacture and ripening, lactic starter cultures, together with other microorganisms, milk enzymes, and coagulant enzymes, degrade milk proteins into peptides, which are further hydrolyzed to free amino acids (FAA). Bacterial decarboxylases are responsible for the conversion of precursor amino acids into monoamines and diamines (8), while polyamines can also be formed by "deureation," an alternative metabolic pathway (9). Tyramine, phenylethylamine, histamine, tryptamine, cadaverine, and putrescine are formed through the decarboxylation of tyrosine, phenylalanine, histidine, tryptophan, lysine, and ornithine or arginine (via agmatine), respectively. Enterococci and heterofermentative lactobacilli have been considered the main tyramine and histamine formers, respectively, but other lactic acid bacteria (LAB) and some Gram-negative bacteria may also be involved in BA formation in cheese (10, 11).

The main source of decarboxylase-positive bacteria in cheese is raw milk. Bacterial reduction procedures for milk, such as bactofugation, pasteurization, pressurization, or high-pressure homogenization (12–15), may diminish the levels of decarboxylase-positive bacteria and BA in cheese. Also, bacteriocinogenic strains of LAB have been shown to inhibit decarboxylase-positive bacteria, thus hindering BA formation (16). Even irradiation has been investigated to control BA buildup in cheese, decreasing BA contents with respect to a nonirradiated control but showing a deleterious effect on sensory characteristics (17). High-pressure (HP) treatments have been successfully applied to inactivate microbial contaminants in raw milk cheese (18, 19). To impede BA formation, HP treatments have been assayed only on pasteurized goat milk cheese, but the low BA concentration in control cheese did not permit an evaluation of the efficacy of the procedure (20).

Received 31 October 2012 Accepted 6 December 2012
Published ahead of print 14 December 2012
Address correspondence to Manuel Nuñez, nunez@inia.es.

doi:10.1128/AEM.03368-12

In spite of these and other works, there are aspects of BA formation in cheese by decarboxylase-positive bacteria and its control by HP treatments which need to be elucidated. To our knowledge, the fate of bacterial decarboxylases during ripening and the limiting conditions of substrate depletion or availability for decarboxylation reactions have not been investigated. Also, although the inactivation of glycolytic and proteolytic enzymes of milk- and cheese-borne bacteria by HP treatments has been studied, there is no information on the effect of high pressure on amino acid decarboxylases of bacterial origin in cheese. In the present work, the buildup of BA in raw milk cheeses, pressurized or untreated, throughout their ripening and refrigerated storage periods was investigated with regard to the populations of bacterial groups potentially able to form BA, the activity of decarboxylating enzymes present in cheese, and the concentration of amino acids as the substrates required for BA formation.

MATERIALS AND METHODS

Cheeses and high-pressure treatments. Raw ewe milk (mean total viable counts, 4.78 $\log_{10}$ CFU/ml) was used for the manufacture of Casar cheese in duplicate trials, carried out on consecutive days. Milk (400 liters) with no lactic cultures added was coagulated at 30°C for 60 min with cardoon extract. Curd was cut into 1-cm cubes, held at 30°C for 15 min, and distributed into cylindrical molds. Cheeses, 13 cm in diameter and 6 cm high, were pressed for 3 h and salted by rubbing dry salt onto all the surfaces. They were ripened at 8°C and in 92% relative humidity (RH) until day 60 and afterwards held at 4°C until day 240.

Cheeses from each trial were pressurized for 5 min at 400 or 600 MPa, after 3 or 5 weeks of ripening, and coded as cheeses 400W3, 600W3, 400W5, and 600W5. Before high-pressure (HP) treatment, cheeses were vacuum packaged in CN300 bags (Cryovac Grace S.A., Barcelona, Spain). A 120-liter-capacity isostatic press (NC Hyperbaric, Burgos, Spain) was used for HP treatments. Come-up times to reach 400 and 600 MPa were 1.85 and 2.83 min, respectively, and depressurization times were 7 and 8 s. The temperature of the water used as transmitting fluid remained below 14°C during the process. After HP treatments, cheeses were unpackaged, and ripening proceeded under the same conditions as the control cheese.

Microbiological methods. Representative cheese samples (10 g) were homogenized with 90 ml of a sterile 2% (wt/vol) sodium citrate solution at 45°C in a Colworth Stomacher 400 (A. J. Seward Ltd., London, United Kingdom). Decimal dilutions of samples were prepared in a sterile 0.1% peptone solution. Total viable counts and counts of LAB, enterococci, and lactobacilli were determined in duplicate on plates of plate count agar (Biolife, Milano, Italy) incubated for 48 h at 30°C, De Man-Rogosa-Sharpe (MRS) agar (acidified at pH 5.7 with acetic acid; Biolife) incubated for 48 h at 30°C, Kenner Fecal (KF) Streptococcus agar (Oxoid, Basingstoke, United Kingdom) incubated for 48 h at 37°C, and Rogosa agar (acidified at pH 5.4 with acetic acid; Biolife) incubated anaerobically for 48 h at 37°C, respectively. Counts of *Micrococcaceae* were determined in duplicate on plates of mannitol salt agar (MSA; Oxoid) incubated for 72 h at 30°C, counts of coagulase-positive staphylococci were determined on Baird-Parker agar (Oxoid) with rabbit plasma fibrinogen (RPF) supplement II (Biolife) incubated at 37°C for 48 h, counts of Gram-negative bacteria were determined on MacConkey agar (Biolife) incubated for 24 h at 30°C, and counts of coliforms were determined on violet red bile agar (VRBA; Oxoid) incubated for 24 h at 37°C.

Chemical analyses. BA and FAA were simultaneously extracted from duplicate samples according to procedures described previously by Krause et al. (12). Quantitative analysis of BA, after derivatization with dabsyl chloride, was carried out by reverse-phase high-pressure liquid chromatography (RP-HPLC), using a System Gold HPLC apparatus (Beckman Coulter, Palo Alto, CA) equipped with a Nova-pack C_{18} column (Waters, Milford, MA). A standard mixture of BA (Sigma-Aldrich, Alcobendas, Spain) was used for their identification and quantification. Analysis of FAA was carried out by RP-HPLC after derivatization with Waters AccQ Fluor reagent, using a Waters AccQ Tag column. Concentrations of BA and FAA were expressed as mg per g of dry matter (DM). Cheese DM was determined in triplicate, after cheese grinding with sand, by drying to a constant weight in an oven at 102°C. Cheese pH was measured in triplicate directly by means of a Crison penetration electrode (model 52-3,2; Crison Instruments S.A., Barcelona, Spain) coupled to a Crison GPL 22 pH meter.

Enzymatic determinations. Aminopeptidase activity was determined using an extract obtained by homogenizing 10 g of cheese with 20 ml of 100 mM sodium phosphate buffer (pH 7) for 1 min in an Ultra-Turrax T-10 blender (IKA) at high speed on ice, followed by centrifugation (10,000 × *g* for 30 min at 4°C) and filtering through Whatman no. 2 paper. Activity on duplicate samples was measured with lysine *p*-nitroanilide (Lys *p*-NA) and leucine *p*-nitroanilide (Leu *p*-NA) as substrates and expressed as nmol of *p*-nitroaniline produced per min per g of cheese DM.

For the determination of tyrosine decarboxylase (TDC) activity in cheese, a standard curve was obtained by adding up to 32 mAU/ml of the L-TDC apoenzyme (Sigma, Alcobendas, Spain). (One arbitrary unit [AU] of tyrosine decarboxylase liberates 1.0 μmol of CO_2 from tyrosine per min at pH 5.5 and 37°C.) Methods described previously by Børresen et al. (21), with some modifications, were followed. Briefly, each reaction was initiated by adding different volumes of L-TDC solution (0.625 mg/ml apoenzyme stock solution in 0.5 M acetate buffer [pH 5.5]) in a total volume of 2.2 ml acetate buffer (0.5 M, pH 5.5) in the presence of 1.5 mM L-tyrosine (Sigma) and 0.15 mM pyridoxal-5′-phosphate (Sigma). The reaction mixture was incubated at 37°C for 24 h and stopped by adding 0.55 ml of 1 M perchloric acid to the mixture. After centrifugation (10,000 × *g* for 15 min at 25°C), tyramine was quantified by HPLC, as described above, and tyramine concentrations were plotted against concentrations of the added TDC. To determine TDC concentrations in cheese, 10 g of cheese was mixed with 20 ml of a 2% sodium citrate solution, homogenized for 1 min in an Ultra-Turrax T-10 blender (IKA) at high speed on ice, and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was recovered and dialyzed against sterile double-distilled water for 24 h at 5°C in a dialysis tube with a nominal molecular mass cutoff of 10 kDa (Medicell International Ltd., London, United Kingdom). The dialysate was centrifuged at 10,000 × *g* for 20 min at 5°C, and the supernatant was stored at −20°C until analysis. TDC in cheese was assayed on 0.3 ml of this supernatant as described above, without the addition of the apoenzyme. Reaction mixtures were incubated at 37°C, and the tyramine concentration was determined by HPLC after 0 h and 24 h of incubation. TDC activity in cheeses was calculated from the increase in tyramine concentrations after 24 h by means of the TDC standard curve.

Statistical treatment. Analysis of variance with HP treatment (four treatments and control) and cheese age as the main effects was performed on the analytical variables by means of the SPSS Win 14.0 program. Calculation of correlations and comparison of means by Tukey's test, with the significance assigned at a P value of <0.05, were carried out by using the same program.

RESULTS

Cheese microbiota. Levels of total viable counts and LAB were over 7.5 $\log_{10}$ CFU/g on day 1 (Fig. 1) and had increased to almost 9.5 $\log_{10}$ CFU/g by day 21 (data not shown). HP treatments brought about significant ($P < 0.05$) decreases in counts of all microbial groups, which were particularly pronounced at 600 MPa. Immediately after pressurization, total viable counts were 0.88 to 1.33 log units lower and LAB counts were 1.32 to 1.64 log units lower in cheeses treated at 400 MPa than in control cheese, while the decreases in cheeses treated at 600 MPa were 3.99 to 4.43 log units for total viable counts and 6.03 to 6.51 log units for LAB counts. Afterwards, a slight recovery of LAB was recorded for cheeses treated with 600 MPa, with counts 4.14 to 5.17 log units

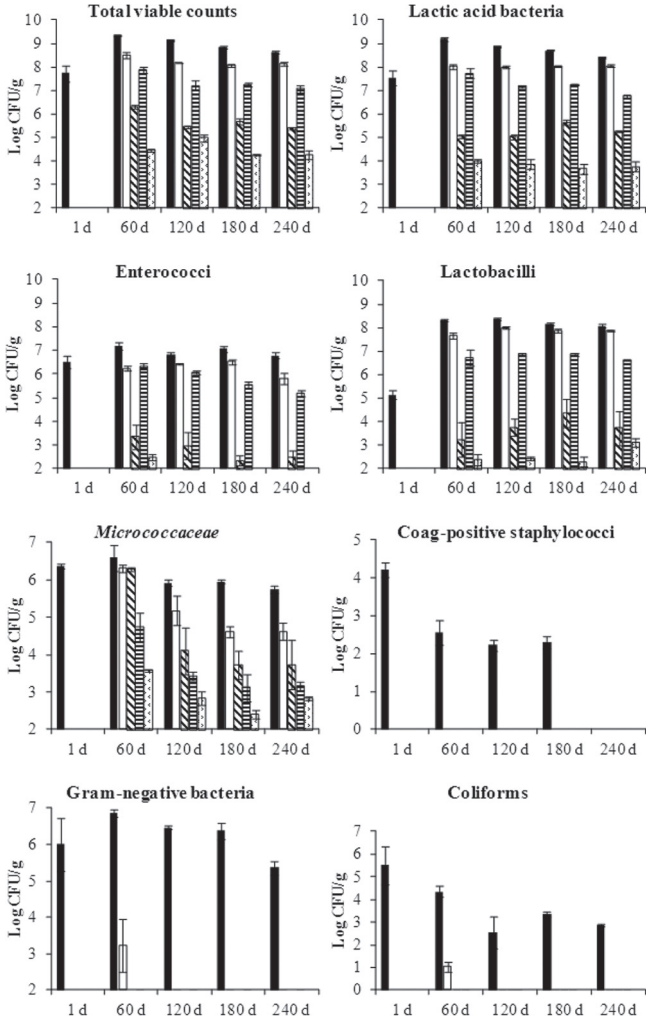


FIG 1 Bacterial counts (log CFU/g) during ripening and storage in control (■) cheeses and cheeses submitted to treatment at 400 MPa (□) or 600 MPa (▨) at 3 weeks and at 400 MPa (▤) or 600 MPa (▦) at 5 weeks. Bars indicate standard errors of the means.

lower in cheeses treated at 600 MPa than in control cheese on day 60. Similar patterns were found for counts of enterococci and lactobacilli. *Micrococcaceae* were more baroresistant than LAB, with decreases immediately after treatment of 1.17 to 1.44 log units in cheeses treated at 400 MPa and 2.78 to 3.89 log units in cheeses treated at 600 MPa. However, on day 60, coagulase-positive staphylococci were below the detection level in all pressurized cheeses. At that time, counts of Gram-negative bacteria and coliforms were more than 3 log units lower in 400W3 cheese than in control cheese and had declined below the detection level in the rest of the HP-treated cheeses. Differences in microbial counts between pressurized and control cheeses persisted throughout refrigerated storage, until day 240. Contrarily, minor differences were recorded for pH values, which ranged from pH 5.33 to 5.42 on day 60 and from pH 5.56 to 5.73 on day 240, and in dry matter content, which ranged from 50.41% to 53.40% on day 60 and from 54.51% to 57.81% on day 240 (data not shown).

Aminopeptidase activity. Cheese pressurization on day 21 reduced significantly ($P < 0.05$) the aminopeptidase activity recorded with Leu *p*-NA as the substrate, by 35% in 400W3 cheese and by 48% in 600W3 cheese, while the respective decreases were 26% and 37% with Lys *p*-NA as the substrate (data not shown). On day 35, the decreases in activity caused by pressurization were 21% in 400W5 cheese and 30% in 600W5 cheese with Leu *p*-NA as the substrate and 24% in 400W5 cheese and 28% in 600W5 cheese with Lys *p*-NA as the substrate (data not shown). Aminopeptidase activity increased in all cheeses during ripening, attaining its maximum values on day 60 (Table 1). Thereafter, a gradual decline of aminopeptidase activity was recorded, with few significant differences between cheeses at the last stages of refrigerated storage.

Free amino acids. Accumulation of FAA during ripening proceeded at different rates in HP-treated and control cheeses from the 0.24 mg/g DM found in control cheese on day 1 (data not shown). Pressurization of cheese at 400 MPa, but not at 600 MPa, enhanced significantly ($P < 0.05$) the formation of FAA during ripening (Fig. 2). Leucine, valine, glutamic acid, and lysine were the most abundant FAA in control cheese on day 60. At the end of refrigerated storage, on day 240, concentrations of FAA totaled 23.31 mg/g DM in control cheese, 25.40 to 26.05 in cheeses treated at 400 MPa, and only 16.47 to 17.47 mg/g DM in cheeses treated at 600 MPa (Fig. 2). At that time, leucine, valine, lysine, and phenylalanine were the most abundant FAA in control cheese.

TABLE 1 Aminopeptidase activity in control and pressurized cheeses during ripening and storage[a]

Substrate	Cheese	Mean aminopeptidase activity (nmol *p*-NA/min per g cheese DM) ± SEM			
		Day 60	Day 120	Day 180	Day 240
Leu *p*-NA	Control	80.67 ± 11.11^{D}	30.95 ± 4.64^{B}	9.41 ± 0.28^{AB}	6.42 ± 0.60^{A}
	400W3	67.45 ± 4.96^{CD}	25.25 ± 0.83^{B}	9.74 ± 1.34^{B}	5.47 ± 0.31^{A}
	600W3	19.05 ± 1.32^{A}	9.70 ± 0.93^{A}	5.88 ± 0.87^{A}	4.45 ± 1.01^{A}
	400W5	41.27 ± 3.26^{BC}	27.58 ± 3.81^{B}	8.07 ± 0.40^{AB}	4.43 ± 0.43^{A}
	600W5	35.17 ± 3.15^{AB}	21.81 ± 1.59^{B}	7.75 ± 0.12^{AB}	5.78 ± 0.90^{A}
Lys *p*-NA	Control	38.10 ± 8.32^{C}	11.34 ± 1.74^{B}	7.07 ± 0.09^{A}	4.67 ± 0.13^{AB}
	400W3	25.69 ± 1.65^{B}	9.04 ± 0.46^{B}	6.76 ± 0.50^{A}	5.04 ± 0.83^{B}
	600W3	7.22 ± 0.70^{A}	4.15 ± 0.50^{A}	5.57 ± 1.19^{A}	2.81 ± 0.37^{A}
	400W5	9.42 ± 2.64^{A}	8.91 ± 1.02^{B}	6.73 ± 0.38^{A}	4.28 ± 0.49^{AB}
	600W5	14.15 ± 0.21^{AB}	8.24 ± 0.69^{AB}	6.64 ± 0.95^{A}	3.82 ± 0.14^{AB}

[a] On day 1, aminopeptidase activities in control cheese with Leu *p*-anilide and Lys *p*-nitroanilide as substrates were 60.93 ± 1.45 and 44.67 ± 0.83 nmol *p*-nitroaniline/min per g cheese DM, respectively. Mean values in the same column for the same substrate bearing the same superscript did not differ significantly ($P > 0.05$).

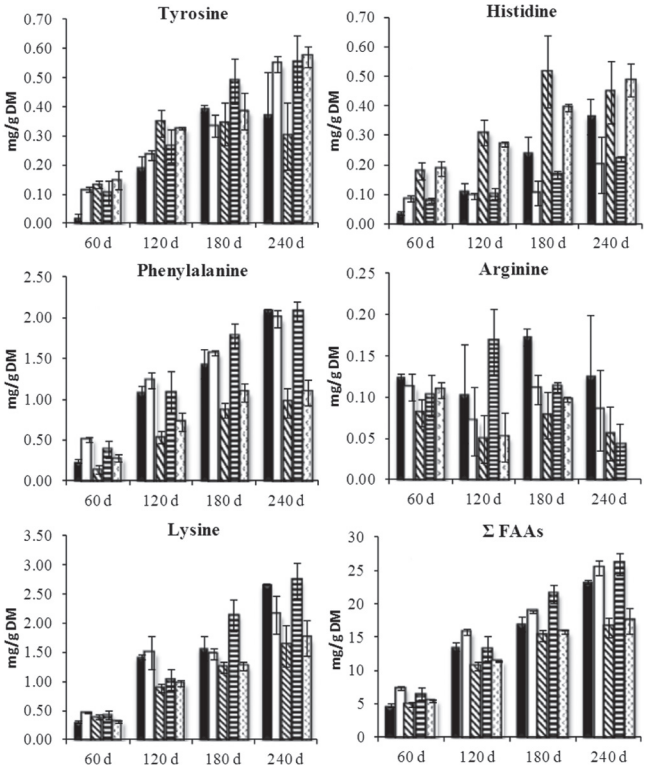


FIG 2 Selected free amino acids (mg/g DM) during ripening and storage in control (■) cheeses and cheeses submitted to treatment at 400 MPa (□) or 600 MPa (▨) at 3 weeks and at 400 MPa (▤) or 600 MPa (▦) at 5 weeks. Bars indicate standard errors of the means.

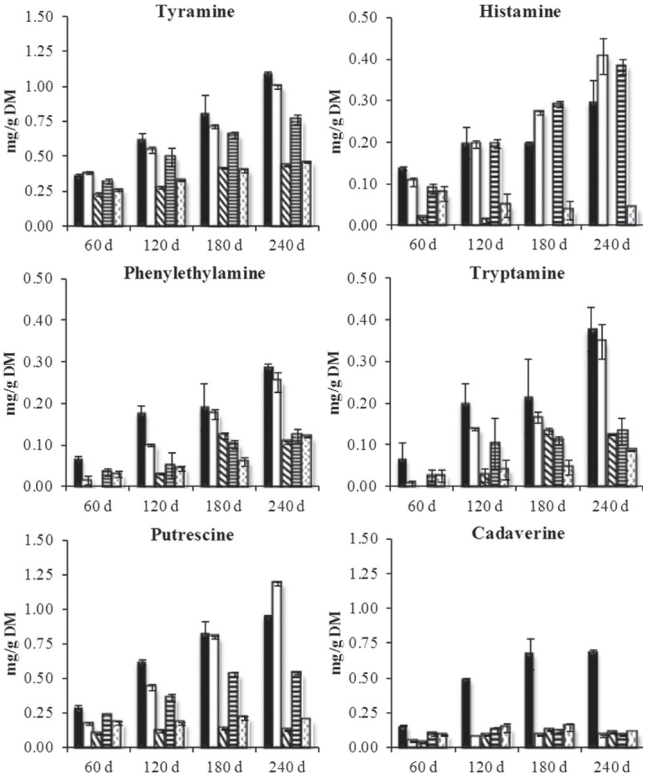


FIG 3 Biogenic amines (mg/g DM) during ripening and storage in control (■) cheeses and cheeses submitted to 400 MPa (□) or 600 MPa (▨) at 3 weeks and at 400 MPa (▤) or 600 MPa (▦) at 5 weeks. Bars indicate standard errors of the means.

Biogenic amines. None of the BA was detected in control cheese on day 1. However, by day 21, the concentration of total BA in control cheese had reached 0.371 mg/g DM, and by day 35, it reached 0.740 mg/g DM (data not shown). On day 60, the most abundant BA in control cheese were tyramine, putrescine, cadaverine, and histamine (Fig. 3). The concentration of total BA increased up to 1.089 mg/g DM in control cheese on day 60, while it remained at 0.728 to 0.794 mg/g DM in cheeses treated at 400 MPa and 0.377 to 0.656 mg/g DM in cheeses treated at 600 MPa. Formation of BA progressed during refrigerated storage, and on day 240, the concentration of total BA reached 3.690 mg/g DM in control cheese, 2.022 to 3.276 mg/g DM in cheeses treated with 400 MPa, and only 0.896 to 1.011 mg/g DM in cheeses treated with 600 MPa. The most abundant BA in control cheese on day 240 were tyramine, putrescine, cadaverine, and tryptamine (Fig. 3). Spermidine and spermine were not found in any of the cheeses at any stage of ripening or refrigerated storage. Concentrations of all individual BA in control cheese correlated significantly ($P < 0.05$) with ripening time. Values of r^2 ranged from 0.891 for histamine to 0.984 for tyramine when concentrations of individual BA in control cheese from day 1 to day 240 were plotted against time.

Tyramine concentrations in control and experimental cheeses on days 60, 120, 180, and 240 correlated significantly ($P < 0.05$) with log counts of enterococci in the same cheeses. Some significant correlations were also found between tyramine concentrations and log counts of LAB or lactobacilli although with lower r^2 values than for enterococci. Correlations of histamine concentrations on days 120, 180, and 240 with log counts of lactobacilli in the same cheeses were significant ($P < 0.05$). Significant correlations between histamine concentrations and log counts of LAB or enterococci were occasionally found, with lower r^2 values than for lactobacilli. Log counts of *Micrococcaceae* did not correlate significantly with tyramine or histamine concentrations at any of the sampling times. Coagulase-positive staphylococci, Gram-negative bacteria, and coliforms were below detection levels in pressurized cheeses, and their correlations with BA could not be calculated. The correlations of histamine contents to histidine contents ($r^2 = 0.128$) and of tyramine concentrations to tyrosine concentrations ($r^2 = 0.290$) in all cheeses, at all times of ripening and storage, were nonsignificant.

Substrate depletion. The proportion of FAA decarboxylated to the respective BA depended on the amino acid and on the HP treatment. Decarboxylated phenylalanine in control cheese up to day 60 represented 27.82% of the FAA formed during the first 60 days of ripening, while in pressurized cheeses, it ranged from 1.86% to 12.53% of the total free phenylalanine formed (Table 2). Similar proportions were recorded for other FAA such as lysine and tryptophan (data not shown). In the case of histidine, the decarboxylated FAA in control cheese up to day 60 was 82.82% of the free histidine formed until that time, and in pressurized cheeses, it accounted for 14.84% to 62.83% of the free histidine formed. Decarboxylated tyrosine in control cheese up to day 60 reached 96.01% of the free tyrosine formed during the first 2 months, while in pressurized cheeses, it ranged from 68.98% to 81.20%. On day 180, the differences in the availability of FAA between control and pressurized cheeses persisted (Table 2).

TABLE 2 Decarboxylation[a] of phenylalanine, histidine, and tyrosine to the respective biogenic amine in cheese during ripening and storage

	Decarboxylation (%)					
	Phenylalanine		Histidine		Tyrosine	
Cheese	Day 60	Day 180	Day 60	Day 180	Day 60	Day 180
Control	27.82	15.41	82.82	53.44	96.01	72.99
400W3	4.16	13.20	62.83	78.05	81.20	73.75
600W3	1.86	16.13	14.84	5.76	68.98	61.59
400W5	10.85	7.26	61.04	70.81	80.15	63.86
600W5	12.53	7.04	36.21	21.25	69.39	57.69

[a] Expressed as the percentage of decarboxylated FAA of the total amount of FAA formed, which was calculated as the sum of the present amount of FAA plus the maximum (present or past) amount of the respective biogenic amine plus the equimolecular CO_2 resulting from decarboxylation.

TABLE 3 Tyrosine decarboxylase activity in control and pressurized cheeses during ripening and storage

	Mean tyrosine decarboxylase activity (mAU/g cheese DM) ± SEM[a]	
Cheese	Day 60	Day 180
Control	0.738 ± 0.027^{B}	3.008 ± 0.162^{C}
400W3	0.031 ± 0.023^{A}	0.183 ± 0.071^{AB}
600W3	0.052 ± 0.031^{A}	0.080 ± 0.053^{A}
400W5	0.103 ± 0.028^{A}	0.689 ± 0.221^{B}
600W5	ND	0.061 ± 0.044^{A}

[a] Means in the same column bearing the same superscript letter did not differ significantly ($P > 0.05$). On day 1, TDC activity of control cheese was below the detection level. ND, not determined (below the detection level).

Decarboxylase activity. The standard curve for the determination of tyrosine decarboxylase activity fit a linear model within the concentration range of 0 to 0.2 mAU of TDC/ml. It followed the equation $y = 72.185x + 0.22219$, in which y was μg tyramine formed/ml and x was mAU TDC/ml, with an r^2 value of 0.999. By means of this standard curve, TDC activity in control and pressurized cheeses was determined. On day 60, TDC activity was significantly ($P < 0.05$) higher in control cheese than in pressurized cheeses (Table 3). Activity increased during refrigerated storage, by approximately 4-fold in control cheese and 6-fold in cheeses treated at 400 MPa from day 60 to day 180, while a minor increase was observed for cheeses treated at 600 MPa. Differences between the TDC activity of pressurized and control cheeses persisted on day 180 (Table 3).

DISCUSSION

Prerequisites for BA formation are availability of FAA, presence of decarboxylase-positive microorganisms, and environmental conditions allowing decarboxylase activity (8, 22). In the present work, HP treatments influenced the first two prerequisites, while the environmental conditions (pH value and dry matter content) were not substantially affected. pH values for control and pressurized cheeses appeared to be favorable for tyramine formation by LAB, while they might slightly hinder its formation by *Enterobacteriaceae*, according to results reported previously for the decarboxylase activity of various bacterial sources at different pH values (23).

According to our results, the overall availability of FAA did not appear to be a limiting factor for BA formation. On day 60, the concentration of total FAA in control cheese was 4.82 mg/g DM versus 1.09 mg/g DM of total BA, while on day 240, the respective concentrations were 23.31 and 3.69 mg/g DM. These concentrations of FAA and BA exceed considerably the respective levels previously reported for other cheese varieties (13, 24, 25). On day 60, total FAA attained higher concentrations in cheeses treated at 400 MPa than in control cheese, a result in apparent contradiction with the lower aminopeptidase activity recorded for the former cheeses. The enhanced proteolysis of HP-treated cheeses observed in the present work can be explained by the conformational changes in the caseins of pressurized cheeses, revealed by confocal laser scanning microscopy (26), which facilitate the access of enzymes to their substrates. Regarding some particular FAA, the phenylalanine concentration did not seem to restrain phenylethylamine formation, since less than 30% of the free phenylalanine formed in control cheese and less than 15% of that formed in pressurized cheeses had been decarboxylated on day 60, while on day 180, this proportion was below 20% in all cheeses (Table 2). Histidine content might limit histamine formation to a certain extent, particularly in control cheese, in which more than 80% of the free histidine had been decarboxylated by day 60, and in cheeses treated with 400 MPa, with over 60% decarboxylated histidine, while in cheeses treated with 600 MPa, the proportion of decarboxylated histidine remained below 40%. The availability of free tyrosine appeared to be strongly limiting for tyramine formation, with more than 95% of the free tyrosine being decarboxylated by day 60 in control cheese, while in cheeses pressurized at 400 and 600 MPa, more than 80% and almost 70% of tyrosine, respectively, had been decarboxylated. The remarkably higher tyrosine decarboxylase activity recorded for control cheese (Table 3), in which the enzyme had not been inactivated since high pressure had not been applied, than in pressurized cheeses, in particular when treated at 600 MPa, appears to be the main cause for tyrosine becoming practically exhausted in the former cheese.

Decarboxylase-positive microorganisms in cheese were negatively influenced by HP treatments, as expected. Pressurization reduced the populations of all the analyzed microbial groups, with a more marked effect on Gram-negative bacteria. Counts of *Enterococcus*, a genus known to harbor abundant decarboxylase-positive strains (11), suffered important declines in pressurized cheeses. A similar result was obtained for *Lactobacillus*, also rich in strains with decarboxylating activity (27). Decreases in the levels of all microbial groups were significantly more pronounced after treatments at 600 MPa than at 400 MPa, in agreement with results from previous studies regarding the effect of pressurization on the microbiota of raw milk cheeses (18, 19, 28).

Tyramine concentrations in control and experimental cheeses correlated significantly with the respective log counts of enterococci from day 60 onwards, and histamine concentrations correlated significantly with the respective log counts of lactobacilli from day 120 onwards. In spite of these correlations, the possible contribution of microorganisms from other genera to the formation of tyramine and histamine in the present work cannot be excluded. Strains of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, and *Enterobacteriaceae* have been found to produce tyramine (23, 29), and strains of *Enterococcus*, *Lactococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Enterobacteriaceae* have been found to form histamine (30–32). On the other hand, bacterial strains belonging to genera frequently found in cheese, such as *Lactobacillus*, *Micrococcus*, and *Pediococcus*, have been reported

to degrade tyramine and histamine, generally by means of monoamine oxidases and preferably under aerobic conditions (33). Also, the concentration of BA in a minicheese model (34) ripened for 4 months was approximately 95% lower when selected *Lactobacillus casei* strains capable of degrading tyramine and histamine in MRS broth were added to milk as adjunct cultures. This phenomenon was probably enhanced by the small size of the minicheeses, favorable for aeration. In the case of real-size cheeses, the low oxygen concentration present in their interior would probably preclude a significant contribution of oxidases to BA degradation. In another work (16), the use of a nisin-producing *Lactococcus lactis* strain as the main starter or two enterocin-producing *Enterococcus faecalis* strains as adjunct cultures inhibited a histamine-forming *Lactobacillus buchneri* strain and reduced histamine contents in 4-month-old cheese from 177 to 214 mg/kg to less than 10 mg/kg.

In the present work, pressurization had a negative effect on all microbial groups and on TDC activity. Levels of microbial groups decreased further during ripening and storage of all cheeses. On the contrary, TDC activity increased by approximately 4-fold from day 60 to day 180 in control cheese and, unexpectedly, even more in cheeses treated at 400 MPa, by approximately 6-fold. The increase of TDC activity in control cheese can be explained by the production of the enzyme by intact bacterial cells, followed by its release into the surrounding medium, favored by spontaneous cell lysis. In cheeses treated at 400 MPa, counts of bacterial groups after pressurization were lower than in control cheese, but the mild-pressure treatment would most probably enhance the lysis of LAB cells, as shown previously for lactococci (35), without inactivating the enzyme. The minor increase in TDC activity recorded for cheeses treated at 600 MPa might be due to a double effect of high pressure at this level, on the one hand promoting cell lysis, as in the case of cheeses treated at 400 MPa, and on the other hand inactivating the enzyme released into the medium.

In spite of the well-known need for sufficient amounts of FAA for BA formation, weak correlations between the concentrations of BA and the concentrations of their precursor FAA in control and pressurized cheeses of different ages were obtained. Pools of FAA and BA in cheese are subject to considerable variations during ripening. The FAA generated in cheese by microbial peptidases, besides being decarboxylated to BA, may be converted into a wide range of compounds through the catabolism processes initiated by aminotransferases, amino acid lyases, and deaminases (36). The BA formed in cheese through FAA decarboxylation may be degraded by oxidases and possibly by other enzymes of microbial origin. From a public health point of view, it must be taken into account that the increase in the concentration of FAA brought about by some of the manufacturing procedures designed to accelerate cheese ripening may enhance BA formation (37, 38), in particular in the presence of decarboxylase-positive microbiota.

According to the results obtained in the present work, it may be concluded that HP treatments were capable of reducing not only the population of potentially decarboxylating microbiota but also the level of decarboxylating enzymes and the concentrations of BA. Contrary to the results found for some FAA in control cheese, no substrate depletion occurred in pressurized cheeses. In spite of this fact, cheeses treated at 400 and 600 MPa showed total BA concentrations up to 45% and 76% lower, respectively, than those in control cheese.

ACKNOWLEDGMENTS

This work was supported by project AGL2009-07801 of the Spanish Ministry of Science and Innovation (MICINN). Javier Calzada was the recipient of a MICINN fellowship.

REFERENCES

1. **Silla-Santos MH.** 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. **29**:213–231.
2. **Til HP, Falke HE, Prinsen MK, Willems MI.** 1997. Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. Food Chem. Toxicol. **35**:337–348.
3. **Stratton JE, Hutkins RW, Taylor SL.** 1991. Biogenic amines in cheese and other fermented foods. J. Food Prot. **54**:460–470.
4. **Hotchkiss JH, Scanlan RA, Libbey LM.** 1977. Formation of bis(hydroxyalkyl)-n-nitrosamines as products of nitrosation of spermidine. J. Agric. Food Chem. **25**:1183–1189.
5. **Eliassen KA, Reistad R, Risøen U, Rønning HF.** 2002. Dietary polyamines. Food Chem. **78**:273–280.
6. **ten Brink B, Damink C, Joosten HMLJ, Huis in't Veld JHJ.** 1990. Occurrence and formation of biologically-active amines in foods. Int. J. Food Microbiol. **11**:73–84.
7. **Taylor SL.** 1986. Histamine food poisoning: toxicology and clinical aspects. Crit. Rev. Toxicol. **17**:91–128.
8. **Halász A, Baráthá, Simon-Sarkadi L, Holzapfel W.** 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. **5**:42–49.
9. **Komprda P, Burdychová R, Dohnal V, Cwiková O, Sládková P.** 2008. Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese. Eur. Food Res. Technol. **227**:29–36.
10. **Joosten HMLJ, Northolt MD.** 1987. Conditions allowing the formation of biogenic amines in cheese. 2. Decarboxylase properties of some nonstarter bacteria. Neth. Milk Dairy J. **41**:259–280.
11. **Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E.** 2001. Biochemical properties of enterococci relevant to their technological performance. Int. Dairy J. **11**:621–647.
12. **Krause I, Bockhardt A, Klostenmeyer H.** 1997. Characterization of cheese ripening by free amino acids and biogenic amines and influence of bactofugation and heat-treatment of milk. Lait **77**:101–108.
13. **Lanciotti R, Patrignani F, Iucci L, Guerzoni ME, Suzzi G, Belletti N, Gardini F.** 2007. Effects of milk pressure homogenization on biogenic amine accumulation during ripening of ovine and bovine Italian cheeses. Food Chem. **104**:693–701.
14. **Novella-Rodríguez S, Veciana-Nogués MT, Trujillo-Mesa AJ, Vidal-Carou MC.** 2002. Profile of biogenic amines in goat cheese made from pasteurized and pressurized milks. J. Food Sci. **67**:2940–2944.
15. **Ordóñez AI, Ibañez FC, Torre P, Barcina Y.** 1997. Formation of biogenic amines in Idiazábal ewe's-milk cheese: effect of ripening, pasteurization and starter. J. Food Prot. **60**:1371–1375.
16. **Joosten HMLJ, Nuñez M.** 1996. Prevention of histamine formation in cheese by bacteriocin-producing lactic acid bacteria. Appl. Environ. Microbiol. **62**:1178–1181.
17. **Rabie MA, Siliha HI, El-Saidy SM, El-Badawy AA, Malcata FX.** 2011. Effect of γ-irradiation upon biogenic amine formation in blue cheese during storage. Int. Dairy J. **21**:373–376.
18. **Arqués JL, Garde S, Gaya P, Medina M, Nuñez M.** 2006. Inactivation of microbial contaminants in raw milk La Serena cheese by high pressure treatments. J. Dairy Sci. **89**:888–891.
19. **O'Reilly CE, O'Connor PM, Kelly AL, Beresford TP, Murphy PM.** 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl. Environ. Microbiol. **66**:4890–4896.
20. **Novella-Rodríguez S, Veciana-Nogués MT, Saldo J, Vidal-Carou MC.** 2002. Effects of high hydrostatic pressure treatments on biogenic amine contents in goat cheeses during ripening. J. Agric. Food Chem. **50**:7288–7292.
21. **Børresen T, Klausen NK, Larsen LM, Sørensen H.** 1989. Purification and characterization of tyrosine decarboxylase and aromatic-L-amino-acid decarboxylase. Biochim. Biophys. Acta **993**:108–115.
22. **Linares D, Del Rio B, Ladero V, Martinez N, Fernandez M, Martin MC, Alvarez MA.** 2012. Factors influencing biogenic amines accumulation in dairy products. Front. Microbiol. **3**:180. doi:10.3389/fmicb.2012.00180.
23. **Pircher A, Bauer F, Paulsen P.** 2007. Formation of cadaverine, histamine,

putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. Eur. Food Res. Technol. **226**:225–231.
24. **Gaya P, Sánchez C, Fernández-García E, Nuñez M.** 2005. Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation. J. Dairy Res. **72**:287–295.
25. **Mayer HK, Fiechter G, Fischer E.** 2010. A new ultra-pressure liquid chromatography method for the determination of biogenic amines in cheese. J. Chromatogr. A **1217**:3251–3257.
26. **O'Reilly CE, Kelly AL, Oliveira JC, Murphy PM, Auty MAE, Beresford TP.** 2003. Effect of varying high-pressure treatment conditions on acceleration of ripening of cheddar cheese. Innov. Food Sci. Emerg. Technol. **4**:277–284.
27. **Joosten HMLJ, Northolt MD.** 1989. Detection, growth and amine producing capacity of lactobacilli in cheese. Appl. Environ. Microbiol. **55**: 2356–2359.
28. **Rodríguez E, Arqués JL, Nuñez M, Gaya P, Medina M.** 2005. Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on the inactivation of *Escherichia coli* O157:H7 in raw milk cheese. Appl. Environ. Microbiol. **71**:3399–3404.
29. **González de Llano D, Cuesta P, Rodríguez A.** 1998. Biogenic amine production by wild lactococcal and leuconostoc strains. Lett. Appl. Microbiol. **26**:270–274.
30. **Chaves-López C, De Angelis M, Martuscelli M, Serio A, Paparella A, Suzzi G.** 2006. Characterization of the Enterobacteriaceae isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). J. Appl. Microbiol. **101**:353–360.
31. **Connil N, Le Breton Y, Dousset X, Auffray Y, Rincé A, Prévost H.** 2002. Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. Appl. Environ. Microbiol. **68**:3537–3544.
32. **Rossi F, Gardini F, Rizzotti L, La Gioia F, Tabanelli G, Torriani S.** 2011. Quantitative analysis of histidine decarboxylase gene (*hdcA*) transcription and histamine production by *Streptococcus thermophilus* PRI60 under conditions relevant to cheese making. Appl. Environ. Microbiol. **77**:2817–2822.
33. **Leuschner RG, Heidel M, Hammes WP.** 1998. Histamine and tyramine degradation by food fermenting microorganisms. Int. J. Food Microbiol. **39**:1–10.
34. **Herrero-Fresno A, Martínez N, Sánchez-Llana E, Díaz M, Fernández M, Martin MC, Ladero V, Alvarez MA.** 2012. *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental model. Int. J. Food Microbiol. **157**:297–304.
35. **Malone AS, Shellhammer TH, Courtney PD.** 2002. Effects of high pressure on the viability, morphology, lysis, and cell wall hydrolase activity of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **68**:4357–4363.
36. **Yvon M, Rijnen L.** 2001. Cheese flavor formation by amino acid catabolism. Int. Dairy J. **11**:185–201.
37. **Fernández-García E, Tomillo J, Nuñez M.** 1999. Effect of added proteinases and level of starter culture on the formation of biogenic amines in raw milk Manchego cheese. Int. J. Food Microbiol. **52**:189–196.
38. **Leuschner RGK, Kurihara R, Hammes WP.** 1998. Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during Gouda cheese ripening. Int. J. Food Microbiol. **44**:15–20.

Capítulo 6.

High-pressure processing for the control of lipolysis, volatile compounds and off-odours in raw milk cheese.

Fotografía: quesos de Torta del Casar en cámara de maduración.

Food Bioprocess Technol (2014) 7:2207–2217
DOI 10.1007/s11947-013-1206-5

ORIGINAL PAPER

High-Pressure Processing for the Control of Lipolysis, Volatile Compounds and Off-odours in Raw Milk Cheese

Javier Calzada · Ana del Olmo · Antonia Picon · Pilar Gaya · Manuel Nuñez

Received: 1 July 2013 / Accepted: 26 September 2013 / Published online: 12 October 2013

Abstract Build-up of flavour compounds throughout ripening of raw milk cheeses may result in strong over-ripening notes during refrigerated storage. In order to control the formation of free fatty acids (FFAs) and volatile compounds, and the appearance of off-odours, raw milk cheeses were high-pressure-processed (HPP) 21 or 35 days after manufacture at 400 or 600 MPa. Ripening proceeded at 8 °C until day 60 and, afterwards, cheeses were held at 4 °C until day 240. The effect of HPP on the formation of FFAs and volatile compounds was dependent on pressure level and cheese age at the time of treatment. On day 60, acetic and propionic acids, branched-chain FFAs and short-chain FFAs showed the lowest ($p<0.05$) concentrations in cheeses treated at 400 or 600 MPa on day 21, while medium- and long-chain FFAs were at similar levels in all cheeses. HPP influenced significantly ($p<0.05$) 84 out of the 94 volatile compounds found in cheese. On day 60, the lowest ($p<0.05$) concentrations of acids, alcohols and esters were recorded for cheeses treated at 400 or 600 MPa on day 21, and the lowest ($p<0.05$) concentrations of ketones for cheeses treated at 400 MPa on days 21 or 35. On day 240, all HPP cheeses showed lower ($p<0.05$) concentrations of aldehydes, esters and, particularly, sulphur compounds than control cheese, which exhibited putrid and rancid off-odours from day 120 onwards. Principal component analysis combining FFAs and volatile compounds discriminated 240-day control cheese from 120-day control cheese and both from the rest of cheeses.

Keywords High-pressure processing · Lipolysis · Volatile compounds · Cheese

J. Calzada · A. del Olmo · A. Picon · P. Gaya · M. Nuñez (✉)
Departamento de Tecnología de Alimentos, INIA, Carretera de La Coruña Km 7, Madrid 28040, Spain
e-mail: nunez@inia.es

Introduction

Cheese quality is determined by flavour, rheological properties and visual appearance (Fox and Wallace 1997). Flavour, probably the main trait influencing cheese quality, has been the subject of numerous scientific investigations. Compounds responsible for cheese flavour are produced through glycolysis, lipolysis and proteolysis, followed by the secondary reactions which occur during ripening (McSweeney and Sousa 2000; Yvon and Rijnen 2001; Collins et al. 2003). More than 600 volatile compounds have been identified in different cheese varieties and many have been related to particular odour and aroma notes (Molimard and Spinnler 1996; Sablé and Cottenceau 1999; Curioni and Bosset 2002).

The main agents responsible for the biochemical changes taking place during the manufacture and ripening of pasteurized milk cheeses are coagulant enzymes together with starter cultures and their enzymes. Pasteurization inactivates most enzymes and microorganisms present in raw milk. However, the enzymes and microorganisms coming from milk are crucial for the ripening process of raw milk cheeses (Fernández-García et al. 2002; Gaya et al. 2005). In the case of varieties not produced, or produced at a lesser amount, during certain periods of the year, generally due to the seasonality in milk production, refrigerated storage of ripe cheese for several months is a must. Since the action of enzymes and microorganisms persists during refrigerated storage of ripe cheese, over-ripening may happen before the product is consumed. Raw milk cheeses, with pronounced enzyme activity and microbial metabolism associated reactions, are particularly prone to it. Freezing of ripe cheeses has been investigated to prevent over-ripening and prolong their shelf life. However, even though their flavour characteristics remained unchanged at thawing, both texture and visual appearance were negatively affected (Tejada et al. 2000; Van Hekken et al. 2005). In the particular case of mixed milk (ewe + cow or goat + cow)

cheese, curd made from ewe or goat milk in the period of maximum production was frozen, and after several months thawed and mixed with fresh cow milk curd for cheese manufacture with satisfactory results (Picon et al. 2013a, b).

Because of its negligible effect on flavour characteristics, high-pressure processing (HPP) meets the increasing consumer demand for fresher tasting minimally processed foods (Norton and Sun 2008). It has been successfully applied to milk and cheese for the inactivation of pathogenic and spoilage microorganisms (O'Reilly et al. 2000; Shao and Ramaswamy 2011). Some cheese-related enzymes such as proteinases and peptidases lose their activity when subjected to HPP, totally or partially depending on the pressure level applied (Malone et al. 2003; Huppertz et al. 2004; Juan et al. 2007). The effect of HPP on cheese esterase activity depends on the pressure level but also on the type of starter culture used (O'Reilly et al. 2002; Ávila et al. 2007). Formation of volatile compounds in pressurized cheese is influenced by both the pressure level and the age of cheese at the time of treatment (Ávila et al. 2006; Arqués et al. 2007). On the basis of those results, it seemed feasible to apply HPP for preventing over-ripening during refrigerated storage of raw milk cheese.

Casar cheese studied in the present work is made from ewe raw milk coagulated with an aqueous extract of *Cynara cardunculus* L. (cardoon) flowers. The strong activity of cardoon enzymes, the raw milk microbiota and the high cheese pH values may cause over-ripening during refrigerated storage of ripe cheese. In a previous work (Calzada et al. 2013), we achieved a delay in proteolysis during refrigerated storage of Casar cheese by means of HPP treatments on days 21 and 35 after manufacture, at 400 or 600 MPa. In the present work, we have investigated the influence of HPP treatments under the same conditions on the lipolysis, the formation of volatile compounds and the appearance of off-odours throughout ripening and refrigerated storage of Casar cheese.

Materials and Methods

Cheese Manufacture and High-Pressure Processing

The manufacturing procedure of Casar cheese from ewe raw milk was described by Calzada et al. (2013). The experiment was carried out in duplicate, at a protected designation of origin (PDO) dairy, on two batches of cheese made on consecutive days, using an aqueous extract of dry cardoon flowers as milk coagulant.

Cheeses were pressurized at 400 or 600 MPa for 5 min, after 21 or 35 days of ripening, as described by Calzada et al. (2013). They were coded as 400W3, 600W3, 400W5 and 600W5, according to the pressure level applied (400 or 600 MPa) and the age of cheese (3 or 5 weeks) at pressurization. After HPP, they were unpackaged. Control cheeses were vacuum-packaged and unpackaged simultaneously with the cheeses pressurized after 21 days of ripening. Ripening took place at 8 °C and 92 % RH until day 60, and afterwards cheeses were held at 4 °C until day 240.

At each of the sampling times, two 100 g pieces per cheese were wrapped in aluminum foil, vacuum-packaged and frozen at −40 °C for chemical analyses.

Chemical Determinations

Acetic and propionic acids, branched-chain carboxylic acids (BCCAs) and free fatty acids (FFAs) from butyric ($C_{4:0}$) to linolenic acid ($C_{18:3}$) in cheese were extracted in duplicate and determined by gas chromatography (GC) with flame ionization detection, as described by Fernández-García et al. (2006). Frozen cheese pieces were thawed overnight at 4 °C, prior to analysis. At all sampling times, acids were extracted from cheeses using a solid-phase extraction technique, with pentanoic, nonanoic and heptadecanoic acids added as internal standards (IS). Fifteen standard solutions of fatty acids were used for the calculation of calibration curves. Individual FFAs were separated, identified and quantified, and their concentrations expressed in milligram per gram cheese dry matter.

Volatile compounds were extracted in duplicate from cheese using a solid-phase microextraction method (Mallia et al. 2005). Ten grams of cheese was homogenized in a mechanical grinder with 20 g of Na_2SO_4 and 25 μL of an aqueous solution of 1,058 mg/L cyclohexanone as IS. Five grams of the mixture was weighed in a 15-mL headspace glass vial sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA, USA). Vials were submerged in a thermostatic bath at 30 °C (D3 model, HAAKE, Berlin, Germany) for both equilibration (20 min) and extraction (30 min) phases. A SPME manual holder equipped with a 2 cm×50/30 μm StableFlex divinylbenzene/carboxen/polydimethylsiloxane-coated fibre (Supelco) was inserted through the PTFE septum for headspace extraction, after which it was inserted into the GC injection port for desorption (270 °C/10 min in splitless mode). Before use, the fibre was conditioned in the injection port of the GC (270 °C/1 h) as recommended by the manufacturer. After each run, the fibre was cleaned up to avoid carry-over problems, and periodically, fibre sensitivity was tested with an aqueous solution of the internal standard. All analyses were run using the same fibre unit.

Gas chromatography–mass spectrometry was carried out using a HP 6890-MSD HP 5973 apparatus (Agilent, Palo Alto, CA, USA) with a capillary column (60 m long; 0.25 mm i.d.; 0.5 μm film thickness; Zebron-WAX plus, Phenomenex, Torrance, CA, USA) and helium flow at 1.4 mL/min for 1 min followed by 1 mL/min. The temperature program was 7 min at 40 °C, first ramp 2 °C/min to 90 °C, second ramp 3 °C/min to 150 °C, final ramp 9 °C/min to 240 °C, and 8 min at 240 °C.

Detection was performed with electron impact ionization, with 70 eV ionization energy operating in the full-scan mode at 1.74 scans/s. Source and quadrupole temperatures were 230 and 150 °C, respectively. Compound identification was carried out by injection of commercial standards and by spectra comparison using the Wiley7Nist05 Library (Wiley & Sons Inc., Germany). The sum of abundances of characteristic ions was used for semi-quantitation of compounds. The areas have been referred to the IS (compound peak area multiplied by 10^3 and divided by the IS peak area).

Sensory Evaluation

A trained 15-member panel carried out the evaluation of odour intensity and quality (preference) of 60-, 120- and 240-day cheeses, scoring on a 0–10 points scale (Nuñez et al. 1991). Odour was defined as the olfactory sensation felt directly by smelling the cheese (Fernández-García et al. 2002). In addition, panellists were asked to evaluate putrid and rancid odour notes, also scoring on a 0–10 points scale. Cheese samples of the same ripening time from the same experiment (four HPP cheeses and one control cheese) were simultaneously presented to panellists at each of the sensory evaluation dates.

Statistical Analysis

Data obtained were analyzed by a two-way analysis of variance, with HPP treatment and days after manufacture as the main effects. Means were compared using Tukey's test, with p assigned at 0.05. Principal component analysis (PCA) was carried out on individual volatile compounds and on the total levels of groups of FFAs and volatile compounds of 60-, 120- and 240-day cheeses for the discrimination of samples according to the HPP treatment applied and the days after manufacture. The SPSS Win 14.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of data.

Results and Discussion

Acetic, Propionic and Branched-Chain Carboxylic Acids

Acetic acid, which derives from the metabolism of lactose, lactate and citrate by lactic acid bacteria and other microorganisms, could be detected as early as day 1 (Table 1). Production of acetic acid proceeded steadily during ripening of control cheese, reaching a concentration 32 times higher on day 60 than on day 1. Significantly ($p<0.05$) lower concentrations of acetic acid were recorded for 400W3 and 600W3 cheeses than for control cheese at day 35, most probably because of the microbial death and injury caused by HPP of cheeses on day 21, while the acetic acid concentrations of 400W5 and 600W5 cheeses did not differ from that of control cheese at day 35. A certain recovery of microbial metabolism in the 400W3 cheese occurred afterwards, as shown by the increase in its acetic acid concentration from day 35 to day 60 and during refrigerated storage. On the contrary, the acetic acid concentration hardly varied in 600W3 cheese from day 35 onwards. The effect of HPP when applied on day 35 was less marked, with no significant differences in acetic acid concentration between cheeses pressurized on day 35 and control cheese during the rest of ripening and refrigerated storage. Acetic acid, a major odorant of Cheddar, Gruyère and Emmental cheeses (Curioni and Bosset 2002) and of other varieties including most ewes milk cheeses (Fernández-García et al. 2006), with a typical vinegar odour note, plays an important role in cheese flavour and aroma by itself and as a substrate for ester formation through esterification reactions. Concentrations of acetic acid ranging from 3.9 to 6.1 mg/g had been reported for ripe Casar cheese by Delgado et al. (2009).

Propionic acid, generally derived from the microbial metabolism of lactate, was not detected on day 1, but its concentration increased almost sixfold from day 21 to day 60 in control cheese (Table 1). Formation of propionic acid was influenced by HPP of cheeses on day 21, with significantly ($p<0.05$) lower concentrations in 400W3 and 600W3 cheeses than in control cheese not only on day 35 but also during the rest of the ripening period and throughout refrigerated storage until day 240. The different patterns of acetic and propionic acid concentrations from day 35 to day 240 in cheeses pressurized on day 21 were probably due to a higher barotolerance of acetic acid-producing bacteria compared with propionic acid-producing bacteria. Propionic acid, characteristic of Swiss-type cheeses, also with a vinegar odour note, is also present in Camembert and some ewes milk cheeses (Molimard and Spinnler 1996; Fernández-García et al. 2004), although it was not found in a previous work on Casar cheese (Delgado et al. 2009). Besides its direct role in cheese odour and aroma, propionic acid contributes to ester formation.

BCCAs were detected at very low concentration on day 1 and increased by more than eightfold from day 21 to day 60 of ripening in control cheese (Table 2). Their formation was hindered by HPP of cheeses on day 21, as shown by the lower ($p<0.05$) concentrations of 2-methylpropanoic and 3-methylbutanoic acids in 400W3 and 600W3 cheeses than in control cheese from day 35 onwards. On the contrary, differences in BCCAs concentrations between cheeses pressurized at 400 or 600 MPa on day 35 and control cheese remained non-significant during the rest of ripening and refrigerated storage. 2-Methylpropanoic and 3-methylbutanoic acids derive, respectively, from the catabolism of valine and leucine, which starts with a transamination catalysed by aminotransferases, with α-ketoglutarate or other α-ketoacid as acceptor, followed by an oxidative decarboxylation generating acyl-CoAs which are further hydrolysed to carboxylic acids (Yvon and Rijnen 2001). Lactic acid bacteria are not the only

Table 1 Concentrations of acetic and propionic acids during ripening and refrigerated storage of control and HPP cheeses

Acid	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
Acetic[b]	1	0.17±0.06				
	21	2.39±0.12 a	2.86±0.15 a	2.64±0.39 a		
	35	4.85±0.42 b	3.21±0.21 a	2.86±0.30 a	5.17±0.42 b	4.97±0.24 b
	60	5.40±0.82 ab	3.92±0.19 ab	3.47±0.23 a	5.35±0.15 ab	6.01±1.00 b
	120	5.65±0.44 b	4.40±0.36 ab	3.27±0.35 a	5.85±0.49 b	5.41±0.47 b
	180	6.06±1.04 ab	4.83±0.32 ab	3.40±0.21 a	6.35±0.49 b	5.57±0.76 ab
	240	5.82±0.73 b	5.17±0.21 b	3.17±0.24 a	6.23±0.15 b	4.75±0.09 ab
Propionic[b]	1	ND				
	21	0.22±0.08 a	0.18±0.09 a	0.22±0.11 a		
	35	1.53±0.50 b	0.18±0.06 a	0.23±0.11 a	1.37±0.38 b	1.70±0.51 b
	60	1.29±0.37 b	0.20±0.08 a	0.27±0.11 a	1.04±0.41 ab	0.96±0.47 ab
	120	2.03±0.26 b	0.39±0.10 a	0.53±0.02 a	0.48±0.15 a	0.68±0.35 a
	180	1.88±0.42 b	0.23±0.07 a	0.26±0.08 a	0.89±0.30 ab	1.54±0.51 ab
	240	2.99±0.20 b	0.34±0.04 a	0.74±0.31 a	0.82±0.27 a	0.97±0.39 a

[a] Codes for HPP cheeses are 400W3, HPP at 400MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Concentrations are expressed in milligram per gram cheese dry matter, as mean±SEM of duplicate determinations on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly. ND, below detection level

microorganisms capable of BCCAs formation. Gram-negative bacteria such as *Pseudomonas* spp. (Morales et al. 2005) have also been reported to form BCCAs in cheese. In the present work, counts of Gram negative bacteria on McConkey agar reached high levels in control Casar cheese, 7.43 log cfu/g on day 21 and 6.85 log cfu/g on day 35 (data not shown), but declined below detection level after HPP of cheeses at 400 or 600 MPa on days 21 or 35. Early HPP treatment on day 21 was more effective in controlling the formation of BCCAs by raw milk microorganisms than HPP on day 35. The odour of 2-methylpropanoic acid has been described as cheesy, sweaty and sour, and that of 3-methylbutanoic acid as rancid, cheesy, sweaty and putrid. Both BCCAs are flavour compounds characteristic of ewes and goat cheeses (Curioni and Bosset 2002) and have been found in Casar cheese (Delgado et al. 2010) and in La Serena cheese, a similar variety (Carbonell et al. 2002).

Free Fatty Acids

Short-chain (SC, $C_{4:0}$ to $C_{8:0}$) FFAs originate not only from the esterase-mediated hydrolysis of triacylglycerides but also from the fermentation of lactose and lactate, from the degradation of amino acids and from the oxidation of some ketones, esters and aldehydes (Molimard and Spinnler 1996; Curioni and Bosset 2002; Collins et al. 2003). In the present work, the

Table 2 Concentrations of branched-chain carboxylic acids (BCCAs) and short-chain (SC, $C_{4:0}$ to $C_{8:0}$) free fatty acids (FFAs) during ripening and refrigerated storage of control and HPP cheeses

Acid	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
BCCAs[b]	1	0.01±0.00				
	21	0.09±0.02 a	0.14±0.03 a	0.10±0.01 a		
	35	0.51±0.10 b	0.19±0.05 a	0.12±0.03 a	0.48±0.04 b	0.45±0.09 b
	60	0.76±0.08 b	0.33±0.04 a	0.27±0.03 a	0.57±0.16 ab	0.63±0.02 ab
	120	1.04±0.08 b	0.37±0.07 a	0.32±0.06 a	0.69±0.21 ab	0.64±0.09 ab
	180	0.80±0.02 b	0.32±0.03 a	0.32±0.03 a	0.84±0.12 b	0.81±0.07 b
	240	1.15±0.18 b	0.40±0.08 a	0.30±0.06 a	0.76±0.06 ab	0.68±0.15 ab
SC FFAs[b]	1	0.06±0.01				
	21	0.22±0.07 a	0.26±0.07 a	0.29±0.02 a		
	35	1.55±0.05 b	0.36±0.05 a	0.37±0.07 a	1.67±0.06 b	1.41±0.10 b
	60	1.93±0.13 b	0.55±0.02 a	0.72±0.03 a	1.41±0.46 ab	1.52±0.12 ab
	120	5.15±0.49 b	0.92±0.02 a	0.99±0.21 a	0.95±0.09 a	1.01±0.22 a
	180	3.42±1.09 b	0.82±0.04 a	0.89±0.04 a	1.95±0.13 ab	2.55±0.17 ab
	240	8.69±1.54 b	1.11±0.03 a	2.34±0.88 a	1.75±0.09 a	1.25±0.37 a

[a] Codes for HPP cheeses are 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Concentrations are expressed in milligram per gram cheese dry matter, as mean±SEM of duplicate determinations on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly

concentration of SC FFAs increased 32-fold from day 1 to day 60 in control cheese (Table 2), with butanoic acid accounting for 95 % of the total SC FFAs on day 60. The concentrations of SC FFAs were significantly ($p<0.05$) lower in 400W3 and 600W3 cheeses than in control cheese from day 35 onwards, while in 400W5 and 600W5 cheeses, they were significantly ($p<0.05$) lower only on days 120 and 240. Esterases from lactic acid bacteria seem to be resistant to the pressurization of cheese at 400 MPa for 5 min according to Ávila et al. (2007), who did not find differences in the concentration of SC FFAs between HPP cheese and control cheese both made from pasteurized milk. Voigt et al. (2012) reported lower concentrations of SC FFAs in 21-day cheeses made from HPP (400 or 600 MPa) milk than when made from raw milk, but higher SC FFAs concentrations on day 180 for the cheese made from milk treated at 600 MPa. In the present work, butanoic acid was at significantly lower concentrations in 400W3 and 600W3 cheeses than in control cheese from day 35 until day 240 while few significant differences were recorded for $C_{6:0}$ and $C_{8:0}$ FFAs. This result points to the production of butanoic acid in cheese by microorganisms from raw milk, rather than to its formation through the esterase-mediated hydrolysis of triacylglycerides. In a work on pressurized cheese made from goat raw milk, minor differences in $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$ FFAs concentrations during ripening were found between HPP cheeses and control cheese (Delgado et al. 2012). Butanoic acid, with a rancid cheese-like odour, plays an important role in the flavour of many cheese types made from cow and ewe milk, although large amounts, usually associated to the butyric acid fermentation of lactate, become undesirable. Hexanoic and octanoic acids are characteristic flavour compounds of aged Grana Padano and Roncal cheeses (Curioni and Bosset 2002).

Medium chain (MC, $C_{10:0}$ to $C_{14:0}$) FFAs concentration increased at a slow rate during ripening of control cheese, only 1.7-fold from day 1 to day 60 (Table 3). By day 60, HPP of cheeses had not affected the concentration of MC FFAs. Significantly ($p<0.05$) higher levels of MC FFAs in control cheese than in HPP cheeses were recorded during refrigerated storage on days 120 and 240, with the only exception of the 400W3 cheese on day 120 which did not differ from the respective control cheese. Our results seem to agree with the reported barotolerance of the lipoprotein lipase from goat milk (Trujillo et al. 1999). Similar concentrations of $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$ FFAs were found in 60-day cheeses made from raw and from HPP (500 MPa) goat milk, while cheese made from pasteurized milk showed significantly lower concentrations of the three MC FFAs (Buffa et al. 2001). Differences in MC FFAs concentrations on day 60 between HPP cheeses and control cheese made from goat raw milk were non-significant (Delgado et al. 2012), and this also occurred when the MC FFAs concentrations in Cheddar cheeses made from HPP and raw milk were compared, with the only exception of $C_{14:0}$ which behaved similarly to SC FFAs (Voigt et al. 2012). MC FFAs such as $C_{10:0}$ and $C_{12:0}$ contribute to the aroma of Cheddar, Roncal and other cheese varieties because of their relatively low perception thresholds (Curioni and Bosset 2002).

Long chain (LC, $C_{16:0}$ to $C_{18:3}$) FFAs also increased at a slow rate during ripening of control cheese, by 2.6-fold from day 1 to day 60 (Table 3). On day 60, there were no significant differences in the concentration of LC FFAs between HPP cheeses and control cheese. Afterwards, significantly ($p<0.05$) higher levels of LC FFAs in control cheese than in HPP cheeses were recorded at all sampling dates throughout refrigerated storage. Since yeasts increase the concentration of LC FFAs in cheese (Chen et al. 2012), the high yeast counts present in Casar cheese (Poullet et al. 1991) might be involved in LC FFAs formation. Milk lipoprotein lipase was probably the main agent responsible for LC FFAs formation. In cheeses made from pressurized goat or cow milk, milk lipoprotein lipase remained active. Thus, the concentration of LC FFAs in 60-day cheeses made from raw and from HPP (500 MPa) goat milk did not differ and both were significantly higher than in cheese made from pasteurized milk (Buffa et al. 2001). When Cheddar cheeses made from HPP milk and from raw milk were compared, LC FFAs concentrations were significantly higher in raw milk cheese on day 21 and in cheese from milk treated at 600 MPa on day 180 (Voigt et al. 2012). Also, the concentration of LC FFAs in goat raw milk cheese on day 60 was not influenced by HPP treatment of cheese (Delgado et al. 2012). There is no available information on the barotolerance of ewes milk lipoprotein lipase, which is not necessarily coincident with those of cow or goat milk lipoprotein lipases. This might explain the discrepancy between the results obtained in the present work and those reported for cheeses made from cow or goat milk, independently of whether HPP was applied to the milk or to the cheese. The high perception thresholds of LC FFAs limit their contribution to cheese aroma, in spite of the high concentrations usually reached in many cheese types (Curioni and Bosset 2002).

Volatile Compounds

Ninety-four volatile compounds were detected in Casar cheese by SPME followed by GC-MS, namely 5 aldehydes (acetaldehyde, 3-methylbutanal, 3-ethylbenzaldehyde, 4-ethylbenzaldehide, vinylbenzaldehyde), 7 ketones (acetone, 2-butanone, 2-pentanone, 2-heptanone, 3-hydroxybutanone, 1-phenyl-2-propanone, tertbutyl-hydroxy-propiophenone), 12 alcohols (2-propanol, ethanol, 2-butanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, methyl-1-butanol, 3-methyl-3-buten-1-ol, 2-heptanol, 3-methyl-2-buten-1-ol, 1-hexanol, 2,5-dimethyl-3-hexanol), 12 acids (acetic, propanoic, 2-methylpropanoic, butanoic, 3-methylbutanoic, 2-methylbutanoic, pentanoic, 2-butenoic, hexanoic, 4-hexenoic, heptanoic, octanoic), 33 esters (ethylacetate, ethylpropanoate, ethyl-2-methylpropanoate, propylacetate, methylbutyrate, 1-methylpropylacetate, 2-

Table 3 Concentrations of medium-chain (MC, $C_{10:0}$ to $C_{14:0}$) and long-chain (LC, $C_{16:0}$ to $C_{18:3}$) free fatty acids (FFAs) during ripening and refrigerated storage of control and HPP cheeses

Acid	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
MC FFAs[b]	1	0.23±0.03				
	21	0.30±0.03 a	0.28±0.02 a	0.29±0.03 a		
	35	0.32±0.03 a	0.30±0.02 a	0.29±0.04 a	0.30±0.01 a	0.28±0.01 a
	60	0.38±0.01 a	0.37±0.02 a	0.35±0.01 a	0.34±0.01 a	0.36±0.02 a
	120	0.66±0.08 b	0.52±0.06 ab	0.42±0.02 a	0.44±0.03 a	0.42±0.02 a
	180	0.69±0.11 a	0.55±0.03 a	0.56±0.03 a	0.52±0.01 a	0.48±0.03 a
	240	0.88±0.08 b	0.63±0.05 a	0.52±0.03 a	0.55±0.03 a	0.54±0.07 a
LC FFAs[b]	1	0.54±0.11				
	21	1.09±0.07 a	1.04±0.05 a	1.03±0.04 a		
	35	1.14±0.08 a	1.07±0.09 a	1.00±0.06 a	1.11±0.02 a	1.00±0.06 a
	60	1.40±0.03 a	1.39±0.06 a	1.23±0.02 a	1.25±0.04 a	1.36±0.06 a
	120	2.61±0.05 c	2.01±0.03 b	1.50±0.10 a	1.65±0.04 a	1.65±0.01 a
	180	2.95±0.14 b	2.03±0.10 a	1.89±0.06 a	1.86±0.13 a	1.62±0.09 a
	240	3.02±0.09 b	2.14±0.10 a	1.70±0.08 a	1.76±0.05 a	1.74±0.18 a

[a] Codes for HPP cheeses are 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Concentrations are expressed in milligram per gram cheese dry matter, as mean±SEM of duplicate determinations on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly

methylpropylacetate, ethylbutyrate, propylpropanoate, 1-methylpropylpropanoate, ethyl-2-methylbutyrate, ethyl-3-methylbutyrate, butylacetate, isoamylacetate, propylbutyrate, 1-methylpropylbutyrate, ethylpentanoate, butylpropanoate, propyl-3-methylbutyrate, 2-methylpropylbutyrate, amylpropionate, butylbutyrate, ethylhexanoate, butyl-3-methylbutyrate, ethyltrans-4-hexenoate, propylhexanoate, isobutylhexanoate, ethylheptanoate, ethyloctanoate, pentylhexanoate, ethyl-3-hydroxybutyrate, ethyldecanoate, butyrolactone), 9 sulphur compounds (methanethiol, dimethylsulphide, sulphur dioxide, *S*-methyl-thioacetate, dimethyldisulphide, *S*-methylthiobutyrate, dimethyltrisulphide, 2-hydroxyethylmethylsulphide, 3-methylthio-1-propanol), 8 aromatic compounds (toluene, styrene, ethylstyrene, 1,3-ethenylbenzene, benzenemethanol, benzeneethanol, phenol, 4-methylphenol), 5 hydrocarbons (hexane, 1,4-pentadiene, heptane, octane, 3-octene) and 3 ethers (2-butoxyethanol, 2-(2-ethoxyethoxy)ethanol, 3-phenoxyethanol). According to the analysis of variance, 84 compounds were significantly ($p<0.05$) affected by the HPP treatment applied and 71 compounds by the days elapsed after manufacture. The number of volatile compounds found in the present work was higher than the 46 volatile compounds reported by Delgado et al. (2010) for this cheese variety and closer to the 112 volatile compounds found for La Serena cheese, a similar variety, by Carbonell et al. (2002).

Total aldehydes were at similar levels in HPP cheeses and in control cheese on day 60 (Table 4). During refrigerated storage, they increased at a lesser rate in HPP cheeses than in control cheese, remaining on day 240 at significantly ($p<0.05$) lower levels in all the HPP cheeses than in control cheese. Acetaldehyde, the major aldehyde during ripening, mostly formed through the metabolism of lactose but also derived from glycine, kept fairly stable during refrigerated storage. 3-Methylbutanal, formed from leucine, increased in control, 400W5 and 600W5 cheeses during refrigerated storage but declined in the rest. In a previous study on Casar cheese, it was detected only until day 30 (Delgado et al. 2010). It is a potent odorant in Camembert, Cheddar, Emmental and Gruyère cheeses and has a pleasant fruity odour which turns into green malty at high concentrations (Curioni and Bosset 2002). The three aromatic aldehydes found, 3-ethylbenzaldehyde, 4-ethylbenzaldehyde and vinylbenzaldehyde, probably derived from the catabolism of aromatic amino acids by Gram negative bacteria, increased during refrigerated storage. The last one became the dominant aldehyde on day 240. Aromatic aldehydes are not common compounds in cheese, but 4-ethylbenzaldehyde has been found in ready-to-use lettuce (Lonchamp et al. 2009).

Total ketones were at significantly ($p<0.05$) higher levels in control, 600W3 and 600W5 cheeses than in 400W3 and 400W5 cheeses on day 60, but the differences were no longer significant on day 240 (Table 4). 2-Butanone, with a sweet odor reminiscent of butterscotch (Curioni and Bosset 2002), was the major ketone in control cheese, accounting for approximately 90 % of total ketones on day 60, in agreement with a previous work (Delgado et al. 2010). Its levels rose in HPP and control cheeses during refrigerated storage, becoming dominant on day 240 in all the cheeses. 3-Hydroxy-2-butanone (acetoin), with a sour milk odour note, was the dominant ketone in 600W3 and 600W5 cheeses at the end of ripening, accounting for 50–80 % of total ketones, but afterwards declined. 2-Propanone, with hay and wood pulp odour notes, 2-pentanone, with a sweet fruity odour note, and 2-heptanone, with a Blue cheese note and a low perception threshold (Molimard and Spinnler 1996), also reached relatively high levels in HPP and control cheeses.

Table 4 Levels of total volatile aldehydes, ketones, alcohols, acids and esters during ripening and refrigerated storage of control and HPP cheeses

Chemical group	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
Aldehydes[b]	60	10.7±0.7 a	9.2±0.9 a	10.8±0.5 a	9.7±0.4 a	9.3±0.3 a
	120	15.8±1.5 b	12.6±1.5 ab	13.3±0.8 ab	11.2±0.4 a	11.0±0.7 a
	240	17.1±0.8 b	12.5±1.0 a	11.9±0.4 a	13.3±0.5 a	12.3±0.8 a
Ketones[b]	60	1,429±119 b	152±25 a	1,857±135 b	387±113 a	1,523±121 b
	120	1,778±266 b	1,836±254 b	1,411±263 ab	1,439±39 ab	1,095±132 a
	240	2,147±179 a	1,796±55 a	2,475±478 a	2,117±172 a	1,625±254 a
Alcohols[b]	60	9,065±248 b	5,191±276 a	4,866±119 a	9,071±180 b	7,879±876 b
	120	13,490±971 c	10,363±498 bc	6,707±418 a	11,375±528 bc	10,067±810 b
	240	15,914±1,379 b	14,701±414 b	7,450±547 a	14,065±557 b	9,052±113 a
Acids[b]	60	12,275±629 b	5,239±127 a	5,246±302 a	9,878±1,344 b	10,962±529 b
	120	10,444±420 b	6,176±260 a	5,866±306 a	7,571±852 ab	8,662±1,279 ab
	240	9,483±344 a	6,680±358 a	7,201±865 a	8,332±381 a	8,537±1,341 a
Esters[b]	60	3,651±430 b	1,411±154 a	862±45 a	2,753±157 b	1,502±34 a
	120	3,984±372 c	1,394±69 a	1,197±68 a	3,093±408 bc	2,058±81 ab
	240	5,536±361 c	1,533±78 a	1,488±188 a	3,522±256 b	2,403±403 ab

[a] Codes for HPP cheeses are 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Levels are expressed in relative abundances to the internal standard, as mean±SEM of duplicate determinations on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly

Total alcohols were at significantly ($p<0.05$) higher levels in control cheese and 400W5 and 600W5 cheeses than in 400W3 and 600W3 cheeses on day 60 (Table 4). Main alcohols at the end of ripening were ethanol, 2-butanol and 3-methyl-1-butanol, followed by 2-methyl-1-propanol and 1-butanol. The secondary alcohol 2-butanol is formed by the enzymatic reduction of 2-butanone (Molimard and Spinnler 1996), while branched-chain primary alcohols derive from the reduction of the respective aldehydes produced from leucine, isoleucine and valine (Yvon and Rijnen 2001). 3-Methyl-1-butanol has pleasant fresh cheese notes, while primary alcohols have green, alcoholic notes and secondary alcohols fruity, herbaceous notes (Curioni and Bosset 2002). Ethanol declined slightly during refrigerated storage, while 2-butanol increased markedly. On day 240, control cheese and 400W3 and 400W5 cheeses showed the maximum ($p<0.05$) levels of alcohols due to their high contents of 2-butanol, which accounted for more than 60 % of total alcohols.

Total volatile acids reached their highest ($p<0.05$) levels at the end of the ripening period in control cheese and in 400W5 and 600W5 cheeses (Table 4). Butanoic, acetic, 2-methylpropanoic, propanoic and 3-methylbutanoic were the main volatile acids on day 60, while acetic, 3-methylbutanoic, butanoic, decanoic and octanoic had been reported as the major volatile acids in 60-day Casar cheese by Delgado et al. (2010). On day 240, butanoic, acetic, 2-methylpropanoic, 3-methylbutanoic and propanoic remained as the main volatile acids in the present work. They are mostly produced through the microbial metabolism of lactose, lactate, citrate and amino acids (Yvon and Rijnen 2001), but butanoic acid may also derive from the hydrolysis of triglycerides (Collins et al. 2003). Their concentration may decrease in cheese because of ester formation, mainly at late ripening stages and during refrigerated storage.

Total esters reached significantly ($p<0.05$) higher levels in control and 400W5 cheeses than in the other treated cheeses on day 60 (Table 4). Ethyl butanoate was the main ester, accounting for more than 60 % of the total esters in control cheese, followed by ethyl acetate, ethyl hexanoate, ethyl propanoate, 3-methylbutyl acetate and ethyl 3-methylbutanoate. Esters generally show sweet, fruity and floral notes, have a low perception threshold and, by masking the sharpness and bitterness of other compounds, contribute to a pleasant cheese flavour. In particular, ethyl butanoate has been identified as a potent odorant in many cow and ewes milk cheese varieties (Curioni and Bosset 2002). During refrigerated storage, total esters increased, the highest ($p<0.05$) level on day 240 being that of control cheese followed by 400W5 cheese. The increase in esters levels during refrigerated storage was accompanied by a decline in acids levels in control cheese and 400W5 and 600W5 cheeses. However, alcohols did not decline during refrigerated storage in spite of their contribution to ester formation. The only cyclic ester detected in cheeses, γ-butyrolactone, showed the highest ($p<0.05$) levels in control cheese on day 60, and increased afterwards in all cheeses, with no significant differences between them on day 240. On the contrary, γ-butyrolactone was not found in Casar cheese

by Delgado et al. (2010), who reported δ-decalactone as the only lactone present.

Total sulphur compounds were at low levels at the end of ripening in HPP and control cheeses, with no significant differences between cheeses (Table 5). During refrigerated storage, their level increased dramatically in control cheese, by factors of 82 on day 120 and 467 on day 240, and at a lesser degree in 400W3 and 600W3 cheeses, by factors of 9 and 10, respectively, from day 60 to day 240. The number of sulphur compounds also increased, from 4 on day 60 to 9 on day 240. Dimethyldisulphide, methanethiol and dimethyltrisulphide were below detection level on day 60 and accounted for 76.6, 7.6 and 4.1 % of total sulphur compounds in 240-day control cheese, while *S*-methyl-thioacetate accounted for 11.3 %, after a 20-fold increase from day 60 to day 240. Carbon disulphide, dimethylsulphide and dimethyldisulphide were the sulphur compounds detected, at low levels, in a study on La Serena cheese (Carbonell et al. 2002), while 3-methylthio-1-propanol was the only sulphur compound found in Casar cheese, at levels which declined during ripening (Delgado et al. 2010). Sulphur compounds may be produced in cheese by *Lactococcus*, *Lactobacillus*, *Brevibacterium*, *Micrococcus*, *Corynebacterium*, *Pseudomonas* and probably other genera of Gram negative bacteria. Most of them derive from methanethiol, a compound of putrid and faecal-like aroma which is formed from methionine by the action of cystathionine or methionine lyases (Weimer et al. 1999). The concentration of methionine in cheeses ranged from 0.15 to 0.27 mg/g DM on day 60 and from 0.73 to 1.32 mg/g DM on day 240 (Calzada et al. 2013). The characteristic odour notes of sulphur compounds at low concentrations (cowy, feedy, garlic, onion, cooked cabbage and cauliflower, mashed potato) make them essential contributors to the aroma of varieties such as Limburger, Camembert, Cheddar, Blue and ewes milk cheeses (Bonnarme et al. 2000; Fernández-García et al. 2004). However, because of to their very low perception thresholds, from 0.2 to 20 ppb (Weimer et al. 1999), high concentrations of sulphur compounds in cheese may result unpleasant.

Total aromatic compounds were at similar levels in HPP and control cheeses (Table 5). The main aromatic compounds were 2-phenylethanol, toluene, styrene, ethylstyrene and phenol. They generally increased during refrigerated storage in all cheeses. Aromatic compounds probably derive from the catabolism of aromatic amino acids by cheese microbiota other than lactic acid bacteria. The concentration of aromatic amino acids in cheeses ranged from 0.27 to 0.63 mg/g DM on day 60 and from 1.26 to 2.63 mg/g DM on day 240 (Calzada et al. 2013). Only styrene and phenol had been previously detected in Casar cheese (Delgado et al. 2010), while most of the aromatic compounds found in the present work had been previously reported for other ewe raw milk cheeses (Carbonell et al. 2002; Fernández-García et al. 2002, 2004).

Total hydrocarbons levels increased slightly during refrigerated storage in HPP and control cheeses, with no significant differences at any of the sampling times (Table 5). Octane, hexane and 1,4-pentadiene were the main linear hydrocarbons, which are formed through the oxidation of FFAs. Hydrocarbons found in the present work have been found in other ewe raw

Table 5 Levels of total volatile sulphur compounds, aromatic compounds, hydrocarbons and ethers during ripening and refrigerated storage of control and HPP cheeses

Chemical group	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
Sulphur compounds[b]	60	14.1±1.6 a	7.3±0.7 a	7.5±1.2 a	10.2±2.6 a	14.6±2.3 a
	120	1,158±219 b	50.5±17.3 a	68.8±19.7 a	10.1±3.0 a	12.7±3.0 a
	240	6,583±1,005 b	66.6±27.1 a	78.1±21.7 a	30.6±11.0 a	8.0±1.5 a
Aromatic compounds[b]	60	48.0±2.8 a	40.8±1.1 a	36.7±0.9 a	42.7±8.7 a	42.8±4.0 a
	120	55.8±3.7 a	49.0±1.8 a	41.0±1.0 a	43.2±4.5 a	48.2±5.8 a
	240	69.8±9.9 a	63.8±8.1 a	50.7±1.5 a	62.8±4.4 a	54.8±6.0 a
Hydrocarbons[b]	60	12.6±0.5 a	18.9±0.8 a	19.1±1.4 a	19.0±2.8 a	19.6±1.8 a
	120	16.6±3.2 a	16.3±2.4 a	18.4±1.7 a	24.4±3.3 a	21.0±1.8 a
	240	21.8±3.8 a	18.6±3.7 a	17.5±1.8 a	20.8±4.4 a	21.9±1.8 a
Ethers[b]	60	14.4±2.6 a	11.3±0.4 a	12.2±0.9 a	17.1±3.1 a	17.4±0.9 a
	120	20.1±1.2 b	15.5±2.1 ab	11.9±1.7 a	16.1±0.7 ab	21.2±2.6 b
	240	23.7±4.2 a	18.1±1.6 a	16.2±0.8 a	24.3±3.3 a	24.6±4.1 a

[a] Codes for HPP cheeses are 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Levels are expressed in relative abundances to the internal standard, as mean±SEM of duplicate determinations on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly

Table 6 Odour characteristics and off-odours during ripening and refrigerated storage of control and HPP cheeses

Characteristic	Days	Control cheese	HPP cheeses[a]			
			400W3	600W3	400W5	600W5
Odour intensity[b]	60	5.92±0.29 a	5.81±0.37 a	5.60±0.28 a	5.88±0.34 a	5.74±0.41 a
	120	6.65±0.38 a	5.66±0.42 a	5.79±0.35 a	6.07±0.29 a	6.45±0.36 a
	240	7.89±0.33 b	6.42±0.26 a	6.14±0.40 a	6.71±0.35 a	6.53±0.32 a
Odour quality[b]	60	6.42±0.38 a	6.23±0.30 a	5.96±0.28 a	6.45±0.34 a	6.18±0.41 a
	120	4.59±0.44 a	5.36±0.29 ab	5.77±0.35 ab	6.11±0.42 b	5.94±0.30 ab
	240	1.87±0.20 a	4.78±0.47 b	4.90±0.36 b	5.32±0.29 b	5.56±0.43 b
Putrid odour[b]	60	1.02±0.19 a	1.46±0.15 a	0.91±0.23 a	0.98±0.09 a	1.32±0.26 a
	120	2.59±0.31b	1.70±0.28 ab	1.14±0.15 a	1.36±0.23 a	1.19±0.30 a
	240	6.82±0.38 b	2.85±0.26 a	2.40±0.37 a	1.97±0.29 a	2.28±0.33 a
Rancid odour[b]	60	0.53±0.12 a	0.76±0.20 a	0.49±0.08 a	0.38±0.19 a	0.62±0.21 a
	120	1.85±0.23 b	1.20±0.17 a	1.06±0.15 a	1.32±0.10 a	0.91±0.22 a
	240	3.28±0.34 b	1.59±0.26 a	1.80±0.32 a	1.47±0.29 a	1.38±0.16 a

[a] Codes for HPP cheeses are 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21; 400W5, HPP at 400 MPa on day 35; 600W5, HPP at 600 MPa on day 35

[b] Results are expressed as mean±SEM of the scores from a 15-member panel using a 10-point scale on two cheese making trials. Means in the same row at the same sampling date followed by the same letter do not differ significantly

milk cheeses (Carbonell et al. 2002; Fernández-García et al. 2002, 2004), while 3-methylpentane was the only hydrocarbon detected in Casar cheese (Delgado et al. 2010).

Total ethers were at low levels on day 60 in HPP and control cheeses, with no significant differences between them at that time (Table 5). They increased slightly in all cheeses during refrigerated storage, with 600W3 cheese generally exhibiting the lowest values. Main ethers were 2-butoxyethanol and 2-(2-ethoxy-ethoxy)ethanol. The presence of ethers in smoked goat raw milk cheese was reported by Guillén et al. (2004), who attributed their origin to pine needle smoke. The possible sources of ethers in non-smoked cheeses are unknown.

Odour Characteristics

Odour intensity of HPP and control cheeses on day 60 did not differ (Table 6). During refrigerated storage, odour intensity scores rose slightly in HPP cheeses and more markedly in control cheese, which showed a significantly ($p<0.05$) higher value than all the HPP cheeses on day 240. The increase in odour intensity scores from day 60 to day 240 may be associated to the build-up of volatile compounds such as aldehydes, alcohols, esters, ketones and probably sulphur compounds too. Regarding odour quality, HPP and control cheeses did not differ on day 60. Control cheese received the lowest ($p<0.05$) score from the panellists on day 240, while HPP cheeses did not differ between them. The increasing concentrations of some volatile compounds in control cheese during refrigerated storage might be responsible for the observed changes in odour quality. In particular, the outstanding increases of sulphur compounds such as dimethyldisulphide, *S*-methyl-thioacetate, methanethiol and dimethyltrisulphide seem to be associated to the appearance of putrid odour and the loss of odour quality during the refrigerated storage of control cheese.

Odour characteristics of 60-day La Serena cheeses were not significantly affected by HPP at 300 or 400 MPa on days 2 or 50 after manufacture (Arqués et al. 2007). On the contrary, the results obtained in the present work show significant beneficial effects of HPP at 400 or 600 MPa on the odour

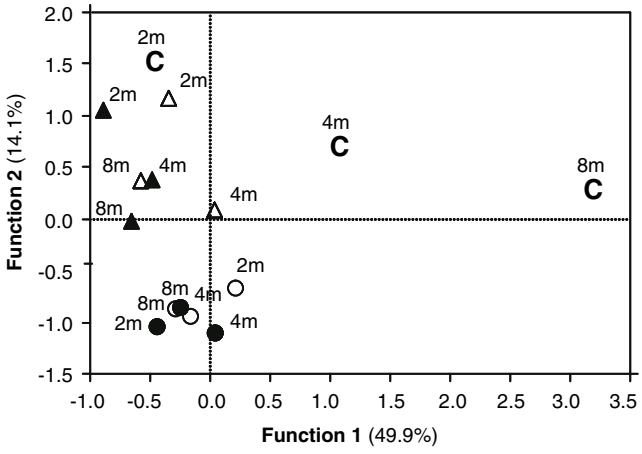


Fig. 1 Distribution of HPP and control cheeses on the plane defined by functions 1 and 2 of the principal component analysis. Each symbol represents the averaged value of the two batches of cheese. Treatments are as follows: control, *C*; 400W3, *open circle*; 600W3, *black circle*; 400W5, *open triangle*; 600W5, *black triangle*. Days after manufacture are as follows: 60 days, *2m*; 120 days, *4m*; 240 days, *8m*

characteristics of Casar cheese when held under refrigeration for a prolonged period.

Principal Component Analysis

Principal component analysis was firstly carried out on 57 volatile compounds, selected by the high statistical significance assigned to them in the analysis of variance. The objective was to discriminate HPP and control cheeses on days 60, 120 and 240 after manufacture, according to pressurization treatment and cheese age, on the basis of their volatile fraction. Function 1, formed by 25 esters, 7 sulphur compounds, 4 alcohols, 2 aldehydes, 2 ketones, 3 aromatic compounds, 1 acid and 1 hydrocarbon, explained 48.2 % of the variance, while function 2, formed by four alcohols, four acids, one ester and one ketone, explained 13.5 % and function 3, formed solely by one alcohol and one acid, explained 8.8 %. When control and HPP cheeses were plotted against functions 1 and 2, two groups of cheeses were obtained, the 240-day control cheese and the rest, which included the 60- and 120-day control cheeses and all the HPP cheeses (data not shown).

A second PCA was performed on the total concentrations of the five groups of acids as determined by GC and the total levels of the nine groups of volatile compounds as determined by GC-MS. The objective was to achieve a better discrimination of cheeses on the basis of their FFAs contents in addition to their volatile fraction. Function 1, formed by all groups of acids (LC FFAs, SC FFAs, MC FFAs, BCCAs, acetic + propionic, in decreasing order of significance) and seven groups of volatile compounds (alcohols, esters, aromatic compounds, sulphur compounds, aldehydes, ethers and ketones, in decreasing order of significance), explained 49.9 % of the variance. Function 2 (formed by hydrocarbons and, with a negative coefficient, by volatile acids) explained 14.1 % of the variance. Function 3 (formed by volatile acids and, with a negative coefficient, by ketones) explained 12.6 % of the variance. Control and HPP cheeses were plotted against functions 1 and 2, which may be respectively associated to over-ripening and to cheese age at the time of HPP treatment. Four groups were thus obtained: (1) 240-day control cheese, (2) 120-day control cheese, (3) 400W3 and 600W3 cheeses at all sampling times, and (4) 60-day control cheese together with 400W5 and 600W5 cheeses at all sampling times (Fig. 1). According to the results of this second PCA, cheeses pressurized at 400 or 600 MPa on day 35 after manufacture maintained during a prolonged refrigerated storage period characteristics similar to those of 60-day control cheese.

Conclusions

Carboxylic acids increased during ripening and refrigerated storage in all cheeses, in particular in control cheese which showed considerably higher concentrations of propionic acid, BCCAs and SC FFAs than HPP cheeses. Also, major changes in the volatile fraction of cheeses were recorded during ripening and refrigerated storage, which were more marked in the case of control cheese. The excessive formation of some impact flavour compounds affected negatively the sensory characteristics of control cheese, which suffered a dramatic loss of odour quality during refrigerated storage. Sulphur compounds appeared as the main causative agents of this phenomenon. HPP was an effective procedure to reduce the formation of carboxylic acids and volatile compounds and to control off-odours, thus maintaining cheese sensory characteristics throughout a prolonged refrigerated storage period.

Acknowledgments This research was funded by AGL 2009-07801 project (Ministry of Science and Innovation, Spain). The authors thank the PDO dairy for providing the cheeses and Hyperbaric for HPP treatments. J. Calzada is the recipient of a FPI grant (Ministry of Science and Innovation, Spain).

References

Arqués, J. A., Garde, S., Fernández-García, E., Gaya, P., & Nuñez, M. (2007). Volatile compounds, odor, and aroma of La Serena cheese high-pressure treated at two different stages of ripening. *Journal of Dairy Science, 90*(8), 3627–3639.

Ávila, M., Garde, S., Fernández-García, E., Medina, M., & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the odor and aroma of Hispánico cheese: correlation of volatile compounds and sensory analysis. *Journal of Agricultural and Food Chemistry, 54*(2), 382–389.

Ávila, M., Calzada, J., Garde, S., & Nuñez, M. (2007). Effect of a bacteriocin-producing *Lactococcus lactis* strain and high-pressure treatment on the esterase activity and free fatty acids in Hispánico cheese. *International Dairy Journal, 17*(12), 1415–1423.

Bonnarme, P., Psoni, L., & Spinnler, H. E. (2000). Diversity of L-methionine catabolism pathways in cheese-ripening bacteria. *Applied and Environmental Microbiology, 66*(12), 5514–5517.

Buffa, M., Guamis, B., Pavia, M., & Trujillo, A. J. (2001). Lipolysis in cheese made from raw, pasteurized or high-pressure-treated goats' milk. *International Dairy Journal, 11*(3), 175–179.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013). Using high-pressure-processing for reduction of proteolysis and prevention of over-ripening of raw milk cheese. *Food and Bioprocess Technology*. doi:10.1007/s11947-013-1141-5.

Carbonell, M., Nuñez, M., & Fernández-García, E. (2002). Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavor characteristics. *Lait, 82*(6), 683–698.

Chen, L., Cui, J., Ding, Q., Ma, Y., Chen, L., Dong, J., Jiang, T., & Maubois, J. (2012). The effect of yeast species from raw milk in China on proteolysis and aroma compound formation in Camembert-type cheese. *Food and Bioprocess Technology, 5*(6), 2548–2556.

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13*(11), 841–866.

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal, 12*(12), 959–984.

Delgado, F. J., González-Crespo, J., Ladero, L., Cava, R., & Ramírez, R. (2009). Free fatty acids and oxidative changes of a Spanish soft cheese (PDO 'Torta del Casar') during ripening. *International Journal of Food Science and Technology, 44*(9), 1721–1728.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J., & Ramírez, R. (2010). Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. *Food Chemistry, 118*(1), 182–189.

Delgado, F. J., González-Crespo, J., Cava, R., & Ramírez, R. (2012). High-pressure treatment applied throughout ripening of a goat cheese caused minimal changes on free fatty acids content and oxidation in mature cheese. *Dairy Science and Technology, 92*(3), 237–248.

Fernández-García, E., Carbonell, M., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. *Journal of Dairy Research, 69*(4), 579–593.

Fernández-García, E., Carbonell, M., Gaya, P., & Nuñez, M. (2004). Evolution of the volatile components of ewes raw milk Zamorano cheese. Seasonal variation. *International Dairy Journal, 14*(8), 701–711.

Fernández-García, E., Carbonell, M., Calzada, J., & Nuñez, M. (2006). Seasonal variation of the free fatty acids contents of Spanish ovine milk cheeses protected by a designation of origin: a comparative study. *International Dairy Journal, 16*(3), 252–261.

Fox, P. F., & Wallace, J. M. (1997). Formation of flavor compounds in cheese. *Advances in Applied Microbiology, 45*, 17–85.

Gaya, P., Sánchez, C., Nuñez, M., & Fernández-García, E. (2005). Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation. *Journal of Dairy Research, 72*(3), 287–295.

Guillén, M. D., Ibargoitia, M. L., Sopelana, P., Palencia, G., & Fresno, M. (2004). Components detected by means of solid-phase micro-extraction and gas chromatography/mass spectrometry in the head-space of artisan fresh goat cheese smoked by traditional methods. *Journal of Dairy Science, 87*(2), 284–299.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71*(4), 496–499.

Juan, B., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. (2007). Effects of high pressure on proteolytic enzymes in cheese: relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science, 90*(5), 2113–2125.

Lonchamp, J., Barry-Ryan, C., & Devereux, M. (2009). Identification of volatile quality markers of ready-to-use lettuce and cabbage. *Food Research International, 42*(8), 1077–1086.

Mallia, S., Fernández-García, E., & Bosset, J. O. (2005). Comparison of purge and trap and solid phase microextraction techniques for studying the volatile aroma compounds of three European PDO hard cheeses. *International Dairy Journal, 15*(6–9), 741–758.

Malone, A. S., Wick, C., Shellhammer, T. H., & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science, 86*(4), 1139–1146.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheese during ripening: a review. *Lait, 80*(3), 293–324.

Molimard, P., & Spinnler, H. E. (1996). Compounds involved in the flavour of surface mold-ripened cheeses: origins and properties. *Journal of Dairy Science, 79*(2), 169–184.

Morales, P., Fernández-García, E., & Nuñez, M. (2005). Volatile compounds produced in cheese by *Pseudomonas* strains of dairy origin belonging to six different species. *Journal of Agricultural and Food Chemistry, 53*(17), 6835–6843.

Norton, T., & Sun, D.-W. (2008). Recent advances in the use of high pressure as an effective processing technique in the food industry. *Food and Bioprocess Technology, 1*(1), 2–34.

Nuñez, M., Guillén, A. M., Rodríguez-Marín, M. A., Marcilla, A. M., Gaya, P., & Medina, M. (1991). Accelerated ripening of ewes' milk Manchego cheese: the effect of neutral proteinases. *Journal of Dairy Science, 74*(12), 4108–4118.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology, 66*(11), 4890–4896.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L., & Beresford, T. P. (2002). Effects of high-pressure treatment on viability and autolysis of starter bacteria and proteolysis in Cheddar cheese. *International Dairy Journal, 12*(11), 915–922.

Picon, A., Alonso, R., Gaya, P., & Nuñez, M. (2013a). High-pressure treatment and freezing of raw goat milk curd for cheese manufacture: effects on cheese characteristics. *Food and Bioprocess Technology, 6*(10), 2820–2830.

Picon, A., Alonso, R., van Wely, K. H. M., & Nuñez, M. (2013b). Microstructural, textural and colour characteristics during ripening of Hispánico cheese made using high-pressure-treated ovine milk curd. *Food and Bioprocess Technology, 6*(11), 3056–3067

Poullet, B., Huertas, M., Sánchez, A., Cáceres, P., & Larriba, G. (1991). Microbial study of Casar de Cáceres cheese throughout ripening. *Journal of Dairy Research, 58*(2), 231–238.

Sablé, S., & Cottenceau, G. (1999). Current knowledge of soft cheeses flavor and related compounds. *Journal of Agricultural and Food Chemistry, 47*(12), 4825–4836.

Shao, Y. W., & Ramaswamy, H. S. (2011). *Clostridium sporogenes*-ATCC 7955 spore destruction kinetics in milk under high pressure and elevated temperature treatment conditions. *Food and Bioprocess Technology, 4*(3), 458–468.

Tejada, L., Gómez, R., Vioque, M., Sánchez, E., Mata, C., & Fernández-Salguero, J. (2000). Effect of freezing and frozen storage on the sensorial characteristics of Los Pedroches, a Spanish ewe milk cheese. *Journal of Sensory Studies, 15*(3), 251–262.

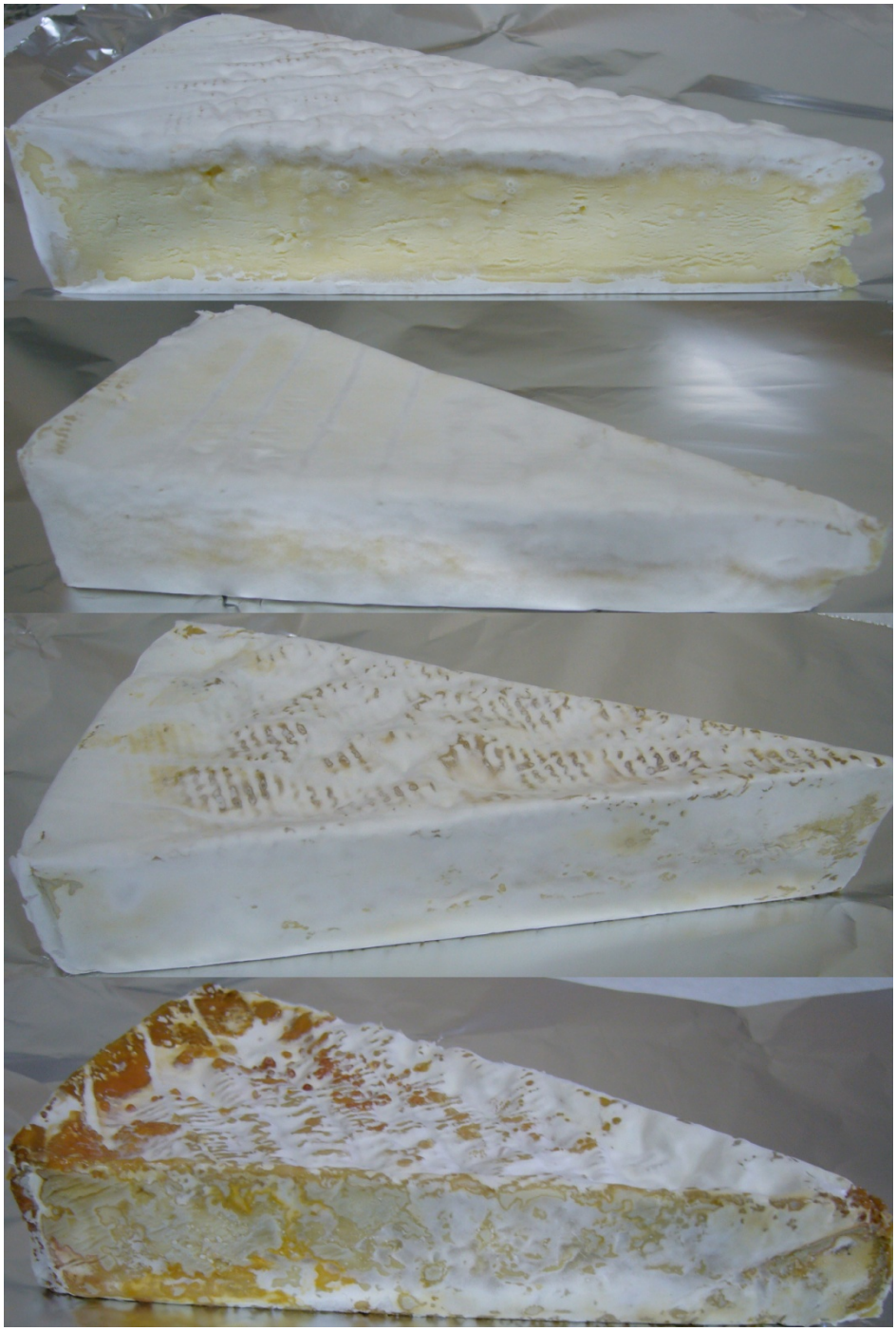
Trujillo, A. J., Royo, C., Ferragut, V., & Guamis, B. (1999). Ripening profiles of goat cheese produced from milk treated with high pressure. *Journal of Food Science, 64*(5), 833–837.

Van Hekken, D. L., Tunick, M. H., & Park, Y. W. (2005). Effect of frozen storage on the proteolytic and rheological properties of soft caprine milk cheese. *Journal of Dairy Science, 88*(6), 1966–1972.

Voigt, D. D., Chevalier, F., Donaghy, J. A., Patterson, M. F., Qian, M. C., & Kelly, A. L. (2012). Effect of high-pressure treatment of milk for cheese manufacture on proteolysis, lipolysis, texture and functionality of Cheddar cheese during ripening. *Innovative Food Science and Emerging Technologies, 13*, 23–30.

Weimer, B., Seefeldt, K., & Dias, B. (1999). Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek, 76*(1–4), 247–261.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11*(4–7), 185–201.



Capítulo 7.

Effect of high-pressure-processing on the microbiology, proteolysis, texture and flavour of Brie cheese during ripening and refrigerated storage.

Fotografía: cuñas de queso Brie control (no presurizado), de arriba abajo: a día 21,30, 60 y 120.

International Dairy Journal 37 (2014) 64–73

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Effect of high-pressure-processing on the microbiology, proteolysis, texture and flavour of Brie cheese during ripening and refrigerated storage

Javier Calzada, Ana del Olmo, Antonia Picon, Pilar Gaya, Manuel Nuñez*

Departamento de Tecnología de Alimentos, INIA, Carretera de La Coruña Km 7, Madrid 28040, Spain

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form
3 March 2014
Accepted 12 March 2014

ABSTRACT

Brie cheeses were high pressure (HP)-treated at 400 or 600 MPa on days 14 or 21 after manufacture to prevent over-ripening. Lactic acid bacteria and *Penicillium camemberti* numbers declined markedly after HP treatment. In control cheese pH increased 2.0 units from day 21 to day 60, but less than 0.3 units in HP-treated cheeses. Cheeses treated at 600 MPa showed the maximum concentrations of residual caseins during refrigerated storage and control cheese the minimum concentrations. A 7.6-fold increase in hydrophobic peptides was recorded from day 21 to day 60 in control cheese and 0.8–1.6-fold increases in HP-treated cheeses. The maximum aminopeptidase activity was detected in control cheese, the highest free amino acid concentrations in cheeses treated at 400 MPa. The firmest texture was recorded for cheeses treated on day 14 at 400 or 600 MPa. HP-treated cheeses showed higher flavour quality scores than control cheese from day 60 onwards.

1. Introduction

Brie cheese, a surface mould-ripened variety, is usually manufactured from pasteurised milk inoculated with a mesophilic lactic starter culture and *Penicillium camemberti* spores, with *Geotrichum candidum* and *Kluyveromyces lactis* as optional adjunct cultures. The colonization of cheese surfaces by *P. camemberti* mycelium reduces the development of spoilage microorganisms and modulates the external appearance (Lessard, Bélanger, St-Gelais, & Labrie, 2012). Most of the scientific knowledge on the ripening process of surface mould-ripened cheeses has been obtained on Camembert cheese (Spinnler & Gripon, 2004), more suitable for experimental work than Brie cheese because of its smaller size. Growth of *P. camemberti* on the surface of Camembert cheese is accompanied by the production of extracellular enzymes and the consumption of lactic acid, with a concomitant rise in pH value (Desmazeaud, Gripon, Le Bars, & Bergère, 1976). Although phenomena occurring during the ripening process of Brie and Camembert cheeses are similar, the lesser surface to volume ratio of the former variety may result in some differences.

Milk native plasmin, clotting enzymes and proteinases produced by microorganisms, mostly lactic acid bacteria and *P. camemberti*, are responsible for the primary proteolysis taking place during the manufacture and ripening of Camembert cheese (Leclercq-Perlat et al., 2004; Trieu-Cuot & Gripon, 1982). The same causative agents are considered to be involved in primary proteolysis of Brie cheese. Afterwards, peptidases from lactic acid bacteria (Gripon, Desmazeaud, Le Bars, & Bergère, 1977; Lane & Fox, 1997) and *Geotrichum candidum* (Auberger, Lenoir, & Bergère, 1997) carry out further hydrolysis of the products resulting from primary proteolysis to free amino acids. Finally, the catabolism of free amino acids by lactic acid bacteria and other microorganisms (Yvon & Rijnen, 2001) results in the formation of the volatile potent odorants (Kubíčková & Grosch, 1997) and the non-volatile flavour compounds (Kubíčková & Grosch, 1998) present in surface mould-ripened cheeses.

A characteristic phenomenon taking place in surface mould-ripened cheeses is the migration of calcium and phosphate from the cheese interior to the surface, associated with the pH gradient created by lactic acid consumption by moulds (Le Graet, Lepienne, Brule, & Ducruet, 1983). The rise in pH value, firstly at the cheese surface and then at the interior, the reduction of the concentration of calcium phosphate, and the proteolysis, bring about the desirable changes in the texture of surface mould-ripened cheeses (Abraham, Cachon, Colas, Feron, & De Coninck, 2007; Vassal, Monnet, Le Bars, Roux, & Gripon, 1986), which becomes softer and mature to the core during ripening. However, the pH increase throughout

* Corresponding author. Tel.: +34 913476799.
E-mail addresses: nunez@inia.es, mng1950@gmail.com (M. Nuñez).

http://dx.doi.org/10.1016/j.idairyj.2014.03.002
0958-6946/

ripening of Brie and Camembert cheeses creates conditions favourable for the growth of undesirable bacteria such as *Escherichia coli, Yersinia enterocolitica*, *Staphylococcus aureus, Bacillus cereus* and *Listeria monocytogenes* (Maisnier-Patin, Deschamps, Tatini, & Richard, 1992; Nooitgedagt & Hartog, 1988).

Chemical reactions associated with cheese ripening continue during refrigerated storage at retail and at homes. Therefore, cheese at the time of consumption may be of a stronger or different flavour than the manufacturer intended (Wick, Nienaber, Anggraeni, Shellhammer, & Courtney, 2004). Brie cheese is particularly prone to over-ripening not long after the optimal date of consumption, because of its pH value close to neutrality and the presence of potent enzymes of fungal origin. Another undesirable event which may occur in cheese if ripening or refrigerated storage are prolonged in excess is the formation of biogenic amines, a group of compounds of notable public health significance (Silla-Santos, 1996). In surface mould-ripened varieties such as Brie cheese, the build-up of biogenic amines would be favoured by their high concentrations of free amino acids, resulting from extensive proteolysis.

High pressure processing (HPP) efficiently inactivates microbial contaminants in cheese (O'Reilly, O'Connor, Kelly, Beresford, & Murphy, 2000). It also affects the activity of cheese enzymes, which may be increased at pressure levels of 400 MPa or lower and is generally decreased if pressures above 500 MPa are applied (Huppertz, Fox, & Kelly, 2004; O'Reilly, O'Connor, Murphy, Kelly, & Beresford, 2002; Wick et al., 2004). In addition, HPP has shown to be useful in preventing the formation of biogenic amines during ripening and refrigerated storage of cheese (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2013a).

The effect of HPP on the characteristics of surface mould-ripened cheeses has been studied on Camembert and Paillardin, a Belgian variety. Enhanced proteolysis was recorded for Camembert cheese treated at 50 MPa for 4 h, accompanied by an increase in pH value up to 0.50 units (Kolakowski, Reps, & Babuchowski, 1998). Also, Paillardin cheese pressurised at 50 MPa for 8 h exhibited higher soluble N contents and slightly increased pH values than control cheese (Messens, Foubert, Dewettinck, & Huyghebaert, 2001). However, there is no information on the effect of HPP on the ripening of surface mould-ripened cheeses under the conditions currently used in the food industry, with higher pressures and shorter process times. The objective of the present work was to investigate the effect of HPP at 400 or 600 MPa for 5 min, applied on days 14 or 21 after manufacture, as a procedure to control over-ripening of Brie cheese. The microbiology, proteolysis, formation of biogenic amines, texture and flavour of high pressure (HP)-treated Brie cheeses during ripening and refrigerated storage were compared with the characteristics of control cheese.

2. Materials and methods

2.1. Manufacture and high pressure processing of Brie cheese

Brie cheese was made from 2100 L pasteurised (73 °C for 20 s) milk, in duplicate trials carried out on consecutive days at a dairy factory in Central Spain. Mesophilic lactic culture (Flora Danica, Chr. Hansen S.L., Tres Cantos, Spain), *P. camemberti* PC V5 (Sacco, Cadorago, Italy), and 0.02% $CaCl_2$ were added to milk at 37 °C, which was coagulated with equal amounts of animal rennet (Ha-Bo, Chr. Hansen) and microbial rennet (Rennilase, Novo Nordisk, Bagsvaerd, Denmark) in 20 min. The curd was cut into 15-mm cubes, stirred for 15 min, and transferred into circular moulds 32 cm in diameter. Cheeses (2.7 kg in weight, approximately 120 per vat) were held at 28 °C for 22 h, and turned over after 1, 2 and 8 h in the moulds. They were salted by immersion in brine on day 1 after manufacture, and ripened at 12 °C and 90% RH. On day 14 after manufacture, cheeses were cut into 200 g wedges that were wrapped in food-grade metalised paper. Ripening proceeded under the same conditions until day 21 and afterwards wedges were held at 4 °C until day 150.

Wedges of cheeses from both trials were pressurised for 5 min at 400 or 600 MPa on days 14 or 21 after manufacture. They were respectively coded as 400W2, 600W2, 400W3, and 600W3. Before HPP treatments, cheese wedges were vacuum packed in BB325 plastic film bags (Cryovac, Barcelona, Spain). A 120-L capacity isostatic press (Hiperbaric, Burgos, Spain) was used for HPP treatments. Times to reach 400 and 600 MPa were 1.79 and 2.61 min, respectively, and depressurisation times, 6 and 8 s. The initial temperature of the water used as transmitting fluid was 9 °C and remained under 14 °C during the process. After treatments, cheese wedges were taken out of the plastic film bags. Ripening and refrigerated storage conditions of HPP cheese wedges were the same as for control cheese.

2.2. Microbiological analysis and chemical determinations

Representative 10 g samples of cheese wedges, including the rind, were used for microbiological analysis. Total viable counts, lactic acid bacteria, staphylococci, *L. monocytogenes*, Gram-negative bacteria, and coliforms were determined as previously described (Arqués, Garde, Gaya, Medina, & Nuñez, 2006). *P. camemberti* and other fungi were enumerated in duplicate on plates of chloramphenicol glucose agar (Scharlab, Barcelona, Spain), incubated for 3–7 days at 25 °C.

Representative samples for chemical determinations were prepared by removing a 2-mm thick layer of rind from all the surfaces of cheese wedges at 4 °C and mincing the rest of the wedge. Dry matter (DM) content and pH value were determined in triplicate as previously described (Garde, Tomillo, Gaya, Medina, & Nuñez, 2002).

Proteins were analysed in triplicate by capillary gel electrophoresis on an automated PACE/MDQ CE controlled by 32 Karat Software (Beckman Instruments España S.A., Madrid, Spain), according to a previously described method (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2013b). Hydrophilic and hydrophobic peptides in the water-soluble fraction were extracted in duplicate and determined by reverse phase-high pressure liquid chromatography (RP-HPLC) at 214 and 280 nm as described by Lau, Barbano, and Rasmussen (1991). Free amino acids (FAAs) and biogenic amines, simultaneously extracted in duplicate as described by Krause, Bockhardt, Neckermann, Henle, and Klostermeyer (1995), were determined by RP-HPLC and quantified as previously described (Calzada et al., 2013b). Overall proteolysis was determined by the *o*-phthaldialdehyde (OPA) method in duplicate and expressed as the absorbance at 340 nm (Church, Swaisgood, Porter, & Catignani, 1983).

Aminopeptidase activity released into the cheese was determined in duplicate with leucine *p*-nitroanilide (Leu-*p*-NA) and lysine *p*-nitroanilide (Lys-*p*-NA) as substrates and expressed in nmol *p*-nitroaniline per min per g (Garde et al., 2002).

2.3. Texture determinations, sensory analysis and statistical analysis

Fracturability (force at fracture, N), elasticity (Young's modulus, N mm^{-2}), and firmness (energy, area under the force-distance curve up to 75% compression, N m) were determined by uniaxial compression testing on six representative samples per cheese wedge after removing the rind, using an Instron Compression Tester 4301 (Instron, High Wycombe, UK), equipped with Instron Bluehill 2.3 software.

A trained 16-member panel carried out the evaluation of flavour intensity, flavour quality (preference) and five flavour attributes

(acid, bitter, salty, sweet and umami) scoring on a 10-point scale as previously described (Picon et al., 2010). Panellists were also asked to report on the presence of off-flavours.

Statistical analysis of results was carried out by means of SPSS Win 14.0 program (SPSS Inc. Chicago, IL). Two-way analysis of variance included the main effects treatment (four experimental cheeses and one control cheese) and cheese age, as well as the "treatment × age" interaction. Means were compared using Tukey's test, with the significance assigned at $P < 0.05$.

3. Results and discussion

3.1. Microbial groups

Lactic acid bacteria counts in Brie cheese were 8.99 log cfu g^{-1} on day 1, a similar population to that of total viable counts. They decreased by 1.65, 3.71, 0.96 and 3.45 log cfu g^{-1} respectively in 400W2, 600W2, 400W3 and 600W3 cheeses immediately after HPP (Fig. 1). Further decrease in counts during ripening and refrigerated storage occurred only in 600W2 cheese, which might be associated with the particular physiological status of sub-lethally injured cells of lactic acid bacteria in this cheese, which are unable to recover and grow on a selective medium. The recorded inactivation of lactic acid bacteria was in agreement with the results obtained for other cheese varieties submitted to HPP (Calzada et al., 2013a; Voigt, Chevalier, Qian, & Kelly, 2010; Wick et al., 2004).

Counts of *P. camemberti* declined significantly ($P < 0.05$) immediately after HPP (Fig. 1), by 2.25 and 3.29 log cfu g^{-1} in 400W2 and 400W3 cheeses and by at least 5 log cfu g^{-1}, to counts below the detection limit, in 600W2 and 600W3 cheeses. This does not exclude the presence of sublethally injured cells of *P. camemberti* on the cheese rind. There is no information on the resistance of *P. camemberti* to high pressure. Treatment at 400 MPa lowered counts of *Penicillium roqueforti* in cheese slurry by more than 2 log cfu g^{-1} at 10 °C and by 6 log cfu g^{-1} at 20 °C (O'Reilly et al., 2000), and in blue-veined cheese by 0.74–5.33 log cfu g^{-1} depending on cheese age at treatment (Calzada et al., 2013b; Voigt et al., 2010). Those results point to the influence of the strain, its physiological status, growth substrate and HPP temperature on its baroresistance. Non-*Penicillium* fungi, mostly yeasts, were not detected until day 90, at levels ranging from 6.52 to 6.92 log cfu g^{-1} in control cheese and from 2.30 to 3.79 log cfu g^{-1} in cheeses treated at 400 MPa (data not shown). Prior to day 90, the detection of other fungi was most probably hindered by the higher counts of *P. camemberti*, colonies of which spread on chloramphenicol glucose agar plates.

Microbial contaminants tested for such as staphylococci, *L. monocytogenes*, Gram-negative bacteria and coliforms were not detected throughout ripening or refrigerated storage of Brie cheese, indicating satisfactory hygiene conditions during manufacture.

3.2. Dry matter and pH

Dry matter (DM) content of Brie cheese was 44.31% on day 1 (data not shown) and did not vary with HPP immediately after treatments (Fig. 2). On day 21, at the end of ripening, there were no significant ($P < 0.05$) differences in DM content between cheeses but, during refrigerated storage, DM decreased in control cheese and increased in HPP cheeses. The intense proteolysis of control cheese would render it highly hygroscopic as a consequence of the increase in the number of water-binding sites, thus promoting absorption of moisture from the atmosphere of the storage chamber, with a resultant decrease in DM content. The increase in the DM content of HPP cheeses may be directly associated with moisture loss during refrigerated storage. In contrast, previous studies have reported less moisture loss in caprine milk cheese treated at

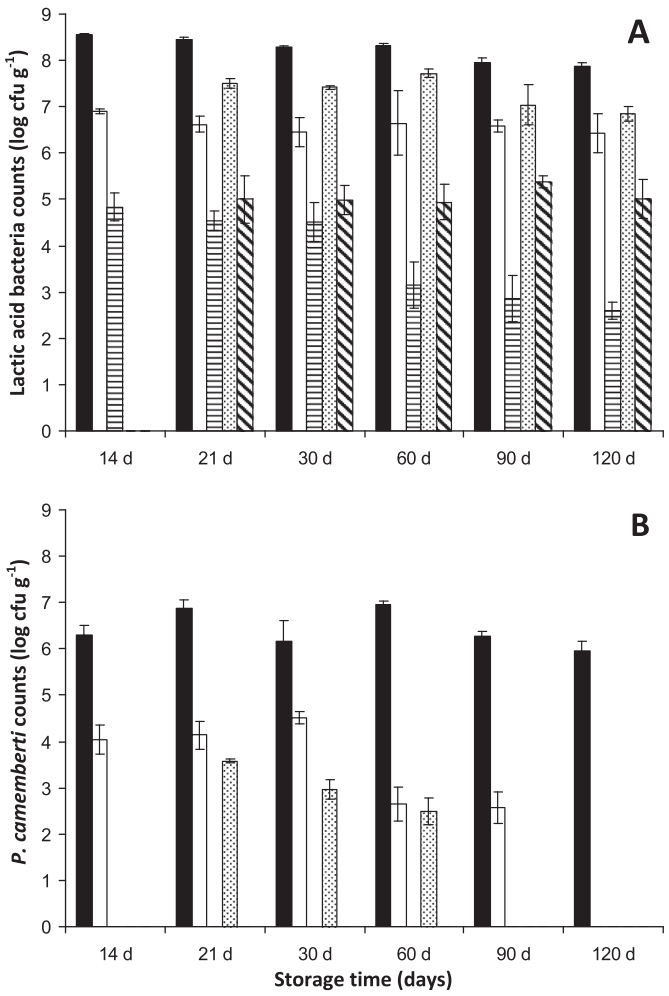


Fig. 1. Counts of the main microbial groups (A, lactic acid bacteria; B, *P. camemberti*) during ripening and refrigerated storage of Brie control cheese (■) and cheeses HP-treated at 400 MPa on day 14 (□), 600 MPa on day 14 (▤), 400 MPa on day 21 (▨) or 600 MPa on day 21 (▧). Bars indicate standard error of the means.

400 MPa than in control cheese, with a difference in DM content of 6.1% on day 28 (Saldo, McSweeney, Sendra, Kelly, & Guamis, 2002), and in ovine milk cheese treated at 500 MPa than in control cheese, with a difference in DM content of 3.3% on day 30 (Juan, Ferragut, Buffa, Guamis, & Trujillo, 2007). These authors ascribed such differences in moisture loss to changes in the structure of the *para*-caseinate network caused by HPP, which would have increased water retention in the cheese. Blue-veined cheeses are more demineralised than hard and semi-hard cheeses, which might influence their water retention ability and DM content after HPP. Thus, the DM content of blue-veined cheese treated at 600 MPa was 2.7% higher than that of control cheese on day 28 after HPP (Voigt et al., 2010), and the DM contents of HP-treated ovine milk blue-veined cheeses was similar to that of control cheese during ripening and refrigerated storage (Calzada et al., 2013b). In the case of Brie cheese, curd demineralisation may also affect water retention ability and DM content after HP treatment.

The pH value declined to 4.81 on day 1 (data not shown), and afterwards rose to 5.35 by day 14 in control cheese (Fig. 2). It was not significantly affected by HPP immediately after treatment, contrarily to the 0.6 pH units increase recorded for caprine milk cheese after treatment on day 4 at 400 MPa (Saldo et al., 2002), which was explained by the release of colloidal calcium phosphate

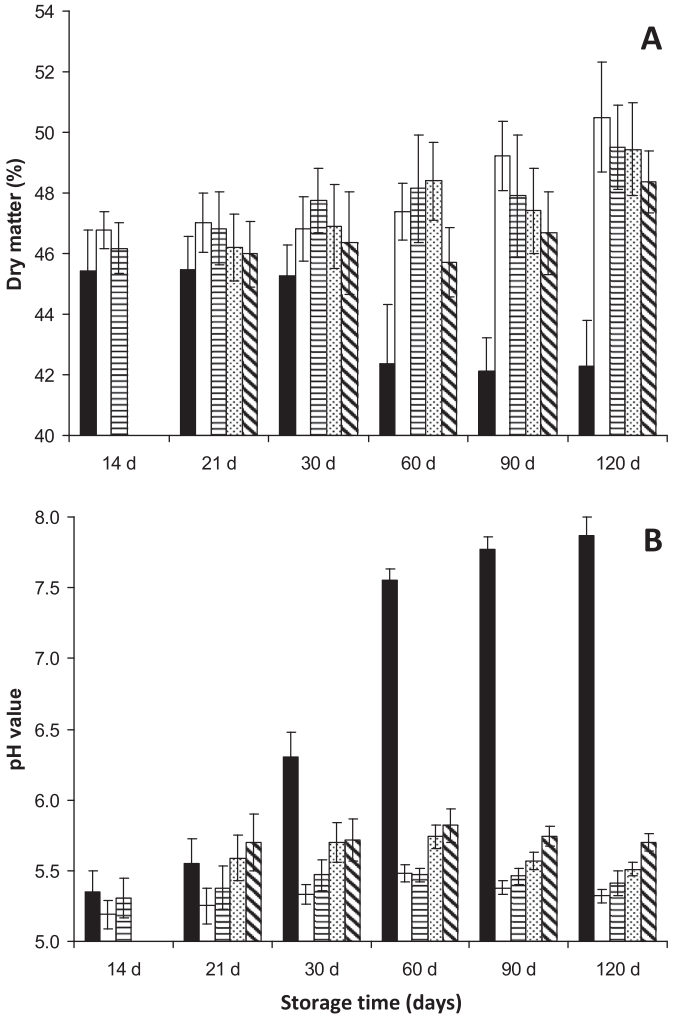


Fig. 2. Dry matter content (A) and pH value (B) during ripening and refrigerated storage of Brie control cheese (■) and cheeses HP-treated at 400 MPa on day 14 (□), 600 MPa on day 14 (▤), 400 MPa on day 21 (▨) or 600 MPa on day 21 (▩). Bars indicate standard error of the means.

into the aqueous phase. The higher demineralisation of the curd during the manufacture of Brie and other soft cheeses in comparison with hard and semi-hard varieties may limit the release of colloidal calcium phosphate, thus reducing the effect of HPP on their pH value. In control cheese, the pH rose sharply from day 21 to day 60, by 2.0 pH units, while in HPP cheeses it increased during the same period by less than 0.3 pH units. The markedly lower *P. camemberti* counts in HPP cheeses than in control cheese (Fig. 1), which may be associated with lower lactic acid consumption, seem to be responsible for the more moderate increase in their pH values. Significant ($P < 0.05$) differences in pH between HPP and control cheeses persisted until the end of the refrigerated storage. A similar phenomenon was recorded for blue-veined cheeses, although the differences between the pH values of control and HPP cheeses did not exceed 0.8 pH units at any of the sampling dates (Calzada et al., 2013b; Voigt et al., 2010).

3.3. Hydrolysis of proteins

Concentrations of α_S-, β-, κ- and *para*-κ-caseins in control Brie cheese were 170.48, 176.28, 43.94 and 32.94 mg per g of cheese DM, respectively, on day 1 (data not shown). On day 21, concentrations were 55, 60, 64 and 83% of those on day 1 (Table 1). During refrigerated storage, there was a drastic hydrolysis in α_S-, β- and κ-caseins, which resulted in concentrations on day 120 of only 1, 3 and 4% of those on day 1, while *para*-κ-casein concentration was 45%. In addition to the activity of rennet enzymes and proteinases from the lactic cultures, activity of the heat-tolerant protease milk plasmin persists in pasteurised milk cheeses (Lane & Fox, 1997). Pepsin and chymosin from rennet show acidic optimum pH values, gradually losing their activity at pH values close to and above 6.0. They would be more active during ripening of control Brie cheese, with pH values ranging from 4.81 to 5.55, than during refrigerated storage. Plasmin hydrolyses α_S- and β-caseins but not κ-casein, which is resistant to the enzyme (Eigel, 1977), and has a slightly alkaline optimum pH value (Visser, 1981). Therefore, plasmin would be more active during refrigerated storage, with pH values ranging from 6.30 to 7.87. *P. camemberti* aspartyl proteinase (Trieu-Cuot, Archieri-Haze, & Gripon, 1982a) and metalloproteinase (Trieu-Cuot, Archieri-Haze, & Gripon, 1982b) are both active on α_S- and β-caseins; values of pH close to and above 6.0 enhance the activity of the metalloproteinase and reduce the activity of the aspartyl proteinase. The considerable decline in the concentrations of α_S- and β-caseins from day 30 onwards (Table 1) as the pH rose from 5.55 on day 21 to 7.55 on day 60 (Fig. 2) can be explained by the enhancement of the activity of plasmin and the *P. camemberti* metalloproteinase at the higher pH values. The effect on κ- and *para*-κ-caseins was less marked (Table 1), since plasmin is not active on these proteins and the metalloproteinase probably has a higher affinity for α_S- and β-caseins as substrates, which might retard its action on κ- and *para*-κ-caseins. Concentrations of α_S-, β-, κ- and *para*-κ-caseins were considerably higher in control Brie cheese on day 90 than in control blue-veined cheese of the same age, in which β- and *para*-κ-caseins were 1.22 and 5.94 mg per g of cheese DM, respectively, while α_S- and κ-caseins were below the detection level (Calzada et al., 2013b).

In HPP cheeses, the concentrations of α_S-, β-, κ- and para-κ-caseins at the end of ripening, on day 21, were similar to those of control cheese (Table 1). Afterwards, a slower rate of proteolysis was generally recorded for HPP cheeses than for control cheese and, among HPP cheeses, for 600 MPa cheeses than for 400 MPa cheeses. The lower pH values of HPP cheeses were less favourable for the activity of plasmin and the *P. camemberti* metalloproteinase during refrigerated storage than the pH values of control cheese. The effect of chymosin and lactic acid bacteria proteinases would be considerably diminished in HPP cheeses in comparison with control cheese, since these enzymes are gradually inactivated at pressures above 400 MPa (Huppertz et al., 2004; Malone, Wick, Shellhammer, & Courtney, 2003). However, plasmin activity is stable at 600 MPa (Huppertz et al., 2004; Malone et al., 2003) and would remain active in HPP cheeses. The action of pepsin and *P. camemberti* proteinases on the caseins in HPP cheeses is more difficult to ascertain since there is no available information, to our knowledge, on the baroresistance of these enzymes. The proteolysis of κ- and *para*-κ-caseins in 600 MPa cheeses cannot be ascribed to chymosin and lactic acid bacteria proteinases, which are inactivated at this pressure level, or to plasmin, which is not active on these casein fractions. Therefore, the proteolysis of κ- and *para*-κ-caseins in 600 MPa cheeses must be attributed to pepsin and/or the *P. camemberti* metalloproteinase, which implies residual activity of at least one of these enzymes after HPP at 600 MPa. The hydrolysis of α_S- and β-caseins in 600 MPa cheeses can be related to the action of plasmin together with pepsin and/or the *P. camemberti* metalloproteinase. These results on proteolysis in Brie cheese were difficult to compare with those of Kolakowski et al. (1998) for Camembert cheese, due to the lower pressures (50–200 MPa) and longer treatment time (4 h) applied by these authors.

Table 1
Levels of caseins during ripening and refrigerated storage of control Brie cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.[a]

Casein	Days	Control cheese	400W2	600W2	400W3	600W3
α_S-Casein	21	92.88 ± 5.03^{a}	95.31 ± 3.68^{a}	106.76 ± 4.32^{a}	98.29 ± 4.67^{a}	99.84 ± 3.37^{a}
	30	87.22 ± 3.32^{ab}	87.04 ± 4.60^{ab}	98.46 ± 2.42^{b}	85.17 ± 2.20^{a}	84.69 ± 1.53^{a}
	60	68.30 ± 5.12^{ab}	54.49 ± 1.61^{a}	84.78 ± 7.48^{b}	79.81 ± 5.79^{b}	80.87 ± 6.80^{b}
	90	21.43 ± 4.00^{a}	21.27 ± 4.32^{a}	56.70 ± 2.81^{b}	15.86 ± 1.63^{a}	58.79 ± 4.17^{b}
	120	1.73 ± 0.27^{a}	12.09 ± 2.02^{ab}	25.40 ± 6.45^{b}	5.24 ± 0.38^{a}	17.16 ± 0.82^{ab}
β-Casein	21	105.60 ± 5.39^{a}	108.62 ± 3.18^{a}	112.05 ± 6.27^{a}	118.80 ± 3.89^{a}	113.70 ± 8.41^{a}
	30	101.64 ± 3.44^{a}	104.08 ± 4.31^{a}	108.20 ± 3.66^{a}	95.35 ± 2.85^{a}	99.71 ± 1.10^{a}
	60	62.38 ± 5.53^{a}	72.66 ± 2.82^{ab}	100.99 ± 4.36^{c}	88.32 ± 3.60^{bc}	92.05 ± 10.14^{bc}
	90	37.38 ± 6.19^{a}	41.71 ± 5.24^{a}	75.58 ± 7.25^{b}	26.04 ± 3.77^{a}	84.89 ± 2.83^{b}
	120	5.05 ± 0.78^{a}	36.42 ± 6.65^{bc}	54.51 ± 9.82^{c}	19.47 ± 0.88^{ab}	33.68 ± 2.05^{bc}
κ-Casein	21	27.89 ± 1.42^{a}	28.87 ± 3.29^{a}	31.19 ± 2.83^{a}	30.03 ± 1.51^{a}	29.96 ± 0.82^{a}
	30	28.27 ± 1.02^{a}	27.90 ± 1.31^{a}	28.35 ± 0.95^{a}	25.82 ± 0.75^{a}	25.65 ± 0.61^{a}
	60	21.39 ± 1.96^{a}	21.00 ± 1.75^{a}	26.69 ± 2.24^{a}	24.51 ± 1.53^{a}	22.60 ± 2.27^{a}
	90	18.15 ± 2.84^{ab}	14.89 ± 3.04^{ab}	19.94 ± 1.48^{b}	9.94 ± 1.61^{a}	21.03 ± 1.52^{b}
	120	1.94 ± 0.29^{a}	9.60 ± 1.58^{b}	13.29 ± 2.73^{b}	2.92 ± 0.46^{a}	3.40 ± 0.60^{a}
para-κ-Casein	21	27.26 ± 1.57^{a}	28.45 ± 2.17^{a}	30.20 ± 2.46^{a}	29.63 ± 1.35^{a}	28.95 ± 0.93^{a}
	30	28.18 ± 0.72^{ab}	29.50 ± 1.17^{b}	27.71 ± 0.94^{ab}	28.04 ± 0.69^{ab}	25.57 ± 0.42^{a}
	60	27.91 ± 0.77^{a}	24.85 ± 1.07^{a}	28.03 ± 0.78^{a}	27.06 ± 1.25^{a}	27.11 ± 2.34^{a}
	90	28.63 ± 1.44^{b}	23.48 ± 1.60^{ab}	27.54 ± 0.53^{b}	21.43 ± 4.30^{a}	25.99 ± 1.04^{ab}
	120	14.91 ± 2.21^{a}	19.58 ± 1.85^{a}	20.99 ± 3.14^{a}	14.74 ± 1.09^{a}	18.61 ± 2.25^{a}

[a] Caseins are expressed in mg per g of cheese DM and presented as mean ± SE ($n = 6$) of triplicate determinations in two cheese-making experiments.
Means on the same row followed by different superscript letters differ significantly ($P < 0.05$).

Concentrations of α-lactalbumin, β-lactoglobulin, serum albumin and γ-caseins in control Brie cheese were 3.53, 15.37, 0.22 and 13.44 mg per g of cheese DM, respectively, on day 1 (data not shown). By the end of the ripening period, α-lactalbumin had not varied in control cheese, β-lactoglobulin had declined by 32%, serum albumin was no longer detected and γ-caseins had increased by 71% in comparison with control cheese concentrations on day 1 (Table 2). During refrigerated storage, α-lactalbumin and β-lactoglobulin concentrations in control cheese markedly decreased from day 60 onwards and from day 90 onwards, respectively, which can be related to the high pH values of cheese (Fig. 2), which are favourable for proteolysis. There was a strong increase in γ-caseins from day 30 to day 60 (Table 2), which can be associated to the decline in β-casein concentration (Table 1) during the same period, favoured by the high pH values of control cheese. Afterwards, there was a considerable decrease of γ-caseins from day 90 to day 120, as they were broken down to smaller size peptides. Concentrations of α-lactalbumin, β-lactoglobulin and γ-caseins reached higher levels in control Brie cheese on day 90 than in control blue-veined cheese of the same age (Calzada et al., 2013b).

In HPP cheeses, the breakdown of α-lactalbumin and β-lactoglobulin evolved similarly to control cheese, with no significant differences during ripening and few significant differences during refrigerated storage (Table 2). The concentration of γ-caseins did not differ significantly ($P < 0.05$) between HPP and control cheeses on day 21, and showed few significant differences between cheeses from day 30 to day 90. However, from day 90 onwards, there was a less pronounced decline in the concentration of γ-caseins in HPP cheeses than in control cheese (Table 2), probably due to the lower pH values of the former cheeses, which are less favourable for the activity of plasmin and *P. camemberti* metalloproteinase.

3.4. Levels of peptides, free amino acids and biogenic amines

The formation of hydrophilic peptides was enhanced in Brie cheeses HP-treated on day 14, which showed significantly

Table 2
Levels of whey proteins and γ-caseins during ripening and refrigerated storage of control Brie cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.[a]

Protein	Days	Control cheese	400W2	600W2	400W3	600W3
α-Lactalbumin	21	4.25 ± 0.26^{a}	4.38 ± 0.32^{a}	3.78 ± 0.23^{a}	4.57 ± 0.24^{a}	4.28 ± 0.20^{a}
	30	5.12 ± 0.41^{b}	4.89 ± 0.17^{b}	3.46 ± 0.37^{a}	4.84 ± 0.33^{b}	4.13 ± 0.11^{ab}
	60	5.73 ± 0.84^{a}	5.72 ± 0.97^{a}	4.66 ± 0.92^{a}	4.89 ± 0.18^{a}	4.28 ± 0.51^{a}
	90	1.33 ± 0.27^{b}	0.66 ± 0.12^{ab}	0.52 ± 0.08^{a}	0.52 ± 0.15^{a}	1.03 ± 0.28^{ab}
	120	0.87 ± 0.15^{a}	0.69 ± 0.11^{a}	0.53 ± 0.16^{a}	0.66 ± 0.07^{a}	0.80 ± 0.24^{a}
β-Lactoglobulin	21	10.38 ± 0.70^{a}	11.67 ± 0.57^{a}	12.30 ± 0.40^{a}	11.02 ± 0.73^{a}	11.26 ± 0.54^{a}
	30	10.95 ± 0.51^{a}	14.42 ± 1.00^{b}	13.16 ± 0.44^{ab}	14.49 ± 0.40^{b}	11.83 ± 0.38^{a}
	60	11.37 ± 0.37^{a}	13.22 ± 0.78^{a}	14.03 ± 1.31^{a}	14.04 ± 0.57^{a}	12.16 ± 0.80^{a}
	90	10.25 ± 0.60^{a}	10.07 ± 0.43^{a}	13.04 ± 0.53^{a}	10.10 ± 1.18^{a}	10.77 ± 0.49^{a}
	120	3.82 ± 0.43^{a}	8.32 ± 1.62^{a}	6.80 ± 1.16^{a}	7.47 ± 0.56^{a}	5.09 ± 1.23^{a}
γ-Caseins	21	23.05 ± 1.48^{a}	24.90 ± 1.09^{a}	24.38 ± 1.32^{a}	24.58 ± 0.81^{a}	22.76 ± 0.93^{a}
	30	28.43 ± 1.31^{ab}	31.65 ± 1.19^{b}	27.02 ± 1.23^{ab}	31.89 ± 1.17^{b}	24.97 ± 0.67^{a}
	60	64.80 ± 3.31^{b}	48.74 ± 3.48^{a}	56.13 ± 4.02^{ab}	51.36 ± 2.73^{ab}	50.52 ± 3.49^{ab}
	90	63.49 ± 4.78^{a}	53.91 ± 2.41^{a}	63.54 ± 1.38^{a}	49.39 ± 4.37^{a}	60.07 ± 3.85^{a}
	120	20.91 ± 1.31^{a}	47.60 ± 2.01^{b}	44.35 ± 5.12^{b}	38.80 ± 1.46^{b}	36.31 ± 2.62^{b}

[a] Proteins are expressed in mg per g of cheese DM and presented as mean ± SE ($n = 6$) of triplicate determinations in two cheese-making experiments.
Means on the same row followed by different superscript letters differ significantly ($P < 0.05$).

Table 3
Levels of hydrophilic peptides, hydrophobic peptides and the ratio of hydrophobic peptides/hydrophilic peptides during ripening and refrigerated storage of control Brie cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.[a]

Peptides	Days	Control cheese	400W2	600W2	400W3	600W3
Hydrophilic	21	11.40 ± 0.17^{a}	18.56 ± 0.72^{b}	17.15 ± 0.68^{b}	13.31 ± 0.61^{a}	12.64 ± 0.49^{a}
	30	15.70 ± 0.24^{a}	27.78 ± 2.61^{b}	22.57 ± 0.19^{ab}	17.56 ± 0.34^{a}	17.79 ± 0.16^{a}
	60	23.99 ± 1.36^{a}	37.09 ± 1.55^{bc}	32.25 ± 0.98^{b}	44.48 ± 1.35^{d}	40.12 ± 2.68^{cd}
	90	43.32 ± 3.56^{a}	47.42 ± 0.96^{a}	48.67 ± 3.35^{a}	42.56 ± 4.14^{a}	42.46 ± 0.96^{a}
	120	47.35 ± 1.00^{b}	33.56 ± 1.73^{a}	37.22 ± 3.14^{ab}	33.94 ± 0.41^{a}	37.45 ± 3.07^{ab}
Hydrophobic	21	4.02 ± 0.19^{a}	4.99 ± 0.45^{a}	4.73 ± 0.51^{a}	3.80 ± 0.22^{a}	3.77 ± 0.30^{a}
	30	6.28 ± 0.05^{a}	6.16 ± 0.88^{a}	4.66 ± 0.10^{a}	4.57 ± 0.19^{a}	4.62 ± 0.11^{a}
	60	30.34 ± 3.16^{b}	6.04 ± 0.45^{a}	3.72 ± 0.58^{a}	5.66 ± 0.52^{a}	5.88 ± 1.17^{a}
	90	72.41 ± 3.75^{b}	7.81 ± 0.75^{a}	7.56 ± 0.94^{a}	6.14 ± 0.75^{a}	5.93 ± 0.24^{a}
	120	36.90 ± 1.13^{b}	5.81 ± 0.53^{a}	6.66 ± 1.50^{a}	5.02 ± 0.08^{a}	5.61 ± 0.20^{a}
Ratio	21	0.352 ± 0.011^{b}	0.269 ± 0.014^{a}	0.276 ± 0.019^{a}	0.287 ± 0.008^{a}	0.297 ± 0.012^{a}
	30	0.401 ± 0.009^{c}	0.221 ± 0.005^{a}	0.207 ± 0.006^{a}	0.260 ± 0.006^{b}	0.260 ± 0.008^{b}
	60	1.252 ± 0.077^{b}	0.162 ± 0.006^{a}	0.114 ± 0.015^{a}	0.126 ± 0.008^{a}	0.143 ± 0.020^{a}
	90	1.684 ± 0.052^{b}	0.166 ± 0.019^{a}	0.154 ± 0.006^{a}	0.144 ± 0.003^{a}	0.140 ± 0.004^{a}
	120	0.779 ± 0.007^{b}	0.172 ± 0.008^{a}	0.172 ± 0.021^{a}	0.148 ± 0.004^{a}	0.152 ± 0.009^{a}

[a] Peptides (determined at 280 nm) are expressed as chromatogram area units mg^{-1} of cheese DM and presented as mean ± SE ($n = 4$) of duplicate determinations in two cheese-making experiments.
Means on the same row followed by different superscript letters differ significantly ($P < 0.05$).

($P < 0.05$) higher levels than control cheese on day 21 (Table 3). HP-treatment of cheeses on day 21 had no significant effect on the level of hydrophilic peptides immediately after HPP. However, all HPP cheeses at day 60 had significantly ($P < 0.05$) higher levels of hydrophilic peptides than control cheese. From day 60 onwards, there was a build-up of hydrophilic peptides in control Brie cheese, probably enhanced by its high pH values, while these tended to decrease in HPP cheeses. A much less marked increase in hydrophilic peptides was recorded during ripening of HPP and control blue-veined cheeses (Calzada et al., 2013b). The different pH optima of the proteolytic systems of *P. camemberti* and *P. roqueforti* (Gripon et al., 1977) may partly explain the differences in the formation of hydrophilic peptides in these two cheese varieties.

The levels of hydrophobic peptides did not show significant differences between cheeses at the end of ripening, on day 21 (Table 3). During refrigerated storage, the level of hydrophobic peptides remained fairly unchanged in HPP cheeses. However, a drastic increase occurred in control cheese from day 30 onwards, up to a value of 72.41 on day 90, approximately 10-fold the level in the respective HPP cheeses. This increase can be associated to the decline in the concentration of α_S- and β-caseins (Table 1). In contrast, hydrophobic peptides hardly varied during ripening of HPP and control blue-veined cheese (Calzada et al., 2013b). As for the hydrophilic peptides, differences in the pH optima of the proteolytic systems of *P. camemberti* and *P. roqueforti* might be responsible for the patterns of hydrophobic peptides in the two cheese varieties.

The ratio of hydrophobic peptides/hydrophilic peptides was significantly ($P < 0.05$) higher in control cheese than in HPP cheeses at all sampling times (Table 3). This ratio has been related to cheese bitterness (Gómez, Garde, Gaya, Medina, & Nuñez, 1997). In control cheese, it increased dramatically from 0.352 on day 21 to 1.684 on day 90, while in HPP cheeses it declined gradually from 0.269 − 0.297 on day 21 to 0.140−0.166 on day 90. This ratio varied to a lesser extent during ripening of blue-veined cheese, from 0.31 on day 21 to 0.20 on day 90 in control cheese and from 0.29 on day 21 to 0.20−0.32 on day 90 in HPP cheeses (Calzada et al., 2013b).

Aminopeptidase activity on Leu-*p*-NA as substrate was 1.79 nmol *p*-nitroaniline min^{-1} g^{-1} on day 1 (data not shown) and did not vary immediately after HPP of Brie cheeses. Higher enzymatic activity was generally recorded for control, 400W3 and 600W3 cheeses than for 400W2 and 600W2 cheeses from day 21 to day 90 (Table 4). Aminopeptidase activity on Lys-*p*-NA as substrate was 2.74 nmol *p*-nitroaniline min^{-1} g^{-1} on day 1 (data not shown) and declined immediately after HPP in 400W2 and 600W2 cheeses, which showed generally lower activity than the rest until day 90 (Table 4). Aminopeptidase activities were not correlated with the populations of viable lactic acid bacteria or *P. camemberti* in HPP and control cheeses (Fig. 1). They did not correlate either with the declines in the counts of these microbial groups caused by HPP,

Table 4
Aminopeptidase activity on Leu-*p*-NA and Lys-*p*-NA during ripening and refrigerated storage of control Brie cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.[a]

Substrate	Days	Control cheese	400W2	600W2	400W3	600W3
Leu-*p*-NA	21	3.43 ± 0.09^{b}	2.19 ± 0.15^{a}	2.33 ± 0.26^{a}	4.29 ± 0.28^{b}	3.53 ± 0.32^{b}
	30	4.16 ± 0.31^{c}	2.28 ± 0.15^{a}	2.06 ± 0.05^{a}	3.97 ± 0.41^{bc}	3.05 ± 0.15^{ab}
	60	7.36 ± 1.06^{b}	3.56 ± 0.34^{a}	3.46 ± 0.26^{a}	5.42 ± 0.28^{ab}	3.89 ± 0.49^{a}
	90	5.05 ± 0.10^{c}	2.07 ± 0.34^{a}	1.09 ± 0.16^{a}	4.39 ± 0.08^{bc}	3.43 ± 0.31^{b}
	120	7.88 ± 1.06^{b}	1.62 ± 0.27^{a}	1.56 ± 0.34^{a}	1.50 ± 0.05^{a}	1.74 ± 0.18^{a}
Lys-*p*-NA	21	4.34 ± 0.12^{b}	2.83 ± 0.29^{a}	3.04 ± 0.30^{a}	4.26 ± 0.34^{b}	4.07 ± 0.17^{b}
	30	5.13 ± 0.80^{b}	3.17 ± 0.42^{a}	3.06 ± 0.09^{a}	4.03 ± 0.15^{ab}	4.15 ± 0.06^{ab}
	60	8.26 ± 1.33^{b}	4.08 ± 0.06^{a}	3.75 ± 0.34^{a}	6.43 ± 0.19^{ab}	4.10 ± 0.49^{a}
	90	5.71 ± 0.53^{c}	1.76 ± 0.04^{a}	1.31 ± 0.18^{a}	4.89 ± 0.32^{c}	3.21 ± 0.16^{b}
	120	7.93 ± 0.64^{b}	2.04 ± 0.51^{a}	1.81 ± 0.39^{a}	1.68 ± 0.02^{a}	1.48 ± 0.14^{a}

[a] Aminopeptidase activities are expressed in nmol *p*-nitroaniline min^{-1} g^{-1} and presented as mean ± SE ($n = 4$) of duplicate determinations in two cheese-making experiments.
Means on the same row followed by different superscript letters differ significantly ($P < 0.05$).

Table 5
Levels of free amino acids (FAAs) and overall proteolysis during ripening and refrigerated storage of control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.[a]

Variable	Days	Control cheese	400W2	600W2	400W3	600W3
FAAs	21	3.88 ± 0.15^{a}	9.47 ± 1.09^{b}	7.85 ± 0.66^{b}	7.27 ± 1.28^{b}	5.02 ± 0.19^{a}
	30	10.96 ± 1.69^{a}	27.63 ± 0.39^{c}	17.04 ± 0.35^{b}	18.32 ± 1.94^{b}	18.67 ± 0.80^{b}
	60	28.64 ± 0.90^{a}	71.44 ± 1.12^{c}	49.30 ± 0.37^{b}	74.82 ± 2.03^{c}	47.90 ± 1.92^{b}
	90	53.76 ± 1.65^{a}	89.32 ± 1.35^{c}	68.64 ± 4.87^{b}	110.66 ± 1.89^{d}	73.24 ± 2.10^{b}
	120	78.24 ± 4.78^{a}	101.84 ± 1.82^{b}	78.03 ± 1.75^{a}	123.36 ± 1.33^{c}	94.63 ± 4.79^{b}
Proteolysis	21	0.43 ± 0.01^{a}	0.77 ± 0.06^{c}	0.54 ± 0.05^{b}	0.47 ± 0.00^{ab}	0.47 ± 0.01^{a}
	30	0.55 ± 0.02^{a}	1.76 ± 0.11^{d}	1.30 ± 0.02^{c}	1.39 ± 0.10^{c}	0.96 ± 0.01^{b}
	60	1.74 ± 0.03^{a}	4.42 ± 0.09^{c}	3.27 ± 0.14^{b}	4.45 ± 0.13^{c}	3.63 ± 0.01^{b}
	90	3.46 ± 0.14^{a}	5.43 ± 0.26^{c}	4.25 ± 0.07^{ab}	6.81 ± 0.36^{d}	5.06 ± 0.06^{bc}
	120	5.73 ± 0.53^{a}	7.47 ± 0.12^{a}	5.80 ± 0.46^{a}	9.59 ± 0.50^{b}	7.22 ± 0.19^{a}

[a] Total free amino acids are expressed in mg g^{-1} cheese DM and overall proteolysis (determined by the OPA method) as the absorbance at 340 nm.
Results are presented as mean ± SE ($n = 4$) of duplicate determinations in two cheese-making experiments.
Means on the same row followed by different superscript letters differ significantly ($P < 0.05$).

which enhances the release of intracellular enzymes to the medium after the death and lysis of microbial cells. In blue-veined cheese, aminopeptidase activity was significantly higher in control cheese than in all the HPP cheeses during refrigerated storage, independently of the pressure applied or the age of cheeses at treatment (Calzada et al., 2013b).

The concentration of total FAA reached 1.94 mg g^{-1} DM in control cheese on day 1 (data not shown). From day 21 onwards, higher FAA concentrations were found in HPP cheeses than in control cheese, and in 400 MPa cheeses than in 600 MPa cheeses (Table 5). Differences in FAA between HPP cheeses and control cheese can tentatively be attributed to a higher activity of peptidolytic enzymes at the lower pH value of HPP cheeses, acting preferentially on the hydrophobic peptides. This would explain why the levels of hydrophobic peptides did not increase during the refrigerated storage of HPP cheeses, in spite of the sharp decrease in their α- and β-casein concentrations, while hydrophobic peptides rose considerably in control cheese (Table 3). The concentration of total FAA in 90-day-old control Brie cheese was close to the 61.20 mg g^{-1} DM reported for control blue-veined cheese of the same age by Calzada et al. (2013b), but total FAA concentrations of HPP Brie cheeses on day 90 were markedly higher than the level of 25.21–64.68 mg g^{-1} DM reported for HPP blue-veined cheeses of the same age.

Overall proteolysis, as determined by the OPA method, attained an absorbance value of 0.19 on day 1 (data not shown). As in the case of FAA, higher absorbance values were recorded from day 21 onwards for HPP cheeses than for control cheese, and for 400 MPa cheeses than for 600 MPa cheeses (Table 5). HPP treatment at the lower pressure level might have favoured the release of intracellular enzymes without causing their inactivation, thus enhancing secondary proteolysis reactions in 400 MPa cheeses.

Biogenic amines were not detected in any sample of control, 400W2 and 600W2 Brie cheeses. Cadaverine, found in 400W3 and 600W3 cheeses (data not shown), increased from 19 mg kg^{-1} DM on day 30 to 117 mg kg^{-1} DM on day 120 in 400W3 cheese and from 6 mg kg^{-1} DM on day 60 to 131 mg kg^{-1} DM on day 120 in 600W3 cheese. Putrescine was found only in 600W3 cheese on day 120, at 20 mg kg^{-1} DM (data not shown). The absence or low concentrations of biogenic amines in control and HPP cheeses are in agreement with the above mentioned low levels of bacterial contaminants during ripening and refrigerated storage. Cadaverine and putrescine have been reported to be the most abundant biogenic amines in Camembert and Brie cheeses (Stratton, Hutkins, & Taylor, 1991). Concentrations of 564 mg kg^{-1} cadaverine, 127 mg kg^{-1} putrescine, 185 mg kg^{-1} tyramine and 6 mg kg^{-1} histamine were found in a commercial raw milk Camembert cheese (Kubíčková & Grosch, 1998), and were partly responsible for its bitter taste.

3.5. Texture

The softening of texture recorded during ripening and refrigerated storage of control Brie cheese (Fig. 3) is characteristic of this cheese variety. Fracturability of control cheese decreased gradually from 10.18 N on day 1 (data not shown) to 0 N on day 60. Elasticity of control cheese was 0.122 N mm^{-2} on day 1 (data not shown), increased to 0.171 N mm^{-2} on day 14, and declined afterwards to 0.002 N mm^{-2} on day 60. Firmness of control cheese was 0.075 N m on day 1 (data not shown), remained at that level until day 14, and afterwards declined to 0.002 N m on day 60. Texture parameters of control cheese showed close to zero values from day 60 onwards, typical of mature to the core Brie cheese (Abraham et al., 2007; Vassal et al., 1986).

Brie cheeses HP-treated on day 14 exhibited a firmer texture than control cheese, with significantly ($P < 0.05$) higher values of texture parameters (Fig. 3). Cheeses HP-treated on day 21 showed different patterns from those of 400W2 and 600W2 cheeses, with marked declines in texture parameters immediately after HPP, generally followed by further declines until day 60, and a final recovery of texture parameters at the end of the refrigerated storage period, in particular in the 400W3 cheese, which might be associated with the increase in DM content (Fig. 2).

The concentrations of residual caseins could explain the differences in texture between control cheese and HP-treated cheeses, but they do not justify the differences between cheeses HP-treated on days 14 and 21. To ascertain the factors responsible for these differences in texture, the correlations of texture parameters with residual caseins, DM content and pH value were calculated for control and HP-treated cheeses from days 21–90. It was found that the fracturability was significantly correlated with residual β-casein level ($r = 0.447$; $P < 0.05$) and pH ($r = -0.459$; $P < 0.05$), the elasticity also with residual β-casein level ($r = 0.493$; $P < 0.05$) and pH ($r = -0.648$; $P < 0.01$), and the firmness with residual α_S-casein level ($r = 0.488$; $P < 0.05$), residual β-casein level ($r = 0.541$; $P < 0.05$), pH value ($r = -0.686$; $P < 0.001$) and DM content ($r = 0.468$; $P < 0.05$). These results emphasise the important role of the increase in pH value during ripening and storage on the softening of the texture of Brie cheese and similar varieties, as previously reported by Abraham et al. (2007) and Vassal et al. (1986) who also found significant negative correlations between firmness and pH in Camembert cheeses.

3.6. Sensory characteristics

Flavour quality scores of control and HPP Brie cheeses did not differ significantly ($P < 0.05$) during the first month (Fig. 4).

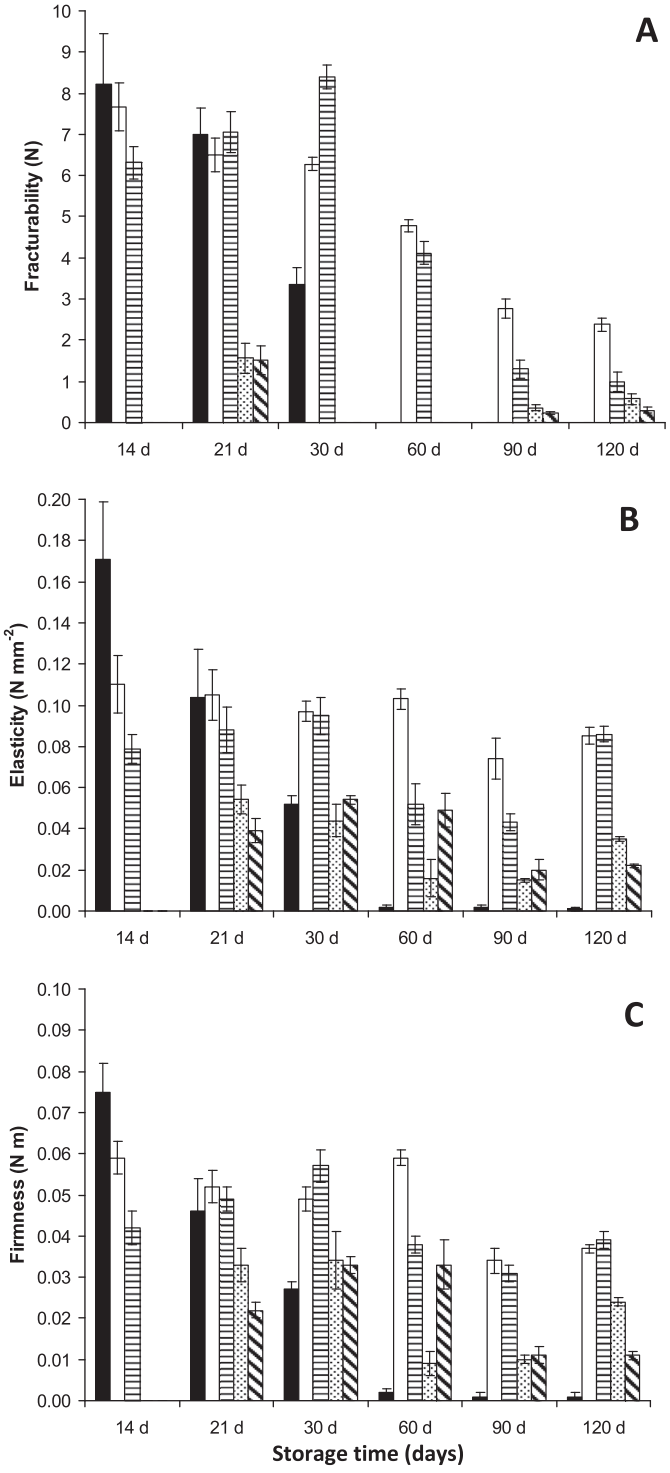


Fig. 3. Texture characteristics (A, fracturability; B, elasticity; C, firmness) during ripening and refrigerated storage of Brie control cheese (■) and cheeses HP-treated at 400 MPa on day 14 (□), 600 MPa on day 14 (▤), 400 MPa on day 21 (▦) or 600 MPa on day 21 (▨). Bars indicate standard error of the means.

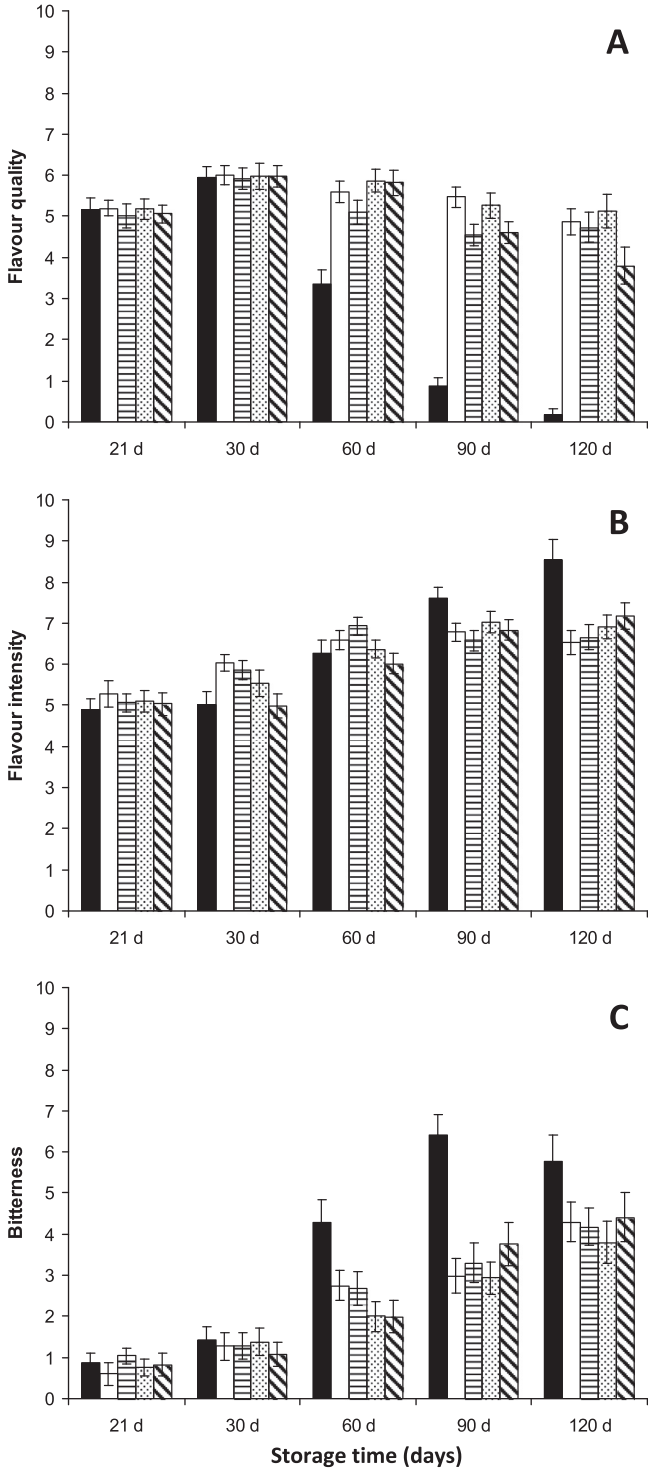


Fig. 4. Sensory scores (A, flavour quality; B, flavour intensity; C, bitterness) during ripening and refrigerated storage of Brie control cheese (■) and cheeses HP-treated at 400 MPa on day 14 (□), 600 MPa on day 14 (▤), 400 MPa on day 21 (▦) or 600 MPa on day 21 (▨). Bars indicate standard error of the means.

However, a drastic decline in flavour quality was recorded for control cheese from day 30 to day 60, while flavour quality scores of HPP cheeses did not vary during this period. The significantly ($P < 0.05$) higher flavour quality scores of HPP Brie cheeses persisted until day 120, a finding in contrast to the results obtained for HPP blue-veined cheeses by Calzada et al. (2013b), who observed acceptable flavour quality scores, above 5.5, for control and HPP blue-veined cheeses until day 360 and no significant differences in bitterness scores between cheeses. In the present work, bitterness evolved more rapidly in control cheese, in which it attained significantly ($P < 0.05$) higher scores than in all HPP cheeses from day 60 onwards (Fig. 4), which most probably impaired flavour

quality. A highly significant ($r = -0.799$; $P < 0.001$) negative correlation was found between flavour quality and bitterness, comparing control and HPP cheeses from day 21 to day 90. The level of hydrophobic peptides in those cheeses correlated significantly with bitterness scores ($r = 0.791$; $P < 0.001$) and, negatively, with flavour quality scores ($r = -0.920$; $P < 0.001$). According to Engel, Tournier, Salles, and Le Quéré (2001), bitterness of Camembert cheese is due mainly to the taste of small peptides released during cheese ripening, modulated by changes in the cheese matrix, which would increase the relative availability of bitter peptides and/or modify texture-taste interactions. Flavour quality of control cheese was most probably also impaired by compounds other than bitter peptides resulting from the secondary degradation of proteolysis and lipolysis products, with unpleasant taste and aroma notes, which conferred over-ripening flavour characteristics such as putrid and rancid to 90- and 120-day control cheeses, according to the remarks from panellists.

Flavour intensity scores showed less marked differences than quality scores between control and HP-treated cheeses (Fig. 4). Some of the HPP cheeses showed higher intensity scores than control cheese on day 30, but intensity scores of control cheese generally exceeded those of HPP cheeses on days 90 and 120. Flavour intensity of control and HP-treated cheeses from days 21 to 90 was highly correlated with levels of hydrophilic peptides ($r = 0.881$; $P < 0.001$) and total FAA ($r = 0.837$; $P < 0.001$), less with levels of hydrophobic peptides ($r = 0.498$; $P < 0.05$), and negatively correlated ($r = -0.491$; $P < 0.05$) with flavour quality. A significant correlation between flavour intensity and total FAA was also found for ovine-milk blue-veined cheeses (Calzada et al., 2013b).

At the end of Brie cheese refrigerated storage period, on day 150, extreme over-ripening of control cheese and over-ripening of all the HPP cheeses was observed. For this reason, the results corresponding to 150-day cheeses are not presented.

4. Conclusions

High-pressure-processing of Brie cheese lowered counts of *P. camemberti* by more than 2 log units at 400 MPa, and by more than 5 log units at 600 MPa. The increase in the pH value of HPP cheeses was retarded in comparison with control cheese, most probably because the reduced fungal population metabolised lactic acid at a considerably slower rate. The lower pH values of HPP cheeses together with the inactivation of proteolytic enzymes by high pressure resulted in higher concentrations of residual caseins, in particular in 600 MPa cheeses, and in markedly lower concentrations of hydrophobic peptides. Firmer texture, higher flavour quality and reduced bitterness of Brie cheese were achieved by means of HPP, a procedure which may be applied to prevent over-ripening.

Acknowledgements

Funding from project AGL2009-07801 from the Ministry of Science and Innovation (MICINN, Madrid, Spain, BES-2010-030444) is acknowledged by the authors. J. Calzada was the recipient of a MICINN fellowship. The authors are grateful to ILAS S.A. (Madrid, Spain) for providing Brie cheeses and to Hiperbaric (Burgos, Spain) for valuable help with HPP.

References

Abraham, S., Cachon, R., Colas, B., Feron, G., & De Coninck, J. (2007). Eh and pH gradients in Camembert cheese during ripening: measurements using microelectrodes and correlations with texture. *International Dairy Journal, 17*, 954–960.

Arqués, J. L., Garde, S., Gaya, P., Medina, M., & Nuñez, M. (2006). Inactivation of microbial contaminants in raw milk La Serena cheese by high-pressure treatments. *Journal of Dairy Science, 89*, 888–891.

Auberger, B., Lenoir, J., & Bergère, J. L. (1997). Partial characterization of exopeptidases produced by a strain of *Geotrichum candidum*. *Sciences des Aliments, 17*, 655–670.

Calzada, J., Del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013a). Reducing biogenic-amine-producing bacteria, decarboxylase activity, and biogenic amines in raw milk cheese by high-pressure treatments. *Applied and Environmental Microbiology, 79*, 1277–1283.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013b). Proteolysis and biogenic amine buildup in high-pressure treated ovine milk blue-veined cheese. *Journal of Dairy Science, 96*, 4816–4829.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*, 1219–1227.

Desmazeaud, M. J., Gripon, J.-C., Le Bars, D., & Bergère, J. L. (1976). Study of the role of microorganisms and enzymes during cheese ripening. III. Influence of microorganisms. *Lait, 56*, 379–396.

Eigel, W. N. (1977). Effect of bovine plasmin on α_{S1}-B and κ-A caseins. *Journal of Dairy Science, 60*, 1399–1403.

Engel, E., Tournier, C., Salles, C., & Le Quéré, J. L. (2001). Evolution of the composition of a selected bitter Camembert cheese during ripening: release and migration of taste-active compounds. *Journal of Agricultural and Food Chemistry, 49*, 2940–2947.

Garde, S., Tomillo, J., Gaya, P., Medina, M., & Nuñez, M. (2002). Proteolysis in Hispánico cheese manufactured using a mesophilic starter, a thermophilic starter, and bacteriocin-producing *Lactococcus lactis* subsp. *lactis* INIA 415 adjunct culture. *Journal of Agricultural and Food Chemistry, 50*, 3479–3485.

Gómez, M. J., Garde, S., Gaya, P., Nuñez, M., & Medina, M. (1997). Relationship between level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk. *Journal of Dairy Research, 64*, 289–297.

Gripon, J.-C., Desmazeaud, M. J., Le Bars, D., & Bergère, J. L. (1977). Role of proteolytic enzymes of *Streptococcus lactis, Penicillium roqueforti*, and *Penicillium caseicolum* during cheese ripening. *Journal of Dairy Science, 60*, 1532–1538.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71*, 496–499.

Juan, B., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. J. (2007). Effects of high pressure on proteolytic enzymes in cheese: relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science, 90*, 2113–2125.

Kolakowski, P., Reps, A., & Babuchowski, A. (1998). Characteristics of pressurized ripened cheeses. *Polish Journal of Food and Nutrition Sciences, 48*, 473–483.

Krause, I., Bockhardt, A., Neckermann, H., Henle, T., & Klostermeyer, H. (1995). Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. *Journal of Chromatography A, 715*, 67–79.

Kubíčková, J., & Grosch, W. (1997). Evaluation of potent odorants of Camembert cheese by dilution and concentration techniques. *International Dairy Journal, 7*, 65–70.

Kubíčková, J., & Grosch, W. (1998). Evaluation of flavour compounds of Camembert cheese. *International Dairy Journal, 8*, 11–16.

Lane, C. N., & Fox, P. F. (1997). Role of starter enzymes during ripening of Cheddar cheese made from pasteurised milk under controlled microbiological conditions. *International Dairy Journal, 7*, 55–63.

Lau, K. Y., Barbano, D. M., & Rasmussen, R. R. (1991). Influence of pasteurization of milk on protein breakdown in Cheddar cheese during aging. *Journal of Dairy Science, 74*, 727–740.

Leclercq-Perlat, M.-N., Buono, F., Lambert, D., Latrille, E., Spinnler, H.-E., & Corrieu, G. (2004). Controlled production of Camembert-type cheeses. Part I. Microbiological and physicochemical evolutions. *Journal of Dairy Research, 71*, 346–354.

Le Graet, Y., Lepienne, A., Brule, G., & Ducruet, P. (1983). Mineral migration in soft cheese during ripening. *Lait, 63*, 317–332.

Lessard, M. H., Bélanger, G., St-Gelais, D., & Labrie, S. (2012). The composition of Camembert cheese-ripening cultures modulates both mycelium growth and appearance. *Applied and Environmental Microbiology, 78*, 1813–1819.

Maisnier-Patin, S., Deschamps, N., Tatini, S. R., & Richard, J. (1992). Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. *Lait, 72*, 249–263.

Malone, A. S., Wick, C., Shellhammer, T. H., & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science, 86*, 1139–1146.

Messens, W., Foubert, I., Dewettinck, K., & Huyghebaert, A. (2001). Proteolysis of a high-pressure-treated mould-ripened cheese. *Milchwissenschaft, 56*, 201–204.

Nooitgedagt, A. J., & Hartog, B. J. (1988). A survey of the microbiological quality of Brie and Camembert cheese. *Netherlands Milk and Dairy Journal, 42*, 57–72.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology, 66*, 4890–4896.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L., & Beresford, T. P. (2002). Effect of high-pressure treatment on viability and autolysis of starter bacteria and proteolysis of Cheddar cheese. *International Dairy Journal, 12*, 915–922.

Picon, A., Alonso, R., Gaya, P., Fernández-García, E., Rodríguez, B., De Paz, M., et al. (2010). Microbiological, chemical, textural and sensory characteristics of Hispánico cheese manufactured using frozen ovine milk curds scalded at different temperatures. *International Dairy Journal, 20*, 344–351.

Saldo, J., McSweeney, P. L. H., Sendra, E., Kelly, A. L., & Guamis, B. (2002). Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening. *International Dairy Journal, 13*, 35–44.

Silla-Santos, M. H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology, 29*, 213–231.

Spinnler, H.-E., & Gripon, J.-C. (2004). Surface mould-ripened cheeses. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.) (3rd ed.)*Major cheese groups: Vol. 2. Cheese. Chemistry, physics and microbiology* (pp. 157–174). London, UK: Elsevier Academic Press.

Stratton, J. E., Hutkins, R. W., & Taylor, S. L. (1991). Biogenic amines in cheese and other fermented foods: a review. *Journal of Food Protection, 54*, 460–470.

Trieu-Cuot, P., Archieri-Haze, M.-J., & Gripon, J.-C. (1982a). Effect of aspartyl proteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on caseins. *Journal of Dairy Research, 49*, 487–500.

Trieu-Cuot, P., Archieri-Haze, M.-J., & Gripon, J.-C. (1982b). Comparative study of the action of metalloproteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on α_{S1}- and β-caseins. *Lait, 62*, 234–249.

Trieu-Cuot, P., & Gripon, J.-C. (1982). A study of proteolysis during Camembert cheese ripening using isoelectric focusing and two-dimensional electrophoresis. *Journal of Dairy Research, 49*, 501–510.

Vassal, L., Monnet, V., Le Bars, D., Roux, C., & Gripon, J.-C. (1986). Relation between pH, chemical composition and texture of Camembert cheese. *Lait, 66*, 341–351.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. *Netherlands Milk and Dairy Journal, 35*, 65–88.

Voigt, D. D., Chevalier, F., Qian, M. C., & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavor compounds in mature blue-veined cheese. *Innovative Food Science and Emerging Technologies, 11*, 68–77.

Wick, C., Nienaber, U., Anggraeni, O., Shellhammer, T. H., & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial Cheddar cheeses treated with high pressure. *Journal of Dairy Research, 71*, 107–115.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11*, 185–201.

Capítulo 8.

Effect of high-pressure-processing on the lipolysis and volatile compounds of Brie cheese during ripening and refrigerated storage.

Fotografía: colonias de *Penicillium camemberti* en agar CG.

International Dairy Journal 39 (2014) 232–239

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Effect of high-pressure-processing on lipolysis and volatile compounds of Brie cheese during ripening and refrigerated storage

Javier Calzada, Ana del Olmo, Antonia Picon, Manuel Nuñez*

Departamento de Tecnología de Alimentos, INIA, Carretera de La Coruña Km 7, Madrid 28040, Spain

ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form
25 June 2014
Accepted 1 July 2014
Available online 29 July 2014

ABSTRACT

Brie cheeses were high pressure (HP)-treated at 400 or 600 MPa, on days 14 or 21 after manufacture, to prevent over-ripening. HP-treatment reduced total free fatty acid content of 120-day-old cheese by up to 88.5%. On day 120, HP-treated cheeses had levels of alcohols, aldehydes, acids, esters and ethers up to 3.4, 1.9, 43.8, 18.7 and 5.6 times higher, respectively, than control cheese, while ketones and hydrocarbons levels were up to 88.6% and 48.9% lower, respectively. Levels of sulphur compounds, pyrazines and amines increased drastically in control cheese from day 60 onwards, resulting in lower odour quality scores. On day 120, HP-treated cheeses had levels of sulphur compounds, pyrazines and amines up to 96.9%, 99.3% and 99.4% lower, respectively, than control cheese. Cheese appearance was impaired by HP-treatment, resulting in lower lightness and slightly more reddish and yellowish colour. These changes might diminish consumer acceptance of HP-treated Brie cheese.

1. Introduction

Soft cheeses ripened by *Penicillium camemberti*, such as Brie and Camembert, are characterised by a thin layer of white mould colonising their rind that metabolises lactic acid with a concomitant increase in cheese pH to values close to neutrality. Extracellular enzymes produced by *P. camemberti*, namely aspartyl proteinase (Trieu-Cuot, Archieri-Haze, & Gripon, 1982a), metalloproteinase (Trieu-Cuot, Archieri-Haze, & Gripon, 1982b) and lipase (Lamberet & Lenoir, 1976), are main contributors to the biochemical changes involved in the ripening process of these cheese varieties. The activity of *P. camemberti* enzymes is generally enhanced at the high pH values of Brie and Camembert cheeses.

Products resulting from the primary hydrolysis of proteins and triglycerides are further degraded, giving rise to the formation of non-volatile flavour compounds, as well as volatile potent odorants responsible for the typical flavour and aroma notes of Brie and Camembert cheeses (Adda & Dumont, 1974; Kubíčková & Grosch, 1997, 1998). The ripening microbiota influences the formation of free fatty acids (FFAs) and volatile compounds in Camembert-type cheeses (Leclercq-Perlat, Corrieu, & Spinnler, 2007; Leclercq-Perlat, Latrille, Corrieu, & Spinnler, 2004). Soft white mould cheeses are rich in FFAs, ketones, esters and sulphur compounds (Adda & Dumont, 1974; Sablé & Cottenceau, 1999). Some of the non-volatile compounds interact with volatile compounds in Camembert cheese, influencing the release of aroma compounds and consumer perception (Pionnier, Engel, Salles, & Le Quéré, 2002). The potent enzymes of fungal origin present in soft cheeses ripened by *P. camemberti* and their high pH values make these cheeses particularly prone to the development of unbalanced flavour profiles, which lead to over-ripening in spite of adequate refrigerated storage conditions.

High pressure processing (HPP) of cheese at sufficiently high pressures, generally above 500 MPa, has been proven to eliminate pathogenic microorganisms (O'Reilly, O'Connor, Kelly, Beresford, & Murphy, 2000), inactivate enzymes (Huppertz, Fox, & Kelly, 2004) and prevent biogenic amine formation (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2013a). It may also affect other cheese characteristics such as flavour and texture (Martínez-Rodríguez et al., 2012). The effect of HPP on the proteolysis, texture and flavour of Brie cheese during ripening and refrigerated storage was reported in a previous paper (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2014b). However, to our knowledge, there is no information available about the influence of HPP on the lipolysis and the formation of volatile compounds in Brie cheese.

In the present study, the formation of FFAs and volatile compounds in the control Brie cheese and in the cheeses HP-treated at 400 or 600 MPa on days 14 and 21 after manufacture (Calzada et al., 2014b), to prevent over-ripening during refrigerated storage, was investigated. The odour and the colour of control and HP-treated Brie cheeses were also compared.

* Corresponding author. Tel.: +34 913476799.
E-mail addresses: nunez@inia.es, mng1950@gmail.com (M. Nuñez).

http://dx.doi.org/10.1016/j.idairyj.2014.07.007
0958-6946/

2. Materials and methods

2.1. Brie cheeses and chemical determinations

The manufacture of Brie cheeses studied in the present work was described previously (Calzada et al., 2014b). In each of two trials, carried out on consecutive days, approximately 120 cheeses, 2.7 kg in weight, were made from 2100 L pasteurised milk. High-pressure (HP) processing of cheeses was performed on days 14 or 21 after manufacture, at 400 or 600 MPa for 5 min. Cheeses HP-treated on day 14 were coded as 400W2 and 600W2, and those HP-treated on day 21 as 400W3 and 600W3. Control and HP-treated cheeses were ripened at 12 °C until day 21 and then held at 4 °C until day 120.

One cheese wedge per trial was used for enzymatic and chemical analyses, after discarding the rind, and another wedge per trial for odour and colour analyses. Esterase activity was determined in duplicate as described by Calzada, del Olmo, Picon, Gaya, and Nuñez (2013b). Free fatty acids were analysed in duplicate by gas-chromatography (GC) according to Fernández-García, Carbonell, Calzada, and Nuñez (2006). Volatile compounds were determined in triplicate by GC coupled to mass-spectrometry (MS), after solid-phase microextraction (SPME), as described by Calzada, del Olmo, Picon, Gaya, and Nuñez (2014a).

2.2. Odour, colour and statistical analysis

Quality (preference) and intensity of odour, defined as the olfactory sensation perceived by smelling the cheese, were evaluated by a trained 16-member panel scoring on a 0–10 point scale as described by Calzada et al. (2014a). Colour parameters of lightness (L^*), redness (a^*) and yellowness (b^*) were determined at the core and the upper rind of cheeses, on each site at four different points, using a CM-2600d spectrocolorimeter (Minolta, Osaka, Japan) with a D65 illuminator at 10° observer angle and specular component included (SCI), at room temperature, according to Picon, Alonso, van Wely, and Nuñez (2013). Statistical analysis of results, including two-way analysis of variance (ANOVA), comparison of means by Tukey's test with the significance assigned at $P < 0.05$ and principal component analysis, was carried out using SPSS Win 14.0 program (SPSS Inc., Chicago, IL, USA), as described by Calzada et al. (2013b).

3. Results and discussion

3.1. Ethanoic acid, propanoic acid and free fatty acids

The concentration of ethanoic acid, a product of microbial origin, was 1.305 mg g^{-1} cheese dry matter (DM) on day 1 (Fig. 1); it decreased in control cheese to a minimum of 0.08 mg g^{-1} on day 30, most probably because of acid consumption by moulds, and then increased gradually. In 400W2 and 400W3 cheeses, the concentration of ethanoic acid was generally higher than in control cheese until day 120, most probably because of lower acid consumption by the smaller population of moulds, whereas in 600W2 and 600W3 cheeses it remained fairly unchanged, which could be associated with slow production and slow consumption by HPP-injured microorganisms. Propanoic acid, a product of microbial origin too, was present at 0.053 mg g^{-1} cheese DM on day 1 (Fig. 1); it declined in control cheese to a minimum on day 60 and afterwards rose to 0.055 mg g^{-1} cheese DM on day 120. In HP-treated cheeses, it also declined to a minimum on day 60, but there was no further recovery probably because of the pressure-induced changes in their microbiota. Ethanoic and propanoic acids, with a typical vinegar odour, are key odorants of Cheddar, Gruyère and Emmental cheeses (Curioni & Bosset, 2002).

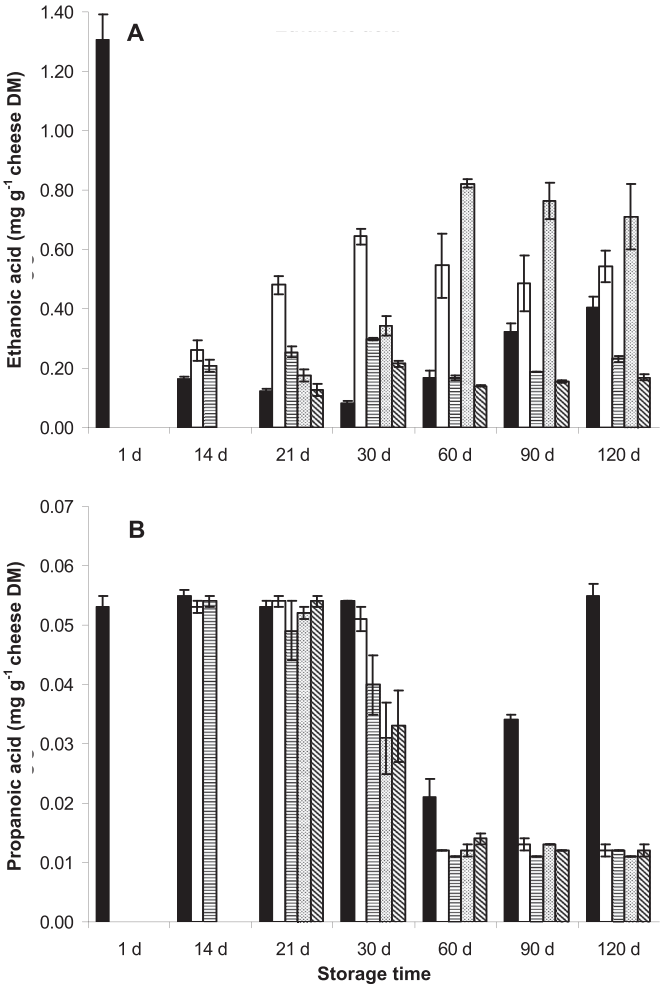


Fig. 1. Levels of (A) ethanoic and (B) propanoic acids during ripening and refrigerated storage of control Brie cheese (black) and cheeses HP-treated at 400 MPa on day 14 (white), 600 MPa on day 14 (horizontally striped), 400 MPa on day 21 (dotted) or 600 MPa on day 21 (obliquely striped). Bars indicate standard error of the means.

Esterase activity increased markedly during ripening and refrigerated storage of control cheese, from 0.83 pmol α-naphthol min^{-1} g^{-1} on day 1 to 9.01 on day 60 and 18.14 on day 120 (Supplementary Fig. S1). HPP of Brie cheese, in particular at 400 MPa, tended to increase esterase activity, probably by enhancing the release of enzymes from dead or injured cells of lactic acid bacteria and *P. camemberti*. Esterase activity in cheeses treated at 400 MPa increased from 15.91–18.37 pmol α-naphthol min^{-1} g^{-1} on day 60 to 33.25–44.07 on day 120, while in cheeses treated at 600 MPa it rose from 8.14–8.92 pmol α-naphthol min^{-1} g^{-1} on day 60 to 22.12–27.61 on day 120. Although treatment at 600 MPa would cause more intense damage to lactic acid bacteria and *P. camemberti* than that at 400 MPa (Calzada et al., 2014a), favouring the release of intracellular enzymes from injured cells, the secreted enzymes outside the microbial cells would presumably be more susceptible to inactivation by HP than those in the interior of the microbial cells.

Short-chain (SC) FFAs were at higher concentrations in 400W3 cheese than in the rest of cheeses from day 21 to day 30 (Fig. 2), which may be associated with its higher esterase activity. However, from day 60 onwards, SC-FFA concentrations were higher in control cheese than in all the HP-treated cheeses. Lactic acid bacteria esterases are responsible for the generation of SC-FFAs in many

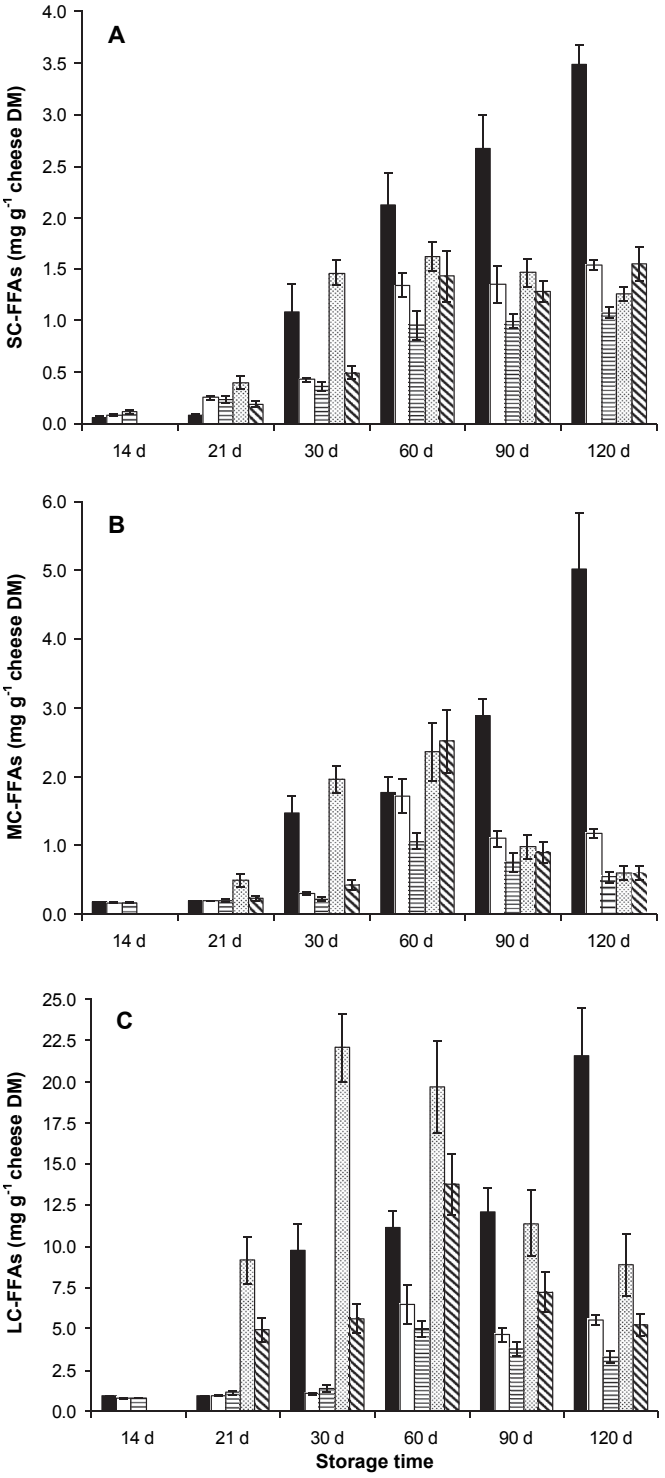


Fig. 2. Levels of (A) short-chain free fatty acids (SC-FFAs, $C_{4:0}$ to $C_{8:0}$), (B) medium-chain FFAs (MC-FFAs, $C_{10:0}$ to $C_{14:0}$) and (C) long-chain FFAs (LC-FFAs, $C_{16:0}$ to $C_{18:3}$) during ripening and refrigerated storage of control Brie cheese (black) and cheeses HP-treated at 400 MPa on day 14 (white), 600 MPa on day 14 (horizontally striped), 400 MPa on day 21 (dotted) or 600 MPa on day 21 (obliquely striped). Bars indicate standard error of the means.

cheese varieties, lipolysis in cheese being related to the autolytic release of intracellular enzymes from lactic acid bacteria (Collins, McSweeney, & Wilkinson, 2003). The higher counts of viable lactic acid bacteria in control Brie cheese than in HP-treated cheeses (Calzada et al., 2014b) would imply less injured cells and less released esterase in control cheese, which seems contradictory in terms of its higher SC-FFA concentrations. Some of the SC-FFAs, in particular branched-chain FFAs, may originate from the breakdown of free amino acids (Urbach, 1993), but there was no correlation between the concentrations of free amino acids in control and HP-treated Brie cheeses (Calzada et al., 2014b) and their SC-FFA content (Fig. 2). SC-FFAs can also derive from ketones, esters and aldehydes by oxidation (Molimard & Spinnler, 1996). However, the more plausible explanation for differences in SC-FFA contents may be the enhancement of *P. camemberti* lipase activity in control cheese. This enzyme, which is capable of hydrolysing SC-fatty acid triglycerides, has optimum activity at alkaline pH (Lamberet & Lenoir, 1976). Consequently, more pronounced lipolysis should be expected to occur during refrigerated storage in control Brie cheese, in which the pH value increased from 5.54 on day 21 to 7.55 on day 60, than in HP-treated cheeses, with pH values which only rose from 5.26–5.70 on day 21 to 5.47–5.82 on day 60 (Calzada et al., 2014b). On day 60, the concentrations of individual SC-FFAs in control Brie cheese were 1.299, 0.050, 0.103, 0.483 and 0.186 mg g^{-1} cheese DM for $C_{4:0}$, iso-$C_{4:0}$, iso-$C_{5:0}$, $C_{6:0}$ and $C_{8:0}$, respectively (data not shown), markedly higher than the levels of these FFAs in commercial Brie cheese reported by Woo, Kollodge, and Lindsay (1984); those authors did not indicate ripening time, which influences FFA concentration.

Medium-chain (MC) FFAs followed a pattern of accumulation similar to that of SC-FFAs, with higher concentrations in control cheese than in HP-treated cheeses from day 90 onwards (Fig. 2). As for SC-FFAs, a lower contribution of lactic acid bacteria esterases than of *P. camemberti* lipase to MC-FFA formation would be expected. Although control cheese had lower esterase activity than 400W2 and 400W3 cheeses from day 60 onwards, its higher pH value (Calzada et al., 2014b) most probably enhanced MC-FFA formation. Concentrations of individual MC-FFAs in 60-day-old control cheese were 0.369, 0.340, 0.014 and 1.051 mg g^{-1} cheese DM, respectively, for $C_{10:0}$, $C_{12:0}$, $C_{13:0}$ and $C_{14:0}$ (data not shown), higher than in commercial Brie cheese (Woo et al., 1984).

Treatment of Brie cheese at 400 MPa on day 21 had a pronounced favourable effect on long-chain (LC) FFA concentrations from day 21 to day 60 (Fig. 2). This can be attributed to the lipase of *P. camemberti*, which is more active than lactic acid bacteria esterases on the triglycerides containing LC-FFAs. Individual LC-FFA concentrations in 60-day-old control cheese were 2.136, 1.038, 6.942, 0.905 and 0.138 mg g^{-1} cheese DM for $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$, higher than in commercial Brie cheese (Woo et al., 1984). From day 90 onwards, LC-FFA concentrations were higher in control cheese than in HP-treated cheeses (Fig. 2), most probably because of the enhancement of the activity of *P. camemberti* lipase at high pH values. Concentrations of LC-FFAs in Camembert-type cheeses were correlated with the levels of *P. camemberti* spores (Leclercq-Perlat et al., 2007).

Previous studies on the lipolysis of HP-treated mould-ripened cheeses dealt only with blue-veined cheeses. No significant effect of HPP at 400 or 600 MPa on the lipolysis in mature blue-veined cheese was recorded after 28 days at 4 °C, probably because of the short storage period (Voigt, Chevalier, Qian, & Kelly, 2010). In contrast, total FFA concentration in 360-day-old ovine milk blue-veined cheese declined by 41% on average when treated at 600 MPa at different stages of ripening, while HPP at 400 MPa had a negligible effect on total FFAs (Calzada et al., 2013b).

FFAs are important contributors to the aroma of surface mould-ripened cheeses such as Camembert and Brie, both directly and as precursors of methyl ketones, alcohols, lactones and esters. They may represent up to 10% of total fatty acids in Camembert cheese, which contains all the even-numbered FFAs, together with iso-$C_{4:0}$

and iso-$C_{5:0}$ acids (Molimard & Spinnler, 1996). Although LC-FFAs are usually the most abundant FFAs in cheeses, they play a minor role in flavour, because of higher perception thresholds than those of SC- and MC-FFAs (Curioni & Bosset, 2002).

3.2. Volatile compounds

One hundred and sixteen compounds were detected in the volatile fraction of Brie cheese by GC–MS, after SPME extraction, including 9 aldehydes, 19 ketones, 20 alcohols, 10 acids, 11 esters, 9 sulphur compounds, 9 pyrazines, 2 amines, 8 ethers, 14 hydrocarbons, and 5 benzenic compounds. This high number of volatile compounds agrees with the results of previous works (Kubíčková & Grosch, 1997; Molimard & Spinnler, 1996). In the present study, 4-methyl-2-pentanone, 2-tridecanone, trimethylamine, 2-ethyl-3,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were found only in control cheese, while 2-propenal, 2-propen-1-ol, octanoic acid, methyl acetate and 3-methyl-1-butanol acetate were detected only in HP-treated cheeses. Terpenes, compounds commonly present in the volatile fraction of cheeses, come from the animal diet (Toso, Procida, & Stefanon, 2002) or are produced by *P. camemberti* and *Penicillium caseifulvum* strains used in cheese manufacture (Jollivet, Belin, & Vayssier, 1993; Larsen, 1998); they were not found in the present study.

The concentration of total aldehydes increased consistently during refrigerated storage in all cheeses, independently of HPP treatment (Table 1). Branched-chain aldehydes, particularly 3-methylbutanal (Supplementary Table S1), predominated in Brie cheese, in agreement with previous findings for Camembert cheese (Leclercq-Perlat et al., 2004; Sablé & Cottenceau, 1999). Ethanal, another abundant aldehyde, can be produced through lactose fermentation by lactic acid bacteria during the early stages of ripening, and is also formed by some *P. caseifulvum* strains (Larsen, 1998) but not by *P. camemberti* strains (Jollivet et al., 1993). Branched-chain aldehydes in cheese generally derive from free amino acids by transamination or by Strecker degradation (Yvon & Rijnen, 2001). Differences in the concentration of precursor amino acids or in lactic acid bacteria counts between HP-treated and control Brie cheeses (Calzada et al., 2014b) do not explain the differences in the concentration of total aldehydes. Resting cells or cellular extracts are capable of degrading amino acids to aroma compounds in cheese models (Yvon & Rijnen, 2001) and abiotic chemical reactions have been suggested as a mechanism of formation of branched-chain aldehydes in blue-veined cheese (Calzada et al., 2013b), what might be also valid for Brie cheese. Some branched-chain aldehydes have been identified as potent cheese odorants; their green, malty, sweet, floral, fruity, nutty, acrid, pungent notes contribute to cheese aroma, generally conferring more pleasant odours at lower levels (Curioni & Bosset, 2002).

The pattern of accumulation of total ketones in Brie cheese differed depending on HPP treatment (Table 1). On day 30, 3-hydroxybutanone was the main ketone in all cheeses (Supplementary Table S2). It is generated from pyruvate by lactic acid bacteria, but *P. camemberti* and *P. caseifulvum* strains do not produce it (Jollivet et al., 1993; Larsen, 1998). On day 120, 3-hydroxybutanone remained as the major ketone in HP-treated cheeses, while 2-pentanone and 2-propanone predominated in control cheese. Formation of methyl ketones by *P. camemberti* ATCC 4845 in a model system was reported by Okumura and Kinsella (1985) and confirmed afterwards for other *P. camemberti* and *P. caseifulvum* strains (Jollivet et al., 1993; Larsen, 1998). In the present study, methyl ketone formation in HP-treated cheeses was most probably hindered by their low *P. camemberti* counts (Calzada et al., 2014b). Methyl ketones contribute to cheese flavour with floral, fruity, musty, earthy, blue-cheese notes, whereas 3-hydroxybutanone has a buttery note (Molimard & Spinnler, 1996; Sablé & Cottenceau, 1999).

Total alcohol levels were significantly ($P < 0.05$) higher in HP-treated cheeses than in control cheese (Table 1). Alcohols contribute to cheese flavour directly and as substrates for ester formation. The main individual alcohols in control cheese were ethanol, 2-propanol and 2-pentanol, whereas ethanol clearly predominated in HP-treated cheeses, followed by 3-methyl-1-butanol (Supplementary Table S3). The higher ethanol levels in HP-treated cheeses cannot be associated with microbial fermentation since the increase persisted after day 30, when carbohydrates were no longer available. In this regard, reduction of ethanal as a mechanism for ethanol formation in blue-veined cheese was suggested by Calzada et al. (2013b). Some *P. caseifulvum* strains are capable of producing ethanol in carbohydrate-containing media (Larsen, 1998), but this alcohol was not detected in *P. camemberti* cultures (Jollivet et al., 1993). 2-Alkanols are derived from the reduction of the respective methyl ketones (Molimard & Spinnler, 1996). Branched-chain alcohols may originate from the respective amino acids through sequential transamination, decarboxylation and reduction (Yvon & Rijnen, 2001). Their formation by *P. camemberti* and *P. caseifulvum* strains has been reported (Jollivet et al., 1993; Larsen, 1998). A key odorant of Brie and Camembert cheeses, 1-octen-3-ol, was found at higher levels in control cheese than in HP-treated cheeses from day

Table 1
Levels of total aldehydes, total ketones, total alcohols, total acids, and total esters in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compounds[a]	Days	Control cheese	400W2	600W2	400W3	600W3
Aldehydes	30	5.75 ± 0.41^{A}	17.02 ± 1.02^{C}	11.05 ± 0.44^{B}	7.45 ± 0.45^{A}	6.69 ± 0.56^{A}
	60	8.74 ± 0.29^{A}	18.69 ± 1.29^{B}	18.85 ± 1.05^{B}	19.37 ± 1.09^{B}	30.74 ± 2.38^{C}
	120	39.13 ± 3.20^{A}	43.72 ± 2.62^{A}	35.03 ± 3.10^{A}	34.55 ± 3.00^{A}	73.72 ± 5.34^{B}
Ketones	30	724.68 ± 84.19^{B}	1458.41 ± 117.46^{C}	632.37 ± 50.90^{AB}	344.47 ± 28.78^{A}	533.13 ± 27.77^{AB}
	60	321.55 ± 22.75^{A}	249.41 ± 26.73^{A}	688.42 ± 27.92^{B}	168.36 ± 8.59^{A}	1252.16 ± 84.64^{C}
	120	2674.07 ± 130.72^{B}	304.85 ± 6.42^{A}	561.79 ± 47.94^{A}	326.77 ± 36.06^{A}	669.40 ± 24.73^{A}
Alcohols	30	181.65 ± 7.17^{A}	656.39 ± 36.98^{BC}	715.22 ± 34.56^{C}	744.83 ± 48.99^{C}	529.25 ± 43.29^{B}
	60	310.19 ± 11.39^{A}	993.30 ± 59.28^{C}	915.22 ± 32.39^{C}	1252.44 ± 29.02^{D}	483.38 ± 18.15^{B}
	120	358.39 ± 24.05^{A}	1119.69 ± 32.42^{D}	809.11 ± 28.41^{C}	1226.36 ± 36.88^{D}	552.77 ± 57.40^{B}
Acids	30	795.51 ± 51.80^{A}	1688.07 ± 82.47^{C}	1271.88 ± 79.12^{B}	956.89 ± 89.88^{A}	833.65 ± 30.99^{A}
	60	70.01 ± 6.12^{A}	2599.83 ± 253.00^{B}	1889.55 ± 27.52^{B}	1909.83 ± 74.37^{B}	1570.19 ± 93.04^{B}
	120	85.04 ± 7.65^{A}	3294.02 ± 161.69^{BC}	2773.47 ± 74.93^{B}	3722.64 ± 180.89^{C}	3206.43 ± 280.71^{BC}
Esters	30	14.37 ± 1.12^{A}	112.39 ± 16.69^{B}	273.80 ± 28.27^{C}	67.11 ± 5.08^{AB}	80.19 ± 5.27^{B}
	60	14.30 ± 0.76^{A}	165.18 ± 10.84^{C}	302.74 ± 17.28^{D}	75.78 ± 3.48^{B}	176.10 ± 8.32^{C}
	120	12.52 ± 0.69^{A}	172.66 ± 5.01^{C}	234.29 ± 16.84^{D}	128.41 ± 5.24^{B}	144.86 ± 5.56^{BC}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^3$); values are means ± SE ($n = 6$) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table 2
Levels of total sulphur compounds, total pyrazines, total amines, total ethers, total hydrocarbons, and total benzenic compounds in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compounds[a]	Days	Control cheese	400W2	600W2	400W3	600W3
Sulphur compounds	30	8.20 ± 0.36^{A}	9.12 ± 0.54^{A}	8.18 ± 0.87^{A}	8.55 ± 0.48^{A}	8.11 ± 0.44^{A}
	60	9.06 ± 0.72^{A}	7.29 ± 0.55^{A}	9.06 ± 0.94^{A}	7.84 ± 0.56^{A}	7.32 ± 0.49^{A}
	120	232.89 ± 39.33^{B}	8.78 ± 0.52^{A}	8.79 ± 0.90^{A}	8.69 ± 0.53^{A}	7.26 ± 0.48^{A}
Pyrazines	30	1.58 ± 0.18^{A}	2.49 ± 0.15^{C}	2.24 ± 0.12^{BC}	2.05 ± 0.12^{ABC}	1.75 ± 0.14^{AB}
	60	2.74 ± 0.21^{A}	3.02 ± 0.29^{A}	2.27 ± 0.14^{A}	4.89 ± 0.18^{B}	4.68 ± 0.18^{B}
	120	408.84 ± 27.32^{B}	4.98 ± 0.23^{A}	2.79 ± 0.20^{A}	5.59 ± 0.55^{A}	7.33 ± 0.97^{A}
Amines	30	1.60 ± 0.23^{B}	0.97 ± 0.05^{A}	0.71 ± 0.13^{A}	0.93 ± 0.11^{A}	0.67 ± 0.12^{A}
	60	1.18 ± 0.23^{C}	1.14 ± 0.20^{BC}	0.54 ± 0.05^{AB}	0.63 ± 0.07^{ABC}	0.52 ± 0.06^{A}
	120	13.26 ± 1.13^{B}	0.70 ± 0.06^{A}	0.14 ± 0.02^{A}	0.16 ± 0.02^{A}	0.08 ± 0.01^{A}
Ethers	30	31.65 ± 3.79^{A}	138.91 ± 12.23^{C}	132.43 ± 10.91^{C}	91.59 ± 7.14^{B}	89.55 ± 4.36^{B}
	60	24.80 ± 3.55^{A}	173.71 ± 5.48^{C}	183.28 ± 6.29^{C}	136.48 ± 8.60^{B}	172.28 ± 2.54^{C}
	120	33.57 ± 2.64^{A}	188.53 ± 7.01^{C}	176.25 ± 3.66^{BC}	161.61 ± 5.60^{B}	159.42 ± 3.63^{B}
Hydrocarbons	30	24.60 ± 1.58^{A}	24.77 ± 1.89^{A}	25.00 ± 1.40^{A}	28.20 ± 1.86^{A}	26.25 ± 1.58^{A}
	60	19.79 ± 1.69^{A}	26.23 ± 2.17^{AB}	28.79 ± 1.76^{B}	27.14 ± 1.82^{B}	24.65 ± 1.27^{AB}
	120	21.56 ± 1.19^{A}	27.58 ± 1.43^{A}	27.82 ± 1.78^{A}	26.57 ± 1.19^{A}	25.74 ± 1.89^{A}
Benzenic compounds	30	18.74 ± 2.01^{A}	16.72 ± 1.53^{A}	16.91 ± 0.98^{A}	15.84 ± 0.91^{A}	15.96 ± 1.50^{A}
	60	16.58 ± 1.45^{A}	15.98 ± 1.44^{A}	13.83 ± 2.13^{A}	17.99 ± 1.93^{A}	17.17 ± 0.87^{A}
	120	31.51 ± 2.26^{B}	16.11 ± 1.54^{A}	16.88 ± 2.56^{A}	16.67 ± 1.72^{A}	21.18 ± 0.69^{A}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^3$); values are means ± SE ($n = 6$) of triplicate determinations in two cheese-making experiments (ND, not detected). Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

30 to day 120 (data not shown). It derives from linoleic and linolenic acids and has green and mushroom-like notes and a low perception threshold (Curioni & Bosset, 2002). Phenylethanol, another key odorant of Brie and Camembert cheeses, with floral notes, generated from phenylalanine (Sablé & Cottenceau, 1999), reached higher levels in control cheese than in HP-treated cheeses on day 120 (data not shown). Nine out of 10 assayed *P. camemberti* strains produced phenylethanol, while only 3 strains formed 1-octen-3-ol (Jollivet et al., 1993).

Total acids in the volatile fraction of 30-day-old Brie cheeses were present at higher levels in 400W2 and 600W2 cheeses than in the rest (Table 1). Afterwards, they declined markedly in control cheese, because of the sharp decrease in butanoic and hexanoic acids (Supplementary Table S4), and increased gradually in HP-treated cheeses. The pattern of accumulation of volatile acids determined by SPME–GC–MS differs from that observed for SCFFAs (Fig. 2). This discrepancy might be explained by competition among volatile compounds for adsorption sites in SPME fibres with solid coatings (Rivas-Cañedo, Juez-Ojeda, Nuñez, & Fernández-García, 2012), which would result in the underestimation of butanoic and other acids in 60- and 120-day-old cheeses. Butanoic was the major acid in the volatile fraction of all Brie cheeses, followed by hexanoic and ethanoic acids (Supplementary Table S4). Some *P. camemberti* and *P. caseifulvum* strains are capable of producing ethanoic, butanoic and hexanoic acids, as well as branched-chain carboxylic acids (Jollivet et al., 1993; Larsen, 1998).

Total ester levels were significantly ($P < 0.05$) higher in all the HP-treated cheeses than in control cheese (Table 1). The major ester in HP-treated cheeses throughout refrigerated storage was ethyl ethanoate (Supplementary Table S5), which correlates well with their higher ethanol and ethanoic acid contents on day 30. Ethyl ester formation in control cheese was most probably limited by its low ethanol content. Esters may be formed by the esterification of alcohols with carboxylic acids or through alcoholysis, the reaction by which fatty acyl groups are transferred to alcohols (Liu, Holland, & Crow, 2004). Most *P. camemberti* and *P. caseifulvum* strains produce ethyl ethanoate, and some may also produce higher ethyl esters (Jollivet et al., 1993; Larsen, 1998). Esters are key cheese odorants, which contribute directly to flavour with their fruity, floral and sweet notes of low perception thresholds, and indirectly by masking unclean off-flavours which might be present (Curioni & Bosset, 2002). The only cyclic ester found in the present work, γ-butyrolactone (Supplementary Table S5), did not vary with HPP or with time of storage, and was more abundant in Brie cheese than in blue-veined cheese (Calzada et al., 2013b). Depending on their concentration in cheese, γ-butyrolactone and other lactones exhibit fruity notes at low levels, or pungent, fetid, buttery notes at higher levels (Molimard & Spinnler, 1996).

Total sulphur compounds remained fairly unchanged from day 30 to day 60, with no significant differences between control and HP-treated cheeses. From day 60 to day 120, they increased 26-fold in control cheese while their levels did not vary in HP-treated cheeses (Table 2). On days 30 and 60, dimethyldisulphide and dimethylsulphone were the principal sulphur compounds in all cheeses (Supplementary Table S6). On day 120, dimethyldisulphide predominated markedly in control cheese, followed by dimethylsulphone, methanethiol and dimethyltrisulphide, while dimethylsulphone and dimethylsulphide were the most abundant in HP-treated cheeses. Methionine γ-elimination produces methanethiol, which can be further chemically oxidised to dimethyldisulphide or dimethyltrisulphide (Yvon & Rijnen, 2001). *Brevibacterium linens* and other microorganisms, including *Lactococcus lactis*, are capable of producing methanethiol from methionine. The contribution of *P. camemberti* and *P. caseifulvum* strains to the formation of sulphur compounds seems less important (Jollivet et al., 1993; Larsen, 1998). The origin of dimethylsulphone, a compound found in milk and in some Italian cheese varieties (Mallia et al., 2005a; Toso et al., 2002), has not been elucidated, although it seems to be influenced by the diet (Urbach, 1990). Sulphur compounds are important key odorants in cheeses, because of their strong garlic, onion, cabbage-like and very ripe cheese odours, together with their low perception thresholds (Engels, Dekker, de Jong, Neeter, & Visser, 1997; Sablé & Cottenceau, 1999).

Total pyrazines followed a pattern of accumulation similar to that of sulphur compounds (Table 2). Their levels rose moderately from day 30 to day 60 in control cheese and in the HP-treated cheeses, excepting the 600W2 cheese. From day 60 to day 120, they increased 149-fold in control cheese and remained relatively constant in the HP-treated cheeses. The major pyrazine in all Brie cheeses until day 60 was 2,3-dimethylpyrazine (Supplementary Table S7). However, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine, followed by trimethylpyrazine and methylpyrazine, predominated on day 120 in control cheese, while 2,3-dimethylpyrazine and 2,6-dimethylpyrazine were the most

abundant pyrazines in HP-treated cheeses. Seven different pyrazines were detected in Parmigiano-Reggiano cheese, which contributed to its nutty, roasted aroma (Qian & Reineccius, 2002). Pyrazines, some of which exhibit earthy, musty, soil odour notes, have also been found in Camembert, Cheddar, Gruyère and Manchego cheeses (Curioni & Bosset, 2002; Fernández-García, Carbonell, & Nuñez, 2002; Mallia, Fernández-García, & Bosset, 2005b).

Total amines were present on day 30 at significantly ($P < 0.05$) lower levels in all the HP-treated cheeses than in control cheese, while on day 60 only the cheeses treated at 600 MPa had lower levels than control cheese (Table 2). From day 60 to day 120, total amines increased 11-fold in control cheese and tended to decline in HP-treated cheeses. Up to 15 amines have been reported in Camembert cheeses of different origins (Molimard & Spinnler, 1996), but only two amines were detected in the present study. They were 4-purinamine, present in all cheeses from day 30, and trimethylamine, found only in 120-day-old control cheese, in which it represented more than 90% of total amines (data not shown). Trimethylamine has a strong fishy odour (Molimard & Spinnler, 1996), but no characteristic odour notes have been reported for 4-purinamine.

Total ethers remained stable in control cheese from day 30 to day 120, while they increased in HP-treated cheeses by 33–78% (Table 2). Their relative abundances were at significantly ($P < 0.05$) higher levels in all the HP-treated cheeses than in control cheese. Eight alkyl–alkyl ethers, with 1-methoxy-2-propanol and 1-ethoxy-2-propanol being the major ones (data not shown), were detected in the present study, a higher number than the 3 ethers found in smoked fresh goat cheese (Guillén, Ibargoitia, Sopelana, Palencia, & Fresno, 2004) or in raw ewes milk cheese (Calzada et al., 2014a). Ethers are components of smoke, but their origin in non-smoked cheeses is not known. Although mild, pleasant, sweet and pungent odour notes have been ascribed to ethers, they are not considered to be key odorants of mould-ripened cheeses (Curioni & Bosset, 2002; Sablé & Cottenceau, 1999).

Total hydrocarbons hardly varied with HPP or with time of refrigerated storage (Table 2). Major hydrocarbons in control and HP-treated Brie cheeses were hexane, octane and undecane (data not shown). Some hydrocarbons are already present in milk (Toso et al., 2002) but hydrocarbons found in the volatile fraction of cheese mostly originate from lipid oxidation (Carbonell, Nuñez, & Fernández-García, 2002). They are not considered to be key odorants in cheese (Thierry, Maillard, & Le Quéré, 1999).

Total benzenic compounds did not vary from day 30 to day 60, and increased only in control cheese from day 60 to day 120 (Table 2). Main benzenic compounds were nitronaphthalene and toluene, which levels doubled in control cheese from day 30 to day 120, followed by xylene (data not shown). Some benzenic compounds are formed through the microbial catabolism of aromatic amino acids (Yvon & Rijnen, 2001). *P. caseifulvum* strains produce higher amounts of benzenic compounds than *P. camemberti* strains (Jollivet et al., 1993; Larsen, 1998), but none of the strains tested by those authors was found to produce nitronaphthalene, toluene or xylene. Naphthalene has been detected in Emmental, La Serena and Zamorano cheeses (Carbonell et al., 2002; Fernández-García, Carbonell, Gaya, & Nuñez, 2004; Thierry et al., 1999) but, to our knowledge, the presence of nitronaphthalene in cheese had not been reported. Toluene and xylene, frequently found in the volatile fraction of some cheese varieties (Fernández-García et al., 2004), are not recognised as important contributors to cheese aroma.

Leclercq-Perlat et al. (2007) selected 12 volatile compounds as markers of the degradation of pyruvate, leucine, phenylalanine, and methionine, the terminal oxidation of FFAs, and the intra-chain oxidation of unsaturated FFAs during ripening of Camembert-type cheeses. Nine of these compounds (3-methylbutyl ethanoate, 3-methylbutanal, 3-methylbutanol, dimethyldisulphide, styrene, ethyl butanoate, ethyl hexanoate, 2-pentanone and 2-octanone) were associated by the authors with *P. camemberti*, while ethyl ethanoate and butyl ethanoate were associated with *Kluyveromyces lactis* and 2-heptanone with *Geotrichum candidum*. Ten of those 12 compounds were found in the present study; exceptions were butyl ethanoate and styrene.

Principal component analysis was performed on 80 individual volatile compounds, selected on the basis of their high statistical significance in the ANOVA. Function 1 (PC1), formed by 14 ketones, 8 pyrazines, 7 alcohols, 5 sulphur compounds, propanoic acid, hexanal, trimethylamine and toluene, and with a negative coefficient by 3-methylhexane, explained 39.4% of the variance. Function 2 (PC2), formed by 8 alcohols, 6 volatile acids, 5 esters, 4 hydrocarbons, 3 aldehydes, 3-methylthio-1-propanol and 1-ethoxy-2-propanol, and with a negative coefficient by 3 ethers and naphthalene, explained 19.0% of the variance.

Fig. 3 depicts the groups obtained when control and HP-treated cheeses of different ages were plotted against functions 1 and 2. Control cheese at days 60 and 120 and 400W3 cheese at day 120 were clearly separated between them and from the rest of cheeses. The 120-day-old control cheese, located at the right side of *x*-axis, was characterised by high amounts of sulphur compounds, amines, pyrazines, ketones and some alcohols, compounds with sulphurous, boiled cabbage, garlic, pungent, fruity, fishy and ammonia unpleasant flavour notes. The 60-day-old control cheese, located at the left side of *x*-axis, was characterised by its low content of alcohols, volatile acids and esters. The 120-day-old 400W3 cheese, located at the upper side of *y* axis, was characterised by its high levels of volatile acids, alcohols, esters, aldehydes and hydrocarbons, compounds which exhibit vinegar, pungent, sour, musty, green, alcohol, sweet, solvent and ethereal flavour notes. The rest of cheeses, located near the origin, showed a balanced flavour profile with cheesy notes, and most of them were grouped with the 30-day-old control cheese.

3.3. Odour and colour

Odour quality (preference) scores did not show significant differences between HP-treated and control cheeses from day 21 to

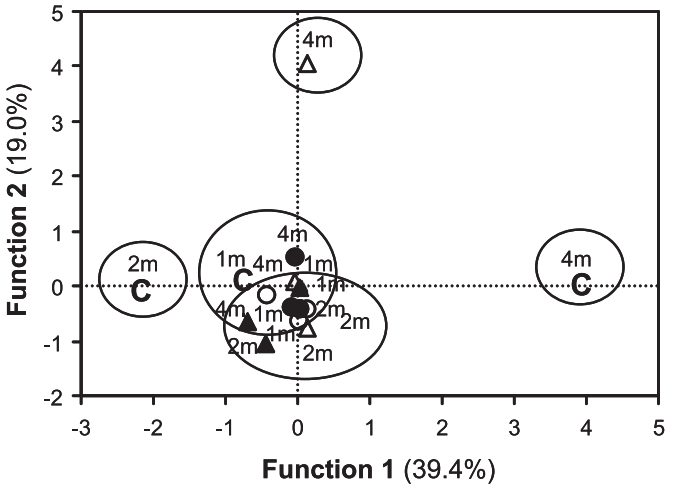


Fig. 3. Distribution of HPP and control Brie cheeses on the plane defined by functions 1 and 2 of principal component analysis. Each symbol represents the averaged value of triplicate determinations on two batches of cheese. Treatments are as follows: control (C), 400W2 (open circle), 600W2 (black circle), 400W3 (open triangle), 600W3 (black triangle). Days after manufacture are as follows: 30 days, 1 m; 60 days, 2 m; 120 days, 4 m.

day 60 (Supplementary Fig. S2). On day 90, the odour quality scores of all the HP-treated cheeses, ranging from 5.73 to 6.11, were significantly ($P < 0.05$) higher than the score 4.02 of control cheese, and by day 120 the difference had increased, with scores of 4.25–4.89 for HP-treated cheeses versus a score of 1.36 for control cheese. There were no significant differences in odour intensity scores (Supplementary Fig. S2) between HP-treated and control cheeses from day 21 to day 90. However, on day 120, control cheese showed a higher ($P < 0.05$) odour intensity score, 7.90, than all the HP-treated cheeses, with scores in the range 6.42–6.81. Differences in the odour characteristics of control and HP-treated cheeses on day 120 can be associated with the marked increase in the levels of volatile compounds with unpleasant aroma notes, in particular sulphurous compounds, pyrazines and amines, in control cheese.

At the core of Brie cheeses, colour parameters L^*, a^* and b^* values (data not shown) showed minor differences between cheeses from day 30 to day 90. On day 120, L^* value reached a significantly ($P < 0.05$) lower value, 75.92, in control cheese (less lightness) than in HP-treated cheeses, in which it ranged from 82.11 to 84.60. The a^* value of control cheese, 2.73, was significantly ($P < 0.05$) higher (more reddish) than those of HP-treated cheeses, which ranged from 0.64 to 1.65. Similarly, the b^* value of control cheese, 22.63, was significantly ($P < 0.05$) higher (more yellowish) than those of HP-treated cheeses, which ranged from 19.00 to 20.96.

Colour parameters at the rind of Brie cheese (data not shown) did not evolve as in the core; significant ($P < 0.05$) differences between the colour parameters of control and HP-treated cheeses were found in most cases. On day 30, L^*, a^* and b^* values were 91.34, 0.12 and 6.80 in control cheese, respectively, while L^* values ranged from 77.10 to 80.42, a^* values from 1.63 to 3.44, and b^* values from 18.44 to 21.26 in HP-treated cheeses. At that time, the visual appearance of the rind of HP-treated cheeses was of lower lightness, together with a slightly more reddish and yellowish colour, than that of control cheese. These changes were associated with impregnation of the mould mycelium at the rind of HP-treated cheeses by water and fat from the interior, resulting in the loss of its typical white mossy aspect, which might diminish consumer acceptance. During the refrigerated storage period, instrumental colour parameters and visual appearance varied on the surface of control cheese, most probably influenced by the growth of red-orange pigmented microorganisms. By day 120, L^* value had decreased to 77.66, a^* value had increased to 2.68 and b^* value had increased to 12.01 at the rind of control cheese. Changes in the colour parameters and visual appearance of the surface of HP-treated cheeses were less marked than in control cheese. Thus, L^* values ranged from 76.68 to 80.52 on day 120, and a^* values from 1.26 to 3.08, close to the corresponding values of HP-treated cheeses on day 30. In contrast, b^* values of HP-treated cheeses increased to levels ranging from 25.12 to 29.36 on day 120, a change which was associated with the growth of yellow-pigmented microorganisms.

4. Conclusions

High-pressure processing modified the accumulation pattern of FFAs and volatile compounds in Brie cheese during ripening and refrigerated storage. Lipolysis was retarded by HPP, although long-chain FFAs reached higher concentrations in some of the HP-treated cheeses than in control cheese until day 60. Alcohols, aldehydes, acids, esters and ethers attained higher levels in HP-treated cheeses than in control cheese. Other groups of volatile compounds, such as sulphur compounds, pyrazines and amines, increased drastically in control cheese from day 60 to day 120, which resulted in strong unpleasant odour notes. The odour quality of Brie cheese was improved by HPP, which yielded a more balanced and pleasant aroma profile and delayed over-ripening. However, the external appearance of Brie cheese became impaired by HPP, which brought about less lightness and a slightly more reddish and yellowish colour, changes that might diminish consumer acceptance.

Acknowledgements

Funding from project AGL2009-07801 from the Ministry of Science and Innovation (MICINN, Madrid, Spain) is acknowledged by the authors. J. Calzada was the recipient of MICINN fellowship BES-2010-030444. The authors are grateful to ILAS S.A. (Madrid, Spain) for providing Brie cheeses and to Hiperbaric (Burgos, Spain) for valuable help with HPP.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2014.07.007.

References

Adda, J., & Dumont, J. P. (1974). Les substances responsables de l'arôme des fromages à pâte molle. *Lait, 74*, 1–21.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013a). Reducing biogenic-amine-producing bacteria, decarboxylase activity, and biogenic amines in raw milk cheese by high-pressure treatments. *Applied and Environmental Microbiology, 79*, 1277–1283.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013b). High-pressure processing decelerates lipolysis and formation of volatile compounds in ovine milk blue-veined cheese. *Journal of Dairy Science, 96*, 7500–7510.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014a). High-pressure processing for the control of lipolysis, volatile compounds and off-odours in raw milk cheese. *Food and Bioprocess Technology, 7*, 2207–2217.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014b). Effect of high-pressure-processing on the microbiology, proteolysis, texture and flavour of Brie cheese during ripening and refrigerated storage. *International Dairy Journal, 37*, 64–73.

Carbonell, M., Nuñez, M., & Fernández-García, E. (2002). Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavor characteristics. *Lait, 82*, 683–698.

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13*, 841–866.

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography–olfactometry. *International Dairy Journal, 12*, 959–984.

Engels, W. J. M., Dekker, R., de Jong, C., Neeter, R., & Visser, S. (1997). A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. *International Dairy Journal, 7*, 255–263.

Fernández-García, E., Carbonell, M., Calzada, J., & Nuñez, M. (2006). Seasonal variation of the free fatty acids contents of Spanish ovine milk cheeses protected by a designation of origin: a comparative study. *International Dairy Journal, 16*, 252–261.

Fernández-García, E., Carbonell, M., Gaya, P., & Nuñez, M. (2004). Evolution of the volatile components of ewes raw milk Zamorano cheese. Seasonal variation. *International Dairy Journal, 14*, 701–711.

Fernández-García, E., Carbonell, M., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. *Journal of Dairy Research, 69*, 579–593.

Guillén, M. D., Ibargoitia, M. L., Sopelana, P., Palencia, G., & Fresno, M. (2004). Components detected by means of solid-phase microextraction and gas chromatography/mass spectrometry in the headspace of artisan fresh goat cheese smoked by traditional methods. *Journal of Dairy Science, 87*, 284–299.

Huppertz, Y., Fox, P. F., & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71*, 496–499.

Jollivet, N., Belin, J.-M., & Vayssier, Y. (1993). Comparison of volatile flavor compounds produced by ten strains of *Penicillium camemberti* Thom. *Journal of Dairy Science, 76*, 1837–1844.

Kubíčková, J., & Grosch, W. (1997). Evaluation of potent odorants of Camembert cheese by dilution and concentration techniques. *International Dairy Journal, 7*, 65–70.

Kubíčková, J., & Grosch, W. (1998). Evaluation of flavor compounds of Camembert cheese. *International Dairy Journal, 8*, 11–16.

Lamberet, G., & Lenoir, J. (1976). Les caractères du système lipolytique de l'espèce *Penicillium caseicolum*. Purification et propriétés de la lipase majeure. *Lait, 56*, 622–644.

Larsen, T. O. (1998). Volatile flavour production by *Penicillium caseifulvum*. *International Dairy Journal, 8*, 883–887.

Leclercq-Perlat, M.-N., Corrieu, G., & Spinnler, H.-E. (2007). Controlled production of Camembert-type cheeses: part III role of the ripening microflora on free fatty acid concentrations. *Journal of Dairy Research, 74*, 218–225.

Leclercq-Perlat, M.-N., Latrille, E., Corrieu, G., & Spinnler, H.-E. (2004). Controlled production of Camembert-type cheese. Part II. Changes in the concentration of the more volatile compounds. *Journal of Dairy Research, 71*, 355–366.

Liu, S.-Q., Holland, R., & Crow, V. L. (2004). Esters and their biosynthesis in fermented dairy products: a review. *International Dairy Journal, 14*, 923–945.

Mallia, S., Carpino, S., Corallo, L., Tuminello, L., Gelsomino, R., & Licitra, G. (2005a). Effects on aroma profile of Piacentinu and Ricotta cheese using different tool materials during cheesemaking. *Royal Society of Chemistry Special Publications, 300*, 23–34.

Mallia, S., Fernández-García, E., & Bosset, J. O. (2005b). Comparison of purge and trap and solid phase microextraction techniques for studying the volatile aroma compounds of three European PDO hard cheeses. *International Dairy Journal, 15*, 741–758.

Martínez-Rodríguez, Y., Acosta-Muñiz, C., Olivas, G. I., Guerrero-Beltrán, J., Rodrigo-Aliaga, D., & Sepúlveda, D. R. (2012). High hydrostatic pressure processing of cheese. *Comprehensive Reviews in Food Science and Food Safety, 11*, 399–416.

Molimard, P., & Spinnler, H. E. (1996). Review: compounds involved in the flavor of surface mold-ripened cheeses: origins and properties. *Journal of Dairy Science, 79*, 169–184.

Okumura, J., & Kinsella, J. E. (1985). Methyl ketone formation by *Penicillium camemberti* in model systems. *Journal of Dairy Science, 68*, 11–15.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology, 66*, 4890–4896.

Picon, A., Alonso, R., van Wely, K. H. M., & Nuñez, M. (2013). Microstructural, textural and colour characteristics during ripening of Hispánico cheese made using high-pressure-treated ovine milk curd. *Food and Bioprocess Technology, 6*, 3056–3067.

Pionnier, E., Engel, E., Salles, C., & Le Quéré, J. L. (2002). Interactions between non-volatile water-soluble molecules and aroma compounds in Camembert cheese. *Food Chemistry, 76*, 13–20.

Qian, M., & Reineccius, G. (2002). Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. *Journal of Dairy Science, 85*, 1362–1369.

Rivas-Cañedo, A., Juez-Ojeda, C., Nuñez, M., & Fernández-García, E. (2012). Volatile compounds in low-acid fermented sausage "espetec" and sliced cooked pork shoulder subjected to high pressure processing. A comparison of dynamic headspace and solid-phase microextraction. *Food Chemistry, 132*, 18–26.

Sablé, S., & Cottenceau, G. (1999). Current knowledge of soft cheeses flavor and related compounds. *Journal of Agricultural and Food Chemistry, 47*, 4825–4836.

Thierry, A., Maillard, M.-B., & Le Quéré, J.-L. (1999). Dynamic headspace analysis of Emmental aqueous phase as a method to quantify changes in volatile flavour compounds during ripening. *International Dairy Journal, 9*, 453–463.

Toso, B., Procida, G., & Stefanon, B. (2002). Determination of volatile compounds in cows' milk using headspace GC–MS. *Journal of Dairy Research, 69*, 569–577.

Trieu-Cuot, P., Archieri-Haze, M.-J., & Gripon, J.-C. (1982a). Effect of aspartyl proteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on caseins. *Journal of Dairy Research, 49*, 487–500.

Trieu-Cuot, P., Archieri-Haze, M.-J., & Gripon, J.-C. (1982b). Comparative study of the action of metalloproteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on α_{s1}- and β-caseins. *Lait, 62*, 234–249.

Urbach, G. (1990). Effect of feed on flavor in dairy foods. *Journal of Dairy Science, 73*, 3639–3650.

Urbach, G. (1993). Relations between cheese flavour and chemical composition. *International Dairy Journal, 3*, 389–422.

Voigt, D. D., Chevalier, F., Qian, M. C., & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavour compounds in mature blue-veined cheese. *Innovative Food Science and Emerging Technologies, 11*, 68–77.

Woo, A. H., Kollodge, S., & Lindsay, R. C. (1984). Quantification of major free fatty acids in several cheese varieties. *Journal of Dairy Science, 67*, 874–878.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11*, 185–201.

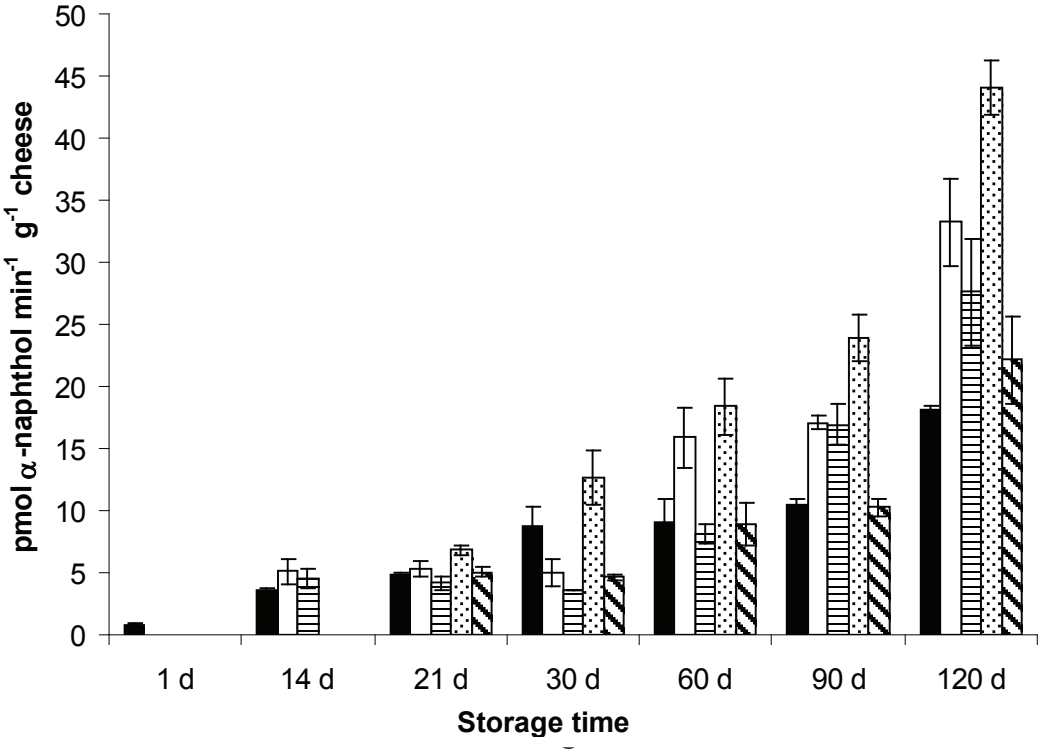


Fig. S1. Esterase activity during ripening and refrigerated storage of control Brie cheese (black) and cheeses HP-treated at 400 MPa on day 14 (white), 600 MPa on day 14 (horizontally striped), 400 MPa on day 21 (dotted) or 600 MPa on day 21 (obliquely striped). Bars indicate standard error of the means.

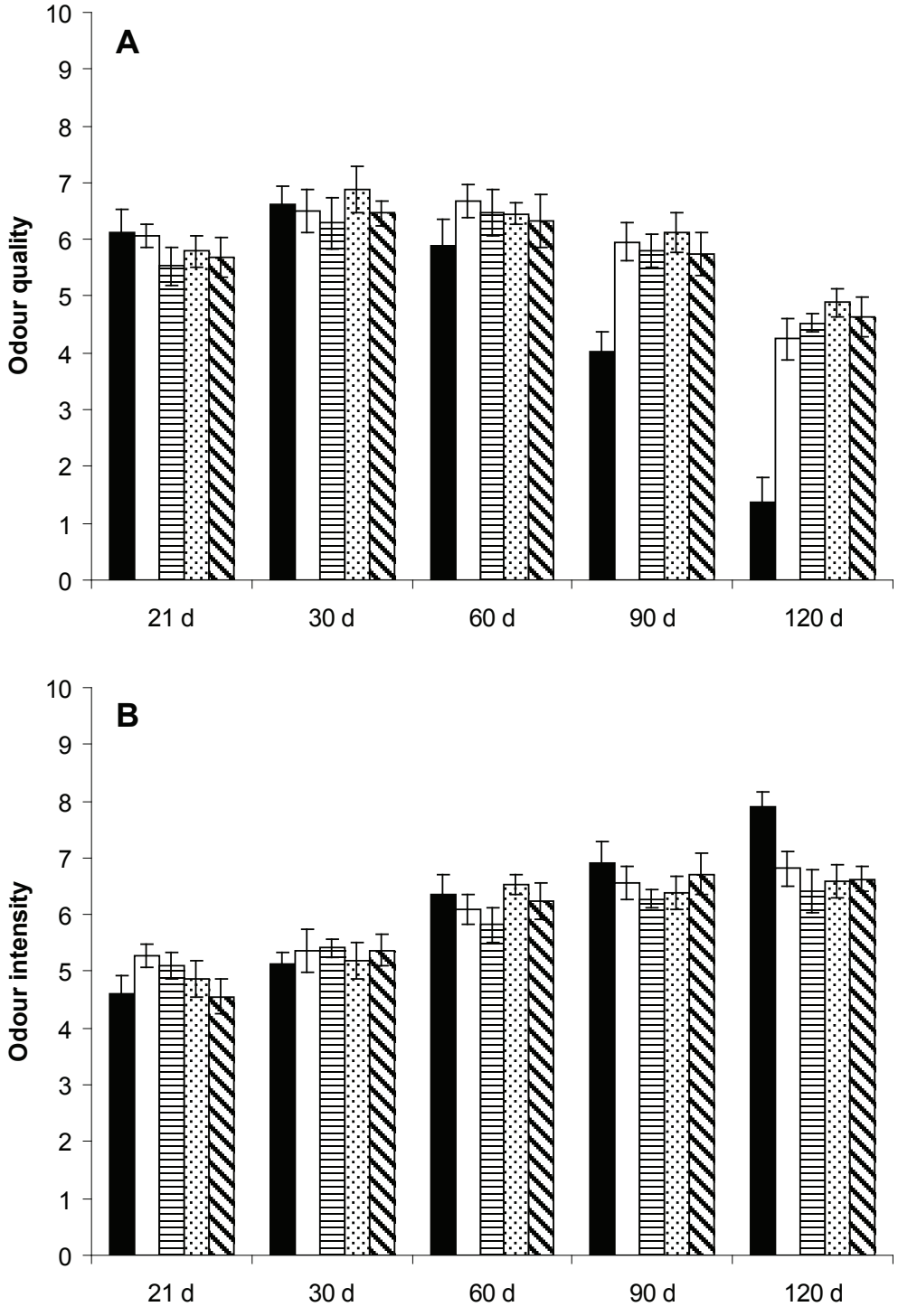


Fig. S2. Odour characteristics (A, quality; B, intensity) (scores from 16 panellists on a 0-10 point scale) during ripening and refrigerated storage of control Brie cheese (black) and cheeses HP-treated at 400 MPa on day 14 (white), 600 MPa on day 14 (horizontally striped), 400 MPa on day 21 (dotted) or 600 MPa on day 21 (obliquely striped). Bars indicate standard error of the means.

Table S1

Principal aldehydes in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compound [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Ethanal	30	1.13±0.17^{a}	1.59±0.14^{ab}	2.15±0.14^{b}	1.61±0.13^{ab}	1.34±0.11^{a}
	60	2.28±0.13^{bc}	1.32±0.10^{a}	1.70±0.15^{ab}	1.65±0.07^{a}	2.31±0.23^{c}
	120	2.16±0.19^{a}	1.43±0.10^{a}	1.72±0.06^{a}	1.85±0.18^{a}	3.41±0.43^{b}
2-Methylpropanal	30	0.44±0.04 ^{a}	1.29±0.17^{c}	1.06±0.16^{bc}	0.87±0.10^{abc}	0.59±0.12^{ab}
	60	0.60±0.04^{a}	1.83±0.13^{b}	1.87±0.13^{b}	1.92±0.08^{b}	3.27±0.34^{c}
	120	4.17±0.55^{a}	3.21±0.28^{a}	2.90±0.22^{a}	3.10±0.30^{a}	5.94±0.45^{b}
2-Methylbutanal	30	0.28±0.06^{a}	1.89±0.20^{c}	0.97±0.21^{b}	0.49±0.05^{ab}	0.50±0.06^{ab}
	60	1.45±0.10^{a}	3.01±0.42^{b}	1.81±0.11^{ab}	2.99±0.24^{b}	4.57±0.42^{c}
	120	5.33±0.32^{a}	4.24±0.44^{a}	5.05±0.62^{a}	4.78±0.48^{a}	11.99±1.83^{b}
3-Methylbutanal	30	2.77±0.24^{a}	11.37±0.99^{c}	6.15±0.41^{b}	3.90±0.41^{ab}	3.61±0.46^{a}
	60	2.62±0.17^{a}	11.80±0.85^{b}	12.67±0.94^{b}	11.98±0.80^{b}	19.82±1.51^{c}
	120	25.16±3.26^{a}	31.96±2.32^{a}	24.32±2.28^{a}	23.75±2.90^{a}	51.23±3.29^{b}
Heptanal	30	0.54±0.04^{b}	0.39±0.03^{a}	0.39±0.03^{a}	0.28±0.02^{a}	0.30±0.04^{a}
	60	1.04±0.19^{b}	0.36±0.03^{a}	0.40±0.03^{a}	0.38±0.03^{a}	0.37±0.04^{a}
	120	1.22±0.10^{b}	0.50±0.03^{a}	0.50±0.06^{a}	0.49±0.03^{a}	0.58±0.05^{a}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table S2

Principal ketones in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compound [a]	Days	Control cheese	400W2	600W2	400W3	600W3
2-Propanone	30	61.25±9.26^{b}	73.75±7.79^{b}	66.63±9.88^{b}	28.54±4.40^{a}	70.01±6.51^{b}
	60	192.54±21.33^{c}	27.58±4.51^{ab}	78.64±7.61^{b}	13.68±1.52^{a}	136.69±20.84^{c}
	120	780.47±82.88^{b}	28.43±1.11^{a}	62.59±5.80^{a}	14.74±2.94^{a}	82.24±6.20^{a}
3-Hydroxybutanone	30	305.36±50.96^{ab}	1123.51±87.20^{c}	424.48±37.54^{b}	158.37±16.70^{a}	296.70±11.07^{ab}
	60	7.26±0.37^{a}	110.02±19.49^{b}	442.42±24.43^{c}	93.07±4.90^{b}	710.37±27.84^{d}
	120	31.38±3.66^{a}	158.32±5.72^{b}	322.49±33.57^{c}	241.85±26.93^{bc}	304.32±27.91^{c}
2-Pentanone	30	218.45±33.86^{b}	185.30±22.10^{b}	71.43±4.34^{a}	96.72±6.91^{a}	87.00±5.01^{a}
	60	60.98±5.53^{ab}	61.98±2.76^{ab}	84.08±4.74^{b}	32.51±1.54^{a}	248.29±26.26^{c}
	120	1311.73±107.50^{b}	49.87±3.11^{a}	77.65±6.82^{a}	28.97±3.04^{a}	140.28±9.62^{a}
2-Heptanone	30	104.14±10.46^{b}	31.46±4.19^{a}	24.29±3.24^{a}	38.15±3.66^{a}	47.26±3.77^{a}
	60	35.80±4.44^{ab}	31.34±2.32^{ab}	39.26±5.92^{b}	15.81±1.63^{a}	102.59±9.79^{c}
	120	330.22±50.80^{b}	39.51±4.05^{a}	58.43±4.80^{a}	25.75±4.36^{a}	100.83±9.62^{a}
2-Nonanone	30	18.08±2.32^{b}	2.46±0.30^{a}	1.01±0.12^{a}	4.79±0.39^{a}	2.70±0.31^{a}
	60	9.19±0.35^{c}	2.27±0.36^{a}	2.41±0.30^{a}	1.16±0.22^{a}	6.73±0.82^{b}
	120	104.44±13.36^{b}	4.47±0.42^{a}	4.23±0.39^{a}	3.18±0.49^{a}	6.44±0.52^{a}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table S3

Principal alcohols in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compound [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Ethanol	30	73.09±5.48^{a}	433.03±25.74^{b}	508.48±27.42^{b}	507.99±48.02^{b}	388.45±36.24^{b}
	60	112.89±7.01^{a}	574.18±55.09^{c}	687.52±21.17^{cd}	734.91±43.66^{d}	266.33±24.84^{b}
	120	57.65±7.25^{a}	672.25±24.38^{c}	623.03±26.95^{c}	759.53±49.59^{c}	404.99±56.86^{b}
2-Propanol	30	23.22±2.52^{a}	21.72±2.25^{a}	30.32±2.69^{a}	48.98±5.37^{b}	23.17±2.14^{a}
	60	140.35±6.24^{c}	69.25±5.50^{b}	54.26±3.30^{b}	71.42±10.7^{b}	18.97±2.60^{a}
	120	116.15±14.60^{c}	60.52±4.58^{b}	23.19±3.61^{a}	69.28±3.02^{b}	12.32±1.17^{a}
2-Butanol	30	5.54±0.91^{a}	18.66±1.12^{bc}	27.49±3.84^{c}	42.61±3.46^{d}	16.59±0.83^{b}
	60	9.86±0.31^{a}	99.42±6.67^{c}	38.92±4.41^{b}	49.20±2.26^{b}	9.85±0.49^{a}
	120	20.77±2.79^{ab}	162.11±16.38^{c}	23.66±2.07^{ab}	48.00±4.73^{b}	9.07±1.13^{a}
3-Methyl-1-butanol	30	28.11±3.66^{a}	112.67±10.54^{d}	88.73±4.24^{cd}	66.71±6.67^{bc}	51.79±4.81^{ab}
	60	1.45±0.12^{a}	134.72±8.88^{c}	73.68±4.27^{b}	233.72±21.71^{d}	111.88±17.20^{bc}
	120	16.48±1.44^{a}	96.93±6.38^{b}	77.12±7.76^{b}	181.61±21.91^{c}	69.87±3.50^{b}
2-Pentanol	30	22.55±1.43^{b}	3.27±0.46^{a}	3.96±0.31^{a}	24.32±3.63^{b}	9.21±0.83^{a}
	60	20.68±1.94^{b}	21.71±2.20^{b}	6.12±0.51^{a}	43.39±2.44^{c}	5.40±0.46^{a}
	120	77.03±5.11^{d}	29.09±2.15^{b}	5.93±0.65^{a}	56.61±3.81^{c}	6.82±0.31^{a}
2-Heptanol	30	6.43±0.81^{a}	4.96±0.41^{a}	6.67±0.49^{a}	17.53±1.95^{b}	6.03±0.66^{a}
	60	7.73±1.52^{a}	15.78±2.15^{b}	7.16±0.66^{a}	19.74±1.12^{b}	2.45±0.19^{a}
	120	15.92±1.35^{b}	15.39±0.67^{b}	4.97±0.67^{a}	30.51±4.47^{c}	2.78±0.43^{a}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table S4

Principal acids in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compound [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Ethanoic	30	29.81±3.42^{a}	378.10±25.82^{d}	163.46±14.63^{c}	108.53±10.98^{bc}	74.60±10.07^{ab}
	60	15.36±1.19^{a}	462.90±38.25^{b}	215.91±15.69^{b}	367.32±21.72^{b}	76.42±4.11^{a}
	120	24.86±2.98^{a}	466.96±50.90^{c}	197.46±15.24^{b}	576.72±28.39^{c}	93.08±7.31^{ab}
Butanoic	30	621.38±46.96^{ab}	982.32±35.37^{c}	791.07±44.08^{bc}	641.40±71.22^{ab}	545.21±24.43^{a}
	60	31.55±3.94^{a}	1458.99±95.26^{d}	1280.18±24.67^{cd}	1099.37±24.48^{bc}	1057.54±15.97^{b}
	120	23.47±2.88^{a}	1945.51±57.46^{b}	1838.89±51.45^{b}	1969.89±79.99^{b}	2062.24±217.36^{b}
3-Methyl butanoic	30	14.88±2.71^{a}	53.14±2.41^{c}	38.64±3.65^{bc}	43.91±5.91^{bc}	31.90±5.24^{ab}
	60	0.73±0.08^{a}	155.01±23.24^{ab}	43.72±3.61^{ab}	104.90±13.62^{ab}	168.55±25.41^{b}
	120	15.92±2.99^{a}	78.14±10.67^{a}	82.43±8.17^{a}	273.24±52.17^{b}	103.31±29.79^{a}
Pentanoic	30	8.16±0.60^{ab}	14.55±0.84^{c}	10.91±1.16^{b}	8.78±0.99^{ab}	7.16±0.67^{a}
	60	1.12±0.19^{a}	22.41±2.01^{c}	18.70±0.26^{ab}	15.89±0.59^{b}	16.26±0.47^{b}
	120	0.74±0.08^{a}	30.12±1.02^{b}	31.48±2.68^{b}	35.25±2.02^{b}	45.99±3.26^{c}
Hexanoic	30	110.60±9.64^{a}	236.65±52.84^{a}	249.84±63.42^{a}	135.08±15.84^{a}	158.99±13.37^{a}
	60	15.99±2.20^{a}	477.82±75.15^{c}	308.82±31.61^{bc}	290.46±42.54^{b}	218.84±32.24^{b}
	120	10.23±1.68^{a}	743.38±67.81^{bc}	597.77±40.06^{b}	815.14±73.61^{bc}	880.67±49.45^{c}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table S5

Principal esters in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compound [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Ethyl ethanoate	30	3.63±0.41^{a}	91.80±16.11^{b}	248.33±27.08^{c}	53.14±5.26^{ab}	66.74±5.84^{b}
	60	4.38±0.47^{a}	125.88±8.91^{c}	262.12±15.51^{d}	53.32±2.93^{b}	145.94±8.44^{c}
	120	4.09±0.47^{a}	109.19±6.17^{c}	180.40±14.17^{d}	61.16±5.19^{b}	105.53±5.47^{c}
Ethyl butanoate	30	1.32±0.11^{a}	3.49±0.27^{c}	3.05±0.38^{c}	2.49±0.38^{bc}	1.37±0.06^{ab}
	60	1.59±0.20^{a}	8.58±0.97^{c}	6.97±0.21^{c}	4.58±0.40^{b}	2.18±0.08^{a}
	120	1.28±0.14^{a}	15.66±0.92^{cd}	14.18±0.85^{c}	18.12±1.45^{d}	6.49±0.76^{b}
Ethyl hexanoate	30	0.52±0.05^{a}	1.89±0.20^{c}	1.49±0.12^{bc}	1.26±0.16^{b}	1.27±0.11^{b}
	60	0.45±0.06^{a}	4.32±0.49^{d}	3.94±0.64^{cd}	2.75±0.21^{bc}	2.13±0.02^{b}
	120	0.47±0.03^{a}	13.47±1.44^{c}	6.41±0.54^{b}	18.24±1.20^{d}	5.68±0.54^{b}
Methylpropyl propanoate	30	4.26±0.34^{a}	4.37±0.31^{a}	4.32±0.25^{a}	3.74±0.34^{a}	3.37±0.43^{a}
	60	4.32±0.34^{b}	3.21±0.22^{ab}	2.52±0.18^{a}	3.52±0.38^{ab}	3.25±0.27^{ab}
	120	3.12±0.35^{a}	2.58±0.26^{a}	2.46±0.42^{a}	2.99±0.40^{a}	3.17±0.17^{a}
γ-Butyrolactone	30	4.45±0.63^{a}	4.27±0.54^{a}	3.63±0.52^{a}	3.52±0.48^{a}	3.26±0.27^{a}
	60	3.53±0.34^{a}	3.24±0.27^{a}	3.55±0.32^{a}	3.43±0.10^{a}	2.96±0.27^{a}
	120	3.17±0.07^{a}	2.77±0.19^{a}	3.02±0.26^{a}	2.89±0.25^{a}	2.78±0.23^{a}

[a] Volatile compounds are expressed as relative abundances on the internal standard (× 10^{3}); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments. Means on the same row followed by different superscripts differ significantly (P <0.05).

Table S6

Principal sulphur compounds in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compounds [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Dimethylsulphide	30	0.40±0.08^{a}	0.70±0.04^{a}	0.68±0.08^{a}	0.55±0.05^{a}	0.64±0.10^{a}
	60	1.75±0.26^{b}	0.92±0.12^{a}	0.87±0.12^{a}	0.63±0.07^{a}	0.61±0.07^{a}
	120	2.69±0.42^{b}	0.93±0.13^{a}	0.82±0.11^{a}	0.53±0.07^{a}	0.66±0.10^{a}
Dimethyldisulphide	30	2.57±0.46^{a}	2.23±0.26^{a}	2.04±0.27^{a}	2.31±0.13^{a}	2.07±0.17^{a}
	60	2.07±0.32^{a}	1.89±0.22^{a}	2.29±0.34^{a}	1.81±0.28^{a}	1.78±0.19^{a}
	120	212.99±37.94^{b}	1.87±0.25^{a}	3.15±0.55^{a}	2.26±0.33^{a}	1.63±0.29^{a}
Dimethyltrisulphide	30	0.12±0.01	ND	ND	ND	ND
	60	0.22±0.03^{a}	0.16±0.03^{a}	ND	ND	ND
	120	4.00±0.28^{b}	0.23±0.03^{a}	0.05±0.02^{a}	0.06±0.03^{a}	0.07±0.03^{a}
3-Penthanethiol	30	1.01±0.06^{a}	1.35±0.12^{ab}	1.13±0.06^{a}	1.76±0.15^{b}	1.24±0.17^{a}
	60	1.12±0.15^{a}	1.16±0.15^{a}	1.12±0.08^{a}	1.55±0.19^{a}	1.18±0.13^{a}
	120	1.23±0.16^{a}	1.34±0.08^{a}	1.04±0.13^{a}	1.21±0.07^{a}	1.28±0.18^{a}
Dimethylsulphone	30	4.09±0.27^{a}	4.83±0.50^{a}	4.32±0.62^{a}	3.92±0.37^{a}	4.17±0.48^{a}
	60	3.89±0.33^{a}	2.77±0.38^{a}	4.25±0.47^{a}	3.09±0.24^{a}	3.34±0.37^{a}
	120	5.91±0.78^{b}	2.59±0.35^{a}	2.10±0.38^{a}	2.53±0.34^{a}	2.12±0.35^{a}

[*] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments (ND, not detected). Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Table S7

Principal pyrazines in the volatile fraction during refrigerated storage of Brie control cheese and cheeses HP-treated at 400 or 600 MPa after 14 days (W2) or 21 days (W3) of ripening.

Compounds [a]	Days	Control cheese	400W2	600W2	400W3	600W3
Methylpyrazine	30	ND	ND	ND	ND	ND
	60	0.26±0.04	ND	ND	ND	ND
	120	21.29±2.61^{b}	0.06±0.01^{a}	0.12±0.01^{a}	0.22±0.03^{a}	0.33±0.07^{a}
2,3-Dimethylpyrazine	30	1.31±0.15^{a}	1.55±0.22^{a}	1.57±0.12^{a}	1.41±0.09^{a}	1.24±0.13^{a}
	60	1.10±0.08^{a}	1.60±0.15^{ab}	1.95±0.12^{b}	1.97±0.13^{b}	1.63±0.22^{ab}
	120	1.08±0.08^{a}	2.49±0.32^{c}	1.48±0.11^{ab}	2.02±0.15^{bc}	1.30±0.12^{ab}
2,5-Dimethylpyrazine	30	ND	ND	ND	ND	ND
	60	ND	ND	ND	ND	ND
	120	193.13±16.57^{b}	ND	ND	ND	0.16±0.02^{a}
2,6-Dimethylpyrazine	30	ND	0.41±0.04^{b}	0.36±0.03^{ab}	0.32±0.04^{ab}	0.23±0.03^{a}
	60	0.69±0.07^{a}	0.61±0.05^{a}	ND	2.04±0.15^{b}	2.18±0.14^{b}
	120	144.98±9.47^{b}	1.18±0.10^{a}	0.84±0.10^{a}	2.50±0.29^{a}	4.13±0.67^{a}
Trimethylpyrazine	30	0.06±0.02^{a}	0.21±0.03^{b}	ND	ND	ND
	60	0.10±0.01^{a}	0.36±0.03^{b}	ND	0.21±0.02^{a}	0.44±0.06^{b}
	120	40.11±5.10^{b}	0.41±0.03^{a}	ND	0.50±0.04^{a}	0.87±0.27^{a}

[a] Volatile compounds are expressed as relative abundances on the internal standard ($\times 10^{3}$); values are means ± SE (n = 6) of triplicate determinations in two cheese-making experiments (ND, not detected). Means on the same row followed by different superscripts differ significantly ($P < 0.05$).

Capítulo 9.

Effect of high-pressure processing on the microbiology, proteolysis, biogenic amines and flavour of cheese made from unpasteurized milk.

Fotografía: cortes de queso Arzúa-Ulloa control (no presurizado) a día 1 (superior, izquierda), 14 (superior derecha), 21 (centro, izquierda), 60 (centro, derecha), 120 (inferior, izquierda) y 240 (inferior, derecha).

Food Bioprocess Technol
DOI 10.1007/s11947-014-1406-7

ORIGINAL PAPER

Effect of High-Pressure Processing on the Microbiology, Proteolysis, Biogenic Amines and Flavour of Cheese Made from Unpasteurized Milk

Javier Calzada · Ana del Olmo · Antonia Picon · Pilar Gaya · Manuel Nuñez

Received: 4 June 2014 / Accepted: 4 September 2014

Abstract Cheese varieties with long ripening periods are prone to form biogenic amines and develop off-flavours. High-pressure processing (HPP), which inactivates microorganisms and enzymes, may be useful in preventing those defects. On this aim, cheeses made from unpasteurized milk were treated at 400 or 600 MPa, after 14 or 21 days of ripening, and their characteristics were compared to those of untreated control cheese throughout a 240-day period. Lactic acid bacteria declined by 2 log units in 400 MPa cheeses and by 6 log units in 600 MPa cheeses after HPP, while Gram-negative bacteria were below detection level in all the HPP-treated cheeses. Aminopeptidase activity was significantly lower in HPP cheeses than in control cheese from day 21 onwards. Hydrolysis of α_s-casein was enhanced in 400 MPa cheeses, but not in 600 MPa cheeses, while a more pronounced hydrolysis of β-, κ- and *para*-κ-caseins was recorded in all the HPP cheeses from day 60 onwards. Levels of hydrophilic and hydrophobic peptides were higher in HPP cheeses than in control cheese on day 60 and thereafter. Total free amino acids were at lower concentrations in 600 MPa cheeses than in the rest from day 60 onwards. The concentration of total biogenic amines was lower in all the HPP cheeses than in control cheese from day 60 onwards. Flavour quality and flavour intensity of cheese made from unpasteurized milk were not significantly affected by HPP.

Keywords High pressure · Cheese · Microbiology · Proteolysis · Biogenic amines · Flavour

J. Calzada · A. del Olmo · A. Picon · P. Gaya · M. Nuñez (✉)
Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA),
28040 Madrid, Spain
e-mail: nunez@inia.es

Introduction

Cheese ripening is a complex phenomenon in which indigenous milk enzymes, enzymes from the coagulant, other added or in situ produced enzymes, lactic acid bacteria (LAB) from starter cultures and other indigenous, added or contaminating microorganisms are involved. The importance of their respective roles in the degradation of milk constituents retained in the curd, mostly proteins and fat, varies among the different cheese varieties, depending on factors such as milk heat treatment, type of starter or manufacturing and ripening parameters.

Milk proteins, mostly caseins, are hydrolyzed by the proteolytic enzymes present in cheese. Weakening of the casein network during manufacture and ripening contributes to the development of cheese texture. A wide range of peptides are generated through the hydrolysis of caseins, most of which positively influence cheese flavour, although some of the β-casein-derived peptides are known to be bitter (Sousa et al. 2001). Free amino acids (FAA) contribute to cheese flavour by themselves and indirectly through the compounds resulting from their catabolism, many of which have higher flavour and aroma impact than their respective precursors (Yvon and Rijnen 2001). Cheese ripening continues during distribution and retail, even at adequate refrigeration temperatures. At the time of purchase, cheese may have developed a stronger or different flavour than that intended by the manufacturer (Wick et al. 2004). Overripening and development of off-flavours limit the shelf-life of cheese.

FAA also serve as substrates for bacterial decarboxylases, giving rise to the formation of biogenic amines (BA), a group of public health significance compounds. Monoamines tyramine, phenylethylamine, histamine and tryptamine are formed through the decarboxylation of tyrosine, phenylalanine, histidine and tryptophan, respectively, while diamines cadaverine

Published online: 16 September 2014

and putrescine are derived from lysine, and ornithine or arginine via agmatine (Linares et al. 2011). Enterococci were traditionally regarded as the main tyramine formers and heterofermentative lactobacilli as the main histamine producers, but other genera of bacteria are also capable of forming BA in cheese (Joosten and Northolt 1987; Pircher et al., 2007). Although the levels of decarboxylase-positive bacteria in cheese can be reduced by means of milk hygienization procedures such as bactofugation, microfiltration and pasteurization, contamination of pasteurized milk and curd by adventitious Gram-positive and Gram-negative bacteria harbouring amino acid decarboxylases (Linares et al. 2011) may occur.

High-pressure processing (HPP) is a treatment which achieves the safety level of heat pasteurization while meeting the consumer demand for fresher-tasting minimally processed foods (Norton and Sun 2008). It may be applied to cheese once the manufacturing process has ended, what offers the advantage that the contamination of the cheese interior is no longer possible. Significant reductions of decarboxylase-positive bacteria counts and biogenic amine concentrations were achieved when HPP was applied to cheese made from ovine raw milk coagulated with vegetable rennet (Calzada et al. 2013a). The procedure was also useful in controlling cheese overripening by lowering microbial counts, proteolytic activity and peptidolytic activity (Calzada et al. 2014a) and had beneficial effects on its volatile profile and odour characteristics (Calzada et al. 2014b). The stage of ripening at which HPP is applied is a relevant parameter in order to retain cheese sensory characteristics. When HPP treatments at 400 MPa on days 2 and 50 of ripening were compared, cheeses treated on day 2 received the lowest scores for taste quality on day 60, while cheeses treated on day 50 did not differ from control cheese (Garde et al. 2007).

The economic importance of cheeses made from cow milk considerably surpasses that of cheeses made from milk of other animal species. In spite of this, the effect of HPP has been studied only on two major cow milk cheese varieties, Gouda (Messens et al. 1999) and Cheddar (O'Reilly et al. 2003; Wick et al. 2004; Rynne et al. 2008), both made from pasteurized milk. Some of those studies were carried out on small cheese samples and pressure levels above 400 MPa were only applied by Wick et al. (2004). To our knowledge, there is no published information on the effect of HPP on cow milk cheeses made from unpasteurized milk, more prone to formation of biogenic amines and generation of off-flavours than cheeses made from pasteurized milk. Tyramine concentrations ranging from 453.7 to 957.6 mg/kg were reported for artisanal raw milk cheeses, while they ranged from 21.7 to 216.8 mg/kg in artisanal pasteurized milk cheeses (Ladero et al. 2010).

The objective of the present work was to investigate the effect of HPP at 400 or 600 MPa applied on days 14 or 21 of ripening on the microbiology, proteolysis, biogenic amines, texture and sensory characteristics of cheese made from unpasteurized cow milk throughout a 240-day ripening and storage period, on the aim of improving its safety while maintaining its flavour traits. Simultaneously, the characteristics of untreated control cheese were studied and compared with those of HPP cheeses.

Materials and Methods

Cheese Manufacture

Cheese was manufactured from 750 L of raw cow milk in each of two trials, carried out on consecutive days at a dairy in NW Spain. Characteristics of the cow milk used (average for the two trials) were 3.14 % protein, 3.65 % fat, 12.49 % dry matter, and 4.10 log cfu/mL viable bacterial counts. Freeze-dried mesophilic starter (25 units of CHN-19, Chr. Hansen S.L., Tres Cantos, Spain), composed of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar diacetylactis and *Leuconostoc*, was added to the milk previously warmed to 32.5 °C. After 15 min, animal rennet (150 mL of Naturen®, 80 % chymosin, Chr. Hansen S.L.) was added to the milk, which was left to coagulate at 32.5 °C for 50 min. The curd was cut into 2-cm cubes, stirred in the vat in its own whey, washed with tap water at 35 °C, and finally salted in the vat by addition of 9 kg dry salt. The curd was dispensed into cylindrical moulds and pressed for 3 h at 20 °C. Total weight of the cheeses out of the press was 95.58 kg (average for the two trials). Cheeses (13.4-cm diameter, 7.0-cm height, 1.15-kg average weight out of the press) were ripened at 8 °C and 72 % RH until day 60 and at 5 °C and 75 % RH afterwards.

High-Pressure Processing and Sampling

Before HPP, cheeses were vacuum-packaged in CN300 bags (Cryovac Grace S.A., Barcelona, Spain). Cheeses, respectively coded as 400W2, 600W2, 400W3 and 600W3, were HPP-treated for 5 min at 400 or 600 MPa after ripening for 14 or 21 days. A 120-L capacity isostatic press (Hiperbaric, Burgos, Spain) was used for HPP. Come-up times to reach 400 and 600 MPa were 1.76 and 2.58 min, respectively, and depressurization times, 5 and 7 s. Initial temperature of the water used as transmitting fluid was 8 °C; it remained below 14 °C during the process. After treatments, HPP cheeses were unpackaged and ripened under the same conditions of control cheese.

From each of the two cheese-making trials, one cheese was analyzed on day 1, three cheeses (control, 400W2, 600W2) on day 14 and five cheeses (control, 400W2, 600W2, 400W3, 600W3) on each of the sampling dates day 21, day 60, day

120, day 180 and day 240, resulting in 29 cheeses analyzed per trial and 58 cheeses analyzed in the overall experiment.

Microbiological Analyses

Representative cheese samples (10 g) were homogenized with 90 mL of a sterile 2 % (*w*/*v*) sodium citrate solution at 45 °C in a Colworth Stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions of milk and cheese homogenate were prepared in sterile 0.1 % peptone solution. Total viable counts, LAB, lactobacilli, enterococci, *Micrococcaceae*, coagulase-positive staphylococci, Gram-negative bacteria, coliforms, moulds and yeasts were determined in duplicate using the culture media and incubation conditions previously described (Calzada et al. 2013b). Analysis of *Listeria monocytogenes* in milk and cheese was performed as indicated by Arqués et al. (2005).

Chemical and Enzymatic Determinations

Caseins and whey proteins were analyzed by capillary gel electrophoresis on triplicate samples, using an automated P/ACE MDQ capillary electrophoresis apparatus controlled by a 32 Karat Software (Beckman Instruments España S.A., Madrid, Spain) according to Calzada et al. (2013b). Commercial standards (Sigma, Alcobendas, Spain) of bovine α-CN, β-CN, κ-CN, α-LA, β-LG, serum albumin and lactoferrin were used for the identification of proteins. Protein peaks were quantified with respect to the standards and expressed as milligrams of protein per gram of cheese dry matter (DM). DM content and pH value of cheeses were determined on triplicate samples according to Garde et al. (2002).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by reverse-phase high-performance liquid chromatography (HPLC) as previously described (Garde et al. 2002), using a Beckman System Gold chromatograph (Beckman Instruments España SA, Madrid, Spain) equipped with a diode array detector module 168, with detection wavelength at 280 nm. Peaks with retention times from 5.5 to 14.6 min were considered to correspond to hydrophilic peptides and those with retention times from 14.6 to 20.5 min to hydrophobic peptides. Results were expressed in arbitrary units (AU), calculated as units of chromatogram area per milligram of cheese DM.

FAA and BA were simultaneously extracted from duplicate samples according to Krause et al. (1995). Analysis of individual FAA after derivatization with Waters AccQ Fluor Reagent (Waters S.A., Barcelona, Spain) and analysis of individual BA after derivatization with dabsyl chloride were carried out by reverse-phase HPLC as previously described (Calzada et al. 2013b). Standard mixtures of FAA and BA (Sigma, Alcobendas, Spain) were used for their identification and quantification.

Aminopeptidase activity released into the cheese was measured on duplicate samples using lysine *p*-nitroanilide (Lys-*p*-NA) and leucine *p*-nitroanilide (Leu-*p*-NA) as substrates according to Garde et al. (2002). One activity unit corresponds to the activity of enzyme(s) producing 1 nmol of *p*-nitroaniline per minute per gram of cheese.

Tyrosine decarboxylase (TDC) activity in cheese was determined in duplicate samples as previously described (Calzada et al. 2013a). Briefly, a cheese homogenate prepared by mixing 10 g of cheese with 20 mL of 2 % sodium citrate solution was centrifuged and the supernatant dialysed (molecular mass cut-off, 10 kDa). The dialysate was centrifuged and the supernatant stored at −20 °C until analysis. A TDC activity standard curve was obtained by means of reaction mixtures containing 1.5 mM L-tyrosine (Sigma), 0.15 mM pyridoxal-5′-phosphate (Sigma) and up to 32 mIU/mL of the L-TDC apoenzyme (Sigma). TDC activity in the supernatants of cheese homogenates was assayed on reaction mixtures without added apoenzyme. TDC activity in cheese was calculated from the increase in tyramine concentration (determined by HPLC after 0 and 24 h of incubation of the reaction mixtures at 37 °C) against the TDC activity standard curve.

Textural Determinations

Eight cylinder-shaped (17-mm height, 17-mm diameter) samples from each cheese were compressed to 75 % of their original height after 10 min at room temperature (20–22 °C) using an Instron Compression Tester 4301 (Instron, High Wycombe, Bucks, UK), with crosshead and chart speeds of 50 and 500 mm/min, respectively. Firmness (work done on the cheese up to 75 % compression, expressed in Newton metre (N m)), elasticity (apparent elastic modulus, expressed in Newton per square millimetre (N/mm^2)) and fracturability (force at breaking point, expressed in Newton (N)) were determined from the compression curves according to Calzada et al. (2014a).

Sensory Evaluation

Seventeen trained panellists evaluated the cheeses after 21, 60, 120, 180 and 240 days of ripening for flavour quality (overall acceptance), flavour intensity (overall intensity) and flavour attributes “acid”, “bitter”, “salty”, “sweet” and “umami” on a 0–10-point scale, using a horizontal line anchored in the middle and at both ends, as previously described (Calzada et al. 2014a). Cheeses were cut into representative cubes (10 to 12 g), which were held for 2 h at 20–22 °C prior to sensory evaluation. Five cheeses per session (one control and four HPP cheeses, manufactured on the same day), coded with

three-digit numbers, were randomly presented to panellists. Bread and water were used as rinsing agents between cheeses.

Statistical Treatment

Analysis of variance with HPP treatment (four HPP treatments and control) and ripening time as main effects was performed on the analytical variables by means of SPSS Win 14.0 program (SPSS Inc., Chicago, USA). Calculation of correlations and comparison of means by Tukey's test, with the significance assigned at $p<0.05$, were carried out using the same program.

Results and Discussion

Microbial Groups

Total viable counts declined gradually during ripening of control cheese, from 9.2 log cfu/g on day 1 (data not shown) to 8.0 log cfu/g on day 240, while LAB counts declined from 8.8 log cfu/g on day 1 to 7.9 log cfu/g on day 240 (Fig. 1). HPP at 400 MPa lowered total viable counts by 2.4 log units on day 14 and by 1.1 log units on day 21, while at 600 MPa the respective declines were 4.0 and 3.3 log units. Decreases of LAB counts after HPP at 400 MPa were 2.3 log units on day 14 and 2.0 log units on day 21 whereas the respective decreases at 600 MPa were 6.0 and 5.8 log cfu/g. The decrease in total viable counts and LAB counts after HPP at 600 MPa should have been similar since LAB accounted for most of the total viable counts before HPP. The higher decline of LAB counts than of total viable counts after HPP at 600 MPa may be due to the use of a selective culture medium for LAB enumeration, which probably hindered the recovery of HPP-injured LAB cells while they were capable of forming colonies on a non-selective medium. The only previously reported result on the decrease of total viable counts and LAB counts in cow milk cheeses treated at 600 MPa was a decrease of *L. lactis* counts by more than 5 log units after HPP of 1-month-old Cheddar cheese at 500–800 MPa (Wick et al. 2004). The decreases of total viable counts and LAB counts after HPP at 600 MPa in the present study were similar to those recorded in ovine milk cheese treated at the same pressure (Calzada et al. 2013a).

Lactobacilli counts increased in control cheese from 4.07 log cfu/g on day 1 (data not shown) to 8.03 log cfu/g on day 240, while enterococci counts decreased from 6.31 log cfu/g on day 1 (data not shown) to 4.71 log cfu/g on day 240 (Fig. 1). HPP at 400 MPa lowered lactobacilli counts by 1.0 log units on day 14 and by 1.1 log units on day 21, while the respective declines at 600 MPa were 4.3 and 4.9 log units. In the case of enterococci, HPP at 400 MPa lowered counts by 0.7 log units on day 14 and by 0.4 log units on day 21, whereas the respective declines at 600 MPa were 2.9 and 3.2 log units. As for LAB, the use of selective culture media for the enumeration of lactobacilli and enterococci after HPP might have underestimated their counts and overestimated the lethality of 600 MPa treatments. In spite of this, lactobacilli and enterococci counts decreased less than after HPP of ovine milk cheese at 600 MPa, in which lactobacilli declined by 5.8–6.1 log units and enterococci by 4.9–5.3 log units (Calzada et al. 2013a). A decrease of lactobacilli counts by more than 4 log units in 1-month-old Cheddar cheese after treatment at 500–800 MPa was reported by Wick et al. (2004).

Micrococcaceae counts (data not shown) reached levels of 6.92 log cfu/g on day 1 in control cheese and decreased to 4.96 log cfu/g on day 60 and 4.08 log cfu/g on day 240. HPP at 400 MPa lowered their counts by 2.1 log units on days 14 and 21, whereas treatment at 600 MPa caused decreases of 3.4 log units on day 14 and 3.3 log units on day 21, a lethal effect similar to the declines of 2.8–3.9 log units after HPP of ovine milk cheese at 600 MPa reported by Calzada et al. (2013a). In the present work, there was no recovery of *Micrococcaceae* after HPP with counts remaining below 3.5 log cfu/g in 400 MPa cheeses and below 2.5 log cfu/g in 600 MPa cheeses until day 240. Coagulase-positive staphylococci counts (data not shown) attained levels of 4.83 log cfu/g on day 1, declined in control cheese to 2.66 log cfu/g on day 14 and to populations below detection level (<1 log cfu/g) during the rest of the ripening period, while they were not found in any of the HPP cheeses after treatments. *L. monocytogenes* was not detected (<1 log cfu/g) in control or HPP cheeses at any stage of manufacture or ripening.

Gram-negative bacteria counts (data not shown) were at 7.52 log cfu/g on day 1 and declined to 6.50, 6.18, 3.90 and 2.04 log cfu/g, respectively, on days 14, 21, 60 and 240 in control cheese. Their population in HPP cheeses was below the detection level (<1.4 log cfu/g) immediately after treatments at 400 or 600 MPa, and they were not found afterwards in any of the HPP cheeses, in agreement with the results obtained by Calzada et al. (2013a) for ovine milk cheese. Similarly, coliform counts declined in the present work from 7.50 log cfu/g on day 1 to 6.08, 5.75, 3.81 and 1.46 log cfu/g, respectively, on days 14, 21, 60 and 240 in control cheese (data not shown). Coliforms were not detected in any of the HPP cheeses during ripening. Yeast counts reached 3.62 log cfu/g on day 1 and declined afterwards in control cheese to 3.18 log cfu/g on day 60 and 2.49 log cfu/g on day 240 (data not shown). Yeasts were not detected in any of the HPP cheeses after treatments.

Dry Matter and pH Value

DM of control cheese increased gradually from 47.78 % on day 1 (data not shown) to 53.13 % on day 60 and 65.43 % on

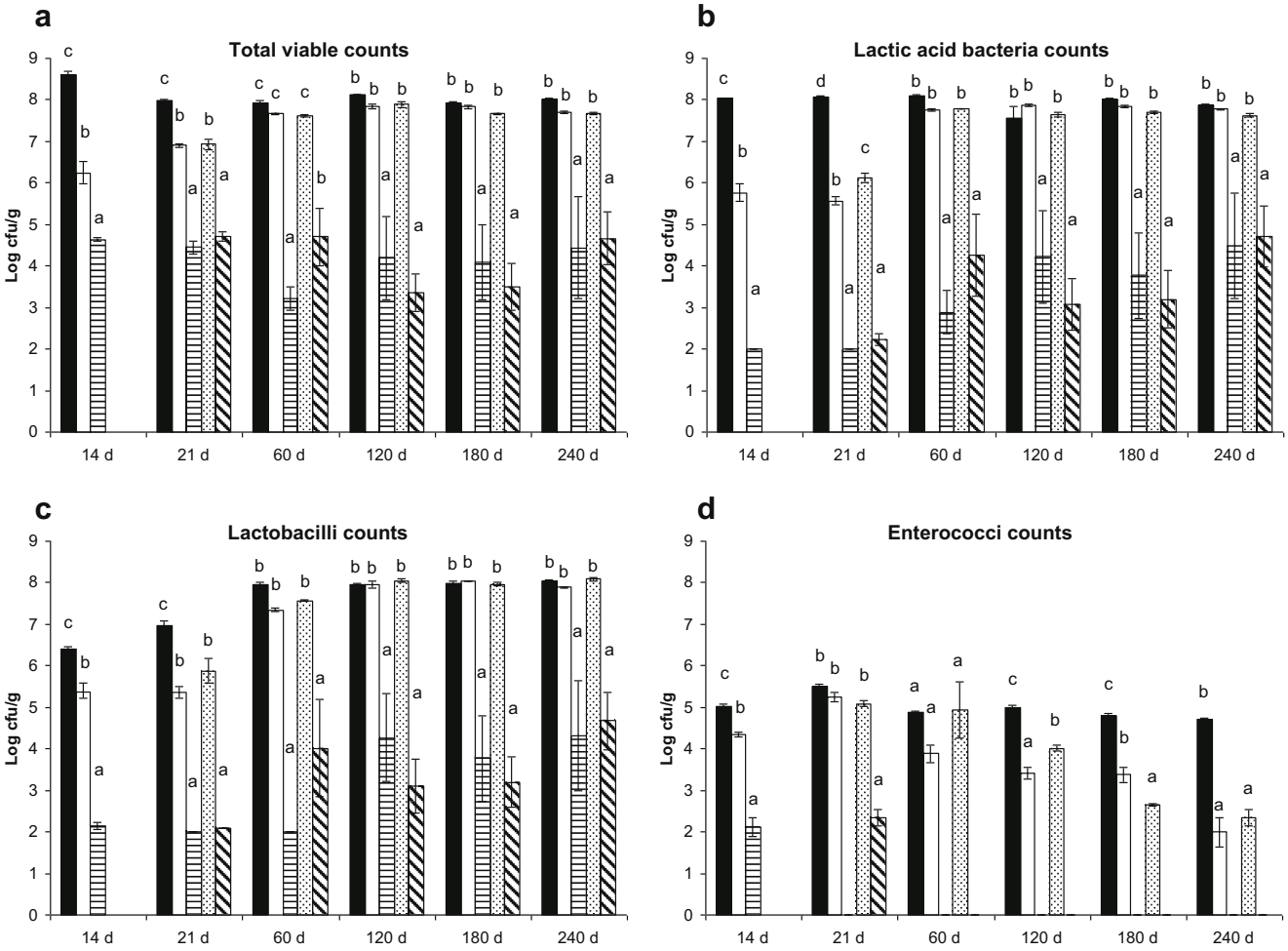


Fig. 1 Counts of the main bacterial groups (**a** total viable bacteria, **b** lactic acid bacteria, **c** lactobacilli, **d** enterococci) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

day 240 (Fig. 2). DM contents of HPP cheeses ranged from 52.84 to 54.05 % on day 60 and from 65.63 to 66.64 % on day 240, with no significant ($p>0.05$) differences between control and HPP cheeses at any stage of ripening. No clear effects of HPP on DM during ripening were recorded in previous works on cow milk cheeses (Messens et al. 1999; Rynne et al. 2008).

Control cheese pH value declined to 5.26 on day 1 (data not shown) and rose gradually afterwards, to 5.36 on day 60 and 5.68 on day 240 (Fig. 2). HPP cheeses had slightly higher pH values than control cheese immediately after treatment, although the differences were below 0.1 pH units. A higher pH increase, with differences of almost 0.2 pH units, had been reported in HPP-treated Gouda cheese (Messens et al. 1999). During ripening, the pH increase in HPP cheeses lagged behind that in control cheese, with values ranging from 5.26 to 5.32 on day 60 and from 5.39 to 5.59 on day 240. These results are in agreement with those obtained by Calzada et al. (2014a), while no changes in pH values during ripening of control or HPP cheeses were recorded by Messens et al. (1999).

Hydrolysis of Proteins

Soluble compounds, including whey proteins, were lost in whey during cheese manufacture. Also, proteolysis took place in the vat and during pressing of cheese. On day 1, casein concentrations were 88.18, 89.61, 23.25, 16.17 and 3.65 mg/g DM for α-casein, β-casein, κ-casein, para-κ-casein and γ-caseins, respectively (data not shown). Concentrations of whey proteins in 1-day cheese were 0.98 mg/g DM for α-lactalbumin and 1.37 mg/g DM for β-lactoglobulin, while bovine serum albumin was not found (data not shown). From day 1 to day 240, the concentration of caseins in control cheese declined by 46, 64, 65 and 35 % for α_s-, β-, κ- and para-κ-caseins, respectively, while a 9.3-fold increase of γ-caseins, products of β-casein hydrolysis, was recorded (Fig. 3). The only whey protein detected during ripening of

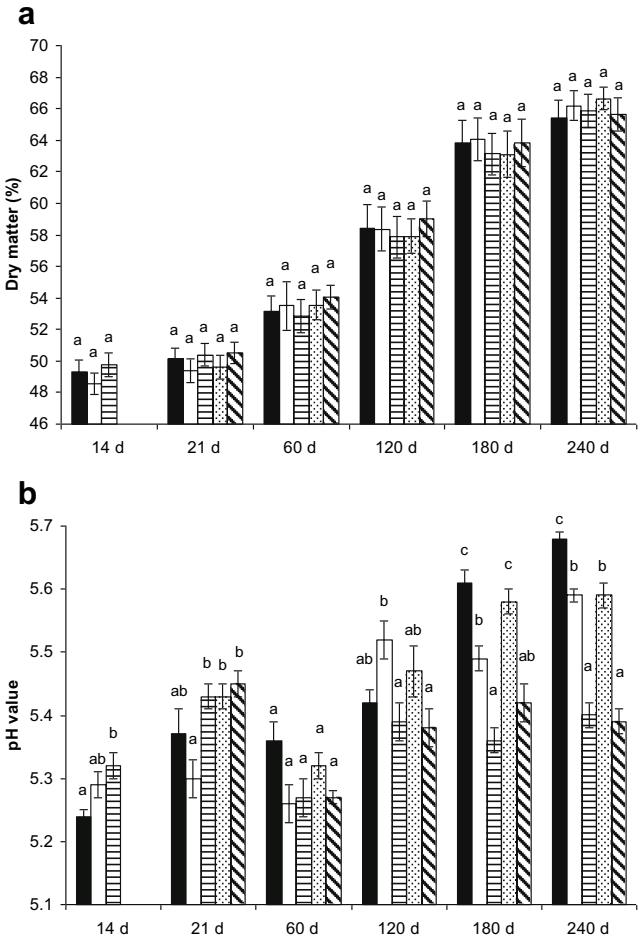


Fig. 2 Dry matter content (**a**) and pH value (**b**) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

control cheese, α-lactalbumin, reached 0.10 mg/g DM on day 14 and was not found afterwards (data not shown).

Proteolysis during ripening of HPP cheeses markedly differed from that of control cheese (Fig. 3). Degradation of α_s-casein was enhanced only in 400 MPa cheeses, with significantly ($p<0.05$) lower concentrations than in control cheese or in 600 MPa cheeses from day 120 onwards. In the case of β-, κ- and para-κ-caseins, concentrations were significantly ($p<0.05$) lower than in control cheese not only in 400 MPa cheeses but also in 600 MPa cheeses from day 60 onwards. From day 1 to day 240, the concentrations of α_s-, β-, κ- and para-κ-caseins in HPP cheeses declined by 41–66, 84–92, 75–81 and 69–88 %, respectively. Chymosin is partially inactivated by HPP at 400 MPa or higher pressures, but plasmin, a more baroresistant enzyme, maintains full activity after pressurization at 500 or 600 MPa (Malone et al. 2003; Huppertz et al. 2004). HPP causes the disruption of linkages between caseins and inorganic constituents of milk, what may enhance the susceptibility of caseins to proteolysis (García-Risco et al. 2000). This might explain the faster degradation of caseins recorded in the present work for HPP cheeses, by the action of chymosin and plasmin in 400 MPa cheeses and mostly by the action of plasmin in 600 MPa cheeses. Bovine pepsin from rennet is also involved in cheese proteolysis, but its contribution to the proteolysis of HPP cheeses is difficult to estimate since, to our knowledge, no information about the baroresistance of this enzyme is available. The concentration of γ-caseins increased by 13.8- to 14.1-fold in HPP cheeses from day 1 to day 240 (data not shown), more than in control cheese, in agreement with the more pronounced degradation of β-casein. As for control cheese, the only whey protein detected during ripening of HPP cheeses was α-lactalbumin, at 0.07–0.08 mg/g DM on day 14 (data not shown). Negligible differences in SDS-PAGE profiles were observed between control and HPP cheeses when Gouda or Cheddar cheeses were treated at 400 MPa (Messens et al. 1999; O'Reilly et al. 2003).

The levels of hydrophilic peptides increased gradually during ripening of control cheese, from 3.53 AU on day 1 (data not shown) to 7.56 AU on day 60 and 8.72 AU on day 240, while the levels of hydrophobic peptides increased from 2.20 AU on day 1 to 7.69 AU on day 60 and then declined to 4.37 AU on day 240 (Fig. 4). All the HPP cheeses showed significantly ($p<0.05$) higher levels of hydrophilic peptides and hydrophobic peptides than control cheese from day 60 onwards, in agreement with the enhanced hydrolysis of caseins (Fig. 3). Contrarily, a decrease in the levels of hydrophilic and hydrophobic peptides with respect to control cheese was reported for pasteurized ewes' milk cheeses treated at 400 or 500 MPa (Juan et al. 2007). The hydrophobic peptides/hydrophilic peptides ratio of control cheese rose from 0.624 on day 1 (data not shown) to a maximum value of 1.125 on day 14 in the present work, and then declined to 0.497 on day 240 (Fig. 4). In HPP cheeses, the ratio tended to be lower than in control cheese until day 60, with few significant differences between cheeses, but from day 120 onwards, it reached significantly ($p<0.05$) higher values in the 600 MPa cheeses than in control cheese.

Aminopeptidase activity on Leu-*p*-NA as substrate (data not shown) increased in control cheese from 1.36 activity units on day 1 to a maximum of 5.87 activity units on day 14 and remained fairly unchanged afterwards, still reaching 5.03 activity units on day 240. With Lys-*p*-NA as substrate, aminopeptidase activity of control cheese rose from 2.25 activity units on day 1 (data not shown) to a maximum of 11.39 activity units on day 21 and declined gradually to 6.85 activity units on day 240 (Fig. 5a). Aminopeptidase activity values during early ripening were considerably lower than those found for ovine milk cheese of the same age by Calzada et al. (2014a), but on day 240, similar activity values were obtained. In addition to the significant ($p<0.05$) decrease in the aminopeptidase activity of HPP cheeses immediately

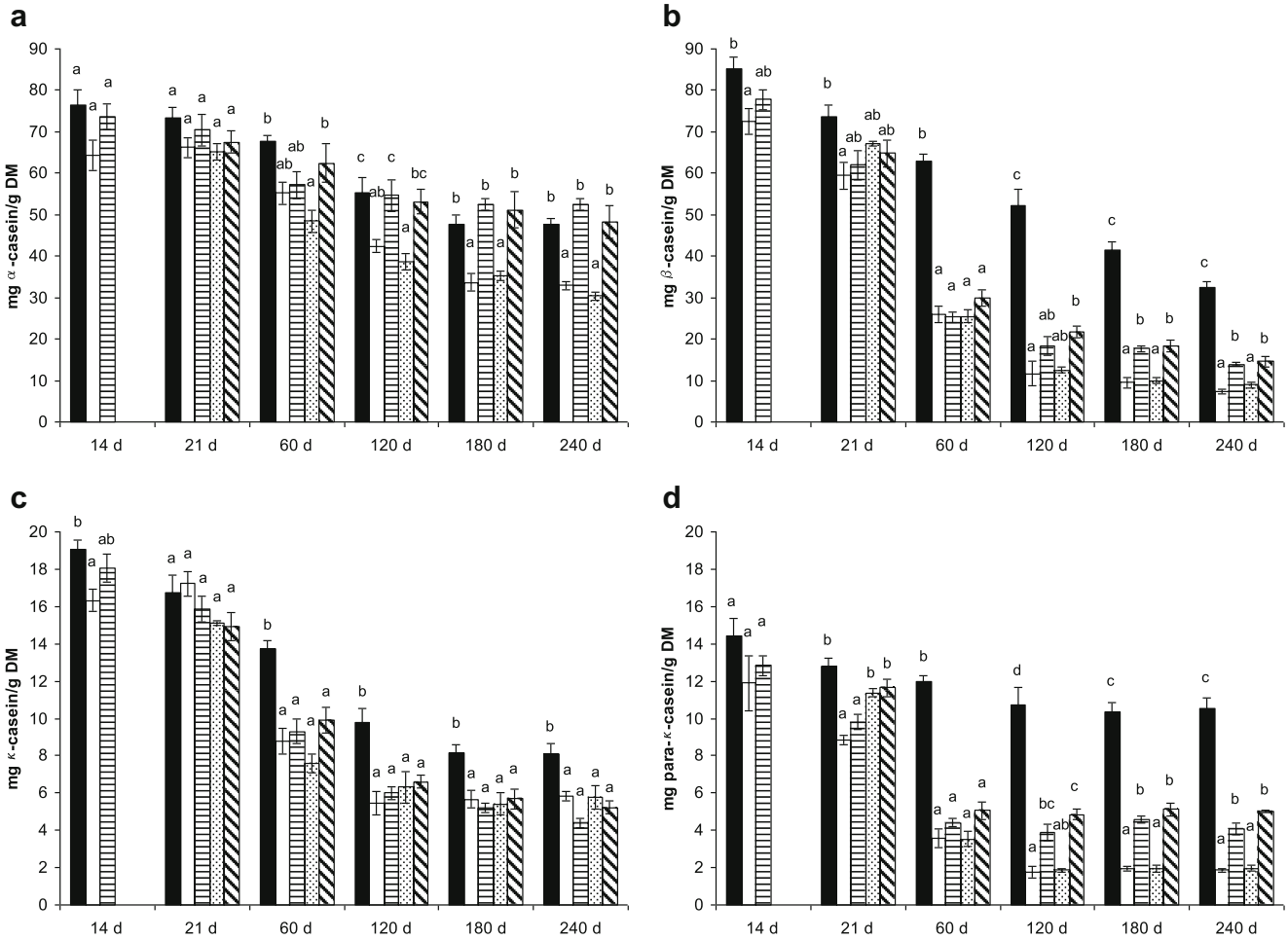


Fig. 3 Levels of α_s-casein (**a**), β-casein (**b**), κ-casein (**c**) and para-κ-casein (**d**) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

after treatment, further decreases were recorded during the rest of the ripening period for most of the HPP cheeses. This pattern is in contradiction with the results obtained by Juan et al. (2007), who observed an increase of the aminopeptidase activity in control cheese and in cheeses treated at 400 or 500 MPa during a 60-day ripening period. The inactivation of bacterial peptidases by HPP depends not only on process parameters, but also on the bacterial species and assay substrates, and the effect may vary for different peptidolytic enzymes within the same bacterial strain (Malone et al. 2003). In the present work, aminopeptidase inactivation immediately after HPP was more dependent on the day of treatment (68–73 % inactivation on day 14 versus 87–89 % on day 21) than on the pressure level (68–88 % at 400 MPa versus 73–89 % at 600 MPa) or the substrate used (68–88 % on Leu-*p*-NA versus 71–89 % on Lys-*p*-NA). Approximately 25 % of the total viable bacteria present in 1-day control cheese remained viable on day 14 while only 6 % were still alive on day 21. Death of bacteria was most probably followed by cell lysis and release of intracellular enzymes, including aminopeptidases, to the surrounding medium. As the released aminopeptidases were no longer protected by cell structures, they were probably more susceptible to HPP inactivation than when inside the cells. This might explain why aminopeptidase inactivation immediately after HPP was higher on day 21, when more intracellular enzymes had been released from the cells, than on day 14.

The concentration of total FAA increased in control cheese from 1.06 mg/g DM on day 1 (data not shown) to 8.79 mg/g DM on day 60 and to 31.68 mg/g DM on day 240 (Fig. 5b). Total FAA concentrations did not vary in HPP cheeses immediately after treatment, and no significant differences in total FAA were found between control and HPP cheeses on day 21. Afterwards, the concentration of total FAA increased in control cheese, which showed 28–31 % higher levels than 400 MPa cheeses and 63–64 % higher levels than 600 MPa cheeses on day 240. The dominant FAA in 240-day control cheese were glutamic acid, leucine, lysine, valine and serine while in the respective HPP cheeses the main FAA were glutamic acid, leucine, lysine, valine and phenylalanine (data

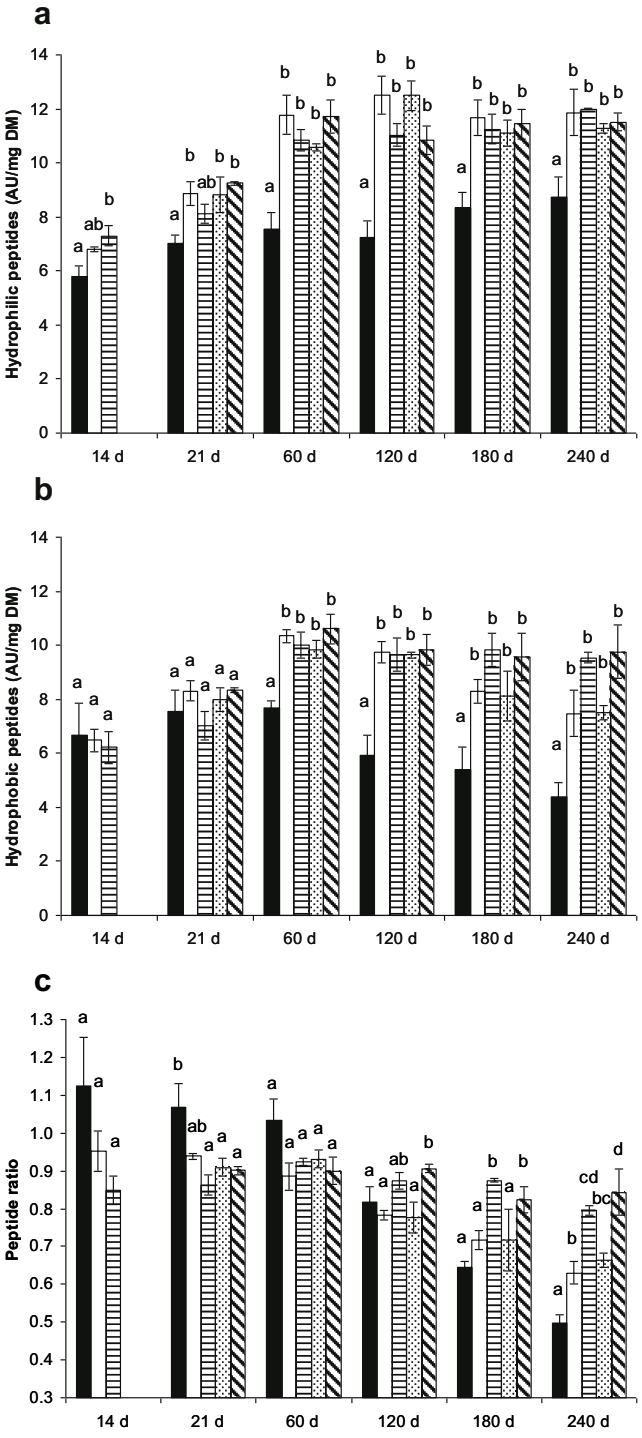


Fig. 4 Levels of hydrophilic peptides (**a**), hydrophobic peptides (**b**) and the ratio of hydrophobic/hydrophilic peptides (**c**) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

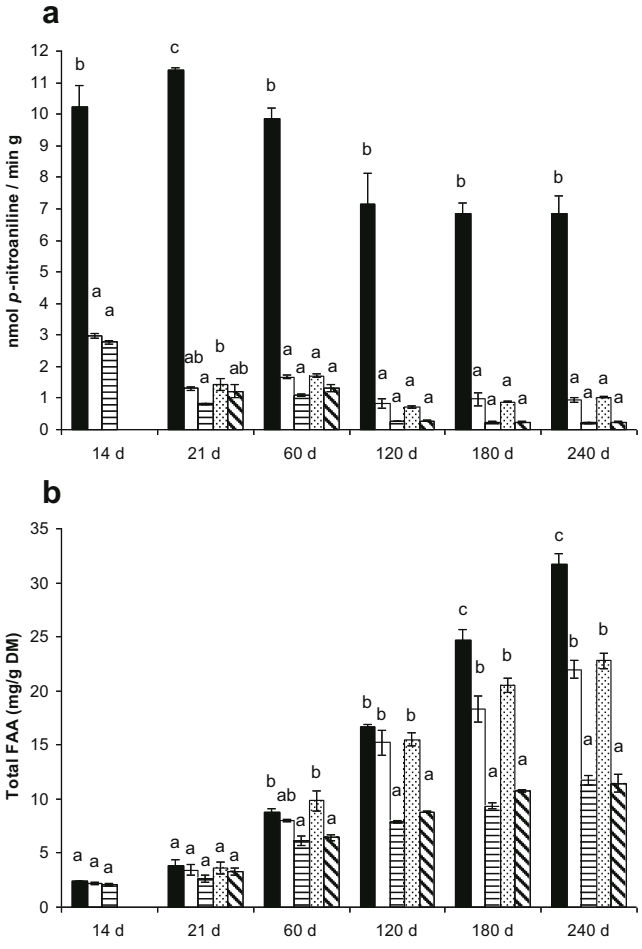


Fig. 5 Aminopeptidase activity on Lys-*p*-NA (**a**) and levels of total free amino acids (**b**) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

not shown). The concentration of total FAA in Cheddar cheese treated at 400 MPa decreased or increased depending on the starter culture used (O'Reilly et al. 2002). HPP of Cheddar cheese at pressure levels of 200 or 300 MPa had no effect on its FAA content, while pressure levels of 400 to 800 MPa retarded the evolution of FAA (Wick et al. 2004). Increases of total FAA concentrations by 9–12 % were reported for ovine milk cheese treated at 400 MPa, after a 240-day ripening and storage period, while HPP at 600 MPa resulted in decreases of 25–29 % (Calzada et al. 2014a). In the present study, total FAA concentrations in 180- and 240-day cheeses were significantly correlated with the respective aminopeptidase activity values, both with Leu-*p*-NA ($r=0.739$, $p<0.05$) and Lys-*p*-NA ($r=0.784$, $p<0.01$) as substrates.

Biogenic Amines

Tyrosine decarboxylase was significantly ($p<0.05$) inactivated by HPP, with lower activity levels in all the HPP cheeses than in control cheese on day 21 (Fig. 6). During the rest of the ripening period, TDC activity increased gradually

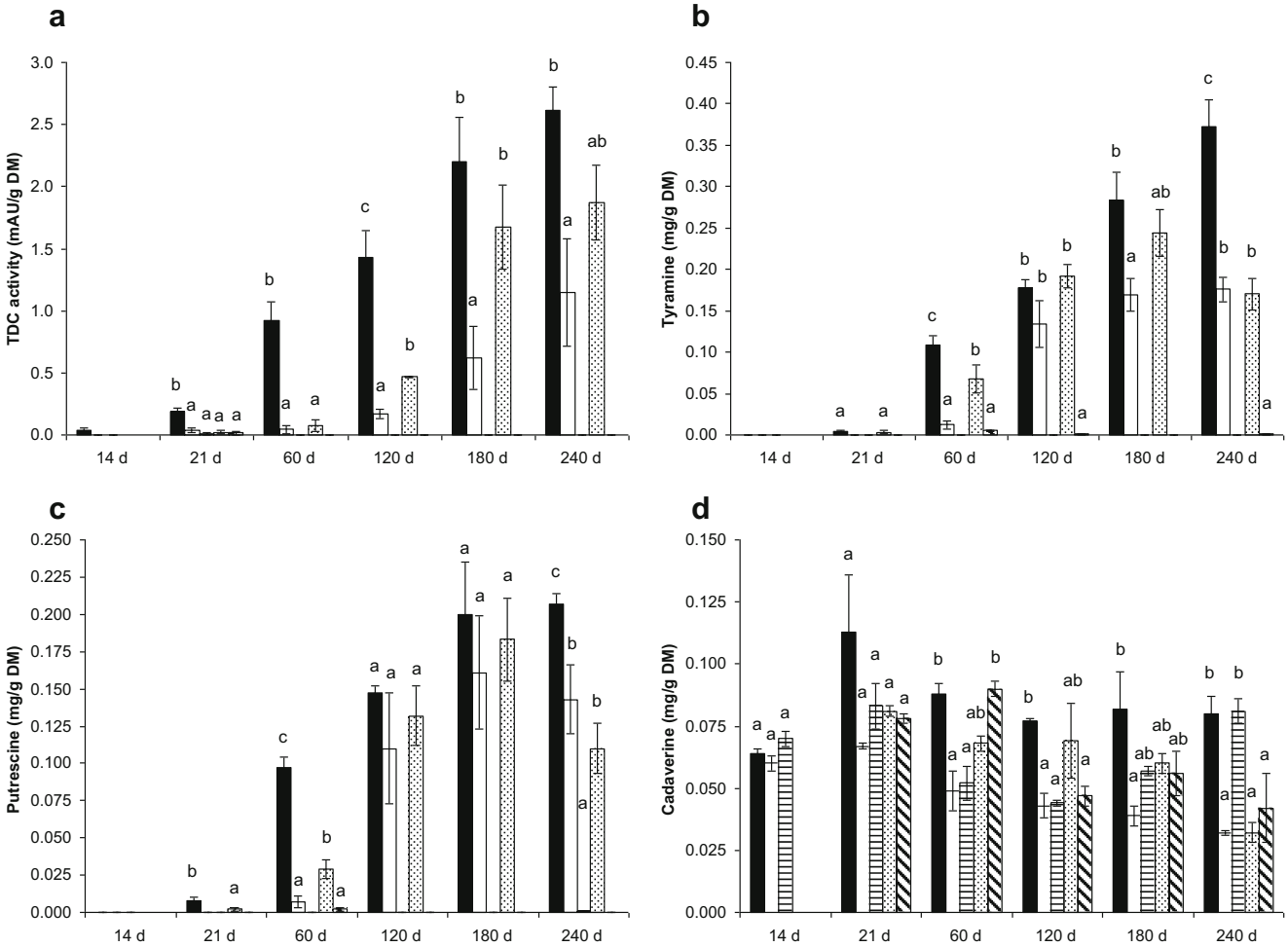


Fig. 6 Tyrosine decarboxylase activity (**a**) and levels of tyramine (**b**), putrescine (**c**) and cadaverine (**d**) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

in control and 400 MPa cheeses while no activity was detected in any of the 600 MPa cheeses. On day 240, it reached 2.619 mAU/g DM in control cheese and 1.148–1.875 mAU/g DM in 400 MPa cheeses. In the present study, TDC activity values during ripening of control cheese were slightly lower than the respective values of control ovine milk cheese while those of 400 MPa cheeses were higher than the respective values of ovine milk cheese treated at 400 MPa (Calzada et al. 2013a), results which can be attributed to differences in cheese microbiota.

Tyramine concentration on day 21 was under 0.010 mg/g DM in control and 400 MPa cheeses and below detection level in 600 MPa cheeses. Afterwards, tyramine accumulated at a faster rate in control cheese than in 400 MPa cheeses while it was only occasionally detected, at levels under 0.010 mg/g DM, in some of the 600 MPa cheeses. On day 240, tyramine concentration attained 0.372 mg/g DM in control cheese, 0.170–0.176 mg/g DM in 400 MPa cheeses and was not detected in 600 MPa cheeses (Fig. 6). Tyrosine concentration was not a limiting factor for tyramine formation, since it reached 0.348 mg/g DM in control cheese, 0.214–0.218 mg/g DM in 400 MPa cheeses and 0.344–0.416 mg/g DM in 600 MPa cheeses on day 240. The low enterococci counts, always below 6 log cfu/g throughout ripening of control cheese and considerably lower in HPP cheeses, in particular in 600 MPa cheeses (Fig. 1), preclude an important contribution of this genus to tyramine formation. In contrast, the population of lactobacilli (Fig. 1), at 8 log cfu/g in control and 400 MPa cheeses from day 60 to day 240, was high enough to actively participate in tyramine formation provided that some of the strains harboured tyrosine decarboxylases. Production of tyramine by lactobacilli isolated from Dutch cheese belonging to different species was reported by Joosten and Northolt (1987) and by lactobacilli from various foods including cheeses by Pircher et al. (2007). Also, decarboxylation of tyrosine by some *L. lactis* strains has been observed (Curtin and McSweeney 2003). In the present work, tyrosine decarboxylase-positive

bacteria were not determined, and no population threshold for tyramine formation could be established. Thresholds for tyramine production in cheese as low as 4 log cfu/g of strains with decarboxylase activity have been suggested (Ladero et al. 2010). Highly significant ($p<0.001$) correlations of tyramine concentrations in 60- to 240-day-old cheeses with LAB counts ($r=0.757$), lactobacilli counts ($r=0.781$) and TDC activity ($r=0.915$) were found in the present work. Histamine was detected only on days 180 and 240, at 0.009–0.026 mg/g DM in control cheese and 0.004–0.005 mg/g DM in some of the 400 MPa cheeses (data not shown). Histidine concentrations, 2.121 mg/g DM in control cheese, 1.265–1.269 mg/g DM in 400 MPa cheeses and 0.630–0.639 mg/g DM in 600 MPa cheeses on day 240, were sufficient for not limiting histamine formation.

On day 21, putrescine was only found in control and 400 MPa cheeses, at concentrations under 0.010 mg/g DM. Afterwards, putrescine increased more rapidly in control cheese than in 400 MPa cheeses whereas it was not detected or was at levels of 0.001–0.002 mg/g DM in 600 MPa cheeses (Fig. 6). On day 240, it reached 0.207 mg/g DM in control cheese, 0.110–0.143 mg/g DM in 400 MPa cheeses and at most 0.001 mg/g DM in 600 MPa cheeses. Gram-negative bacteria are considered to be the main putrescine and cadaverine formers (Pircher et al. 2007). They reached counts above 6 log cfu/g until day 21 and below 4 log cfu/g from day 60 onwards in control cheese and were below the detection level in all samples of HPP cheeses. Low counts of Gram-negative bacteria in control cheese from day 60 onwards precluded a significant contribution of these microorganisms to the biosynthesis of putrescine, which more than doubled from day 60 to day 240. Moreover, putrescine production in 400W2 and 400W3 cheeses took place from day 21 onwards, when Gram-negative bacteria were no longer detected. Therefore, putrescine production in control and HPP cheeses must be ascribed to other microorganisms, in particular to lactic acid bacteria. Putrescine concentrations in 60- to 240-day-old cheeses were highly correlated with counts of lactic acid bacteria ($r=0.786$; $p<0.001$) and lactobacilli ($r=0.808$; $p<0.001$) and at a lower level of significance also with enterococci ($r=0.648$; $p<0.01$). Production of putrescine via the agmatine deamination pathway seems to be a species-level trait of *Enterococcus faecalis* (Ladero et al. 2012). Cadaverine formation was less affected by HPP and time of ripening than tyramine or putrescine (Fig. 6). Its pattern of accumulation was markedly different from that of putrescine. In control cheese, it reached 0.064 mg/g DM on day 14 and a maximum level of 0.113 mg/g DM on day 21. In HPP cheeses, the maximum concentrations were generally reached on day 21, with values ranging from 0.067 to 0.083 mg/g DM. On day 240, it attained 0.080 mg/g DM in control cheese, 0.032–0.034 mg/g DM in 400 MPa cheeses and 0.042–0.081 mg/g DM in 600 MPa cheeses. As counts of Gram-negative bacteria declined, naturally in control cheese and pressure-induced in HPP cheeses, cadaverine accumulation ceased. In our study, the production of cadaverine may be associated with the presence of Gram-negative bacteria at sufficiently high levels. Concentrations of cadaverine in 60- to 240-day-old cheeses were not significantly correlated with counts of lactic acid bacteria ($r=0.049$) or lactobacilli ($r=0.017$). The concentrations of arginine and lysine, precursors for putrescine and cadaverine, were not limiting factors for biogenic amine formation.

Other biogenic amines such as phenylethylamine, tryptamine, spermine and spermidine were not detected in control or HPP cheeses. Strains belonging to some of the bacterial genera commonly found in cheese form biogenic amines through the decarboxylation of FAA, a mechanism which constitutes an alternative energy source in the absence of fermentable carbohydrates (Fernández-García et al. 1999). Some strains of *Lactobacillus*, *Pediococcus* and *Micrococcus* are capable of degrading biogenic amines such as tyramine and histamine by means of monoamine oxidases, preferably under aerobic conditions (Leuschner et al. 1998). The rate of biogenic amine build-up during cheese ripening is the resultant of their formation and degradation in cheese. On day 240, total biogenic amines reached 0.686 mg/g DM in control cheese, while they only attained 0.316–0.351 mg/g DM in 400 MPa cheeses and 0.044–0.081 mg/g DM in 600 MPa cheeses. These levels are considerably lower than in 240-day ovine milk cheeses, in which total biogenic amines attained 3.690 mg/g DM in control cheese, 2.022–3.276 mg/g DM in 400 MPa cheeses and 0.896–1.011 mg/g DM in 600 MPa cheeses (Calzada et al. 2013a). The concentrations of total FAA in control cheese in the present work were 8.79 and 31.68 mg/g DM on days 60 and 240, respectively, higher than the concentrations reported by Calzada et al. (2014a), 4.82 mg/g DM on day 60 and 23.31 mg/g DM on day 240. Since FAA concentrations did not limit BA formation in the present study, the main reason for the differences in BA concentrations between both works seems to be the differences in the BA formation ability of cheese microbiota. Calzada et al. (2013a) reported Gram-negative bacteria counts from day 60 to day 180 above 6 log cfu/g in control cheese and below detection level in HPP cheeses. In their work, tyramine, histamine, phenylethylamine, tryptamine and putrescine increased in control and HPP cheeses from day 60 onwards, even though Gram-negative bacteria were not detected at that time in HPP cheeses. In contrast, phenylethylamine and tryptamine were not detected in control or HPP cheeses in the present work. Also, Calzada et al. (2013a) reported a significant increase in cadaverine concentration in control cheese from day 60 onwards while this biogenic amine did not increase in control cheese from day 60 onwards in the present work. These facts point out differences in the BA formation ability of cheese microbiota between both works.

Textural Characteristics

Firmness of control cheese declined from 0.075 N m on day 1 (data not shown) to 0.026 N m on day 21 and afterwards increased gradually up to 0.141 N m on day 240. HPP cheeses exhibited lower firmness than control cheese on day 21, with values ranging from 0.013 to 0.020 N m, and higher firmness on day 240, with values ranging from 0.151 to 0.200 N m (Fig. 7). Elasticity of control cheese decreased from 0.081 N/mm^2 on day 1 down to 0.027 N/mm^2 on day 21 and rose afterwards to 0.226 N/mm^2 on day 240. HPP cheeses had elasticity values close to those of control cheese on day 21, ranging from 0.016 to 0.027 N/mm^2, and higher values on day 240, ranging from 0.299 to 0.411 N/mm^2 (Fig. 7). Fracturability could not be quantified in control cheese until day 240, time at which it attained 2.43 N, while in HPP cheeses, it could be determined on day 180, with values of 12.05–17.20 N, and on day 240, with values of 18.97–24.03 N, markedly higher than those of control cheese (Fig. 7). The three texture parameters studied were at considerably lower levels in 60-day cheeses of the present work than in 50-day HPP and control Hispánico cheeses (Ávila et al. 2006), in which firmness ranged from 0.21 to 0.34 J, elasticity from 0.14 to 0.72 N/mm^2 and fracturability from 13.58 to 29.28 N. The minor differences in texture parameters caused by HPP observed in the present work should not influence consumer acceptance. Rheological properties of Cheddar cheese were not adversely affected by pressure, according to Wick et al. (2004), while significant increases of fracture strain and fracture stress values, but not of firmness, were reported by Rynne et al. (2008). In the present study, firmness was significantly correlated with DM (r=0.950, p<0.001) and pH value (r=0.524, p<0.01) and negatively correlated with α-casein (r=−0.717, p<0.001) and β-casein (r=−0.702, p<0.001), considering control and HPP cheeses from day 21 to day 240. Elasticity was also significantly correlated with DM (r=0.941, p<0.001) and pH value (r=0.518, p<0.01) and negatively correlated with α-casein (r=−0.723, p<0.001) and β-casein (r=−0.691, p<0.001), considering control and HPP cheeses from day 21 to day 240. Fracturability was significantly correlated only with β-casein (r=−0.762, p<0.05), considering control and HPP cheeses from day 180 to day 240.

The texture of cheese at any specific stage of ripening is determined primarily by its pH and the ratio of moisture to intact casein (Lawrence et al. 1987), although other factors such as the fat content also influence cheese textural characteristics (Oliveira et al. 2011). On the one hand, cheese texture tends to soften when the pH rises from 5.0 to 6.0, as proven for Camembert-type cheeses (Vassal et al. 1986). On the other hand, the protein network weakens when caseins are hydrolyzed during ripening, but as each peptide bond in caseins is cleaved the two new ionic groups compete for the available water in the system, which becomes tied up. The lower the moisture to casein ratio, the firmer the cheese protein matrix will be. These facts would explain why texture strengthened in

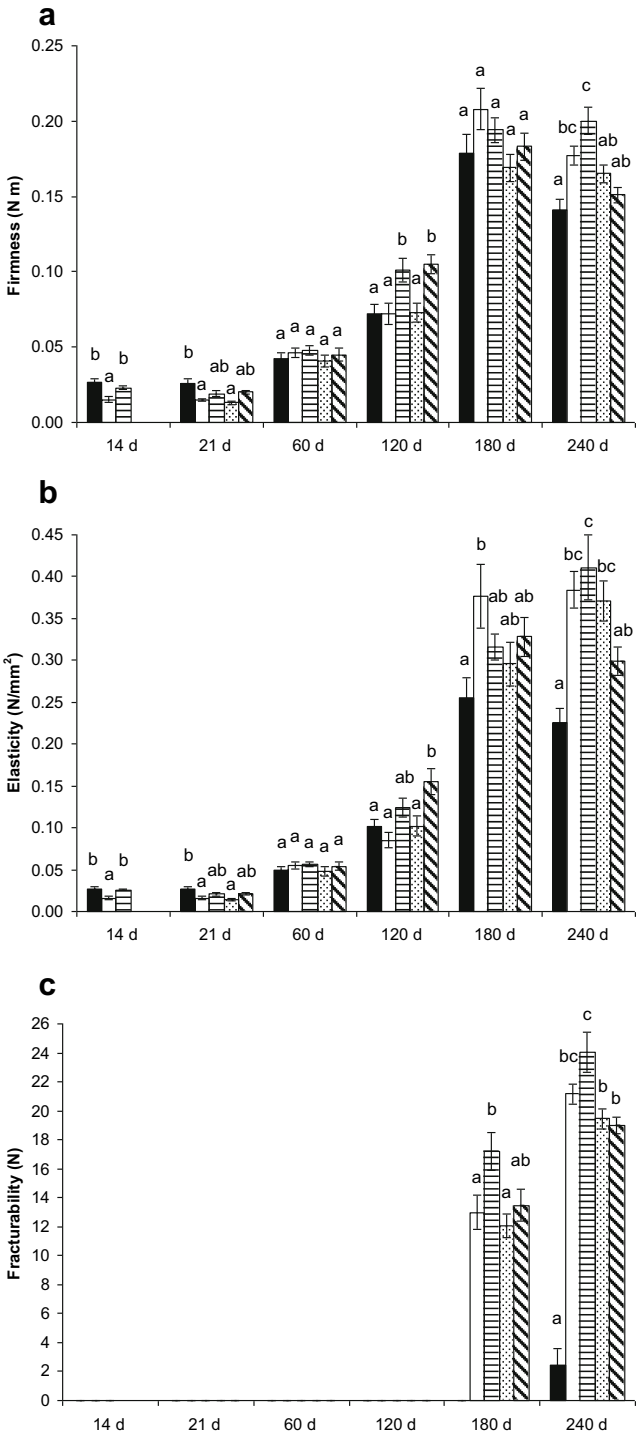


Fig. 7 Texture characteristics (**a** firmness, **b** elasticity, **c**, fracturability) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly (p>0.05)

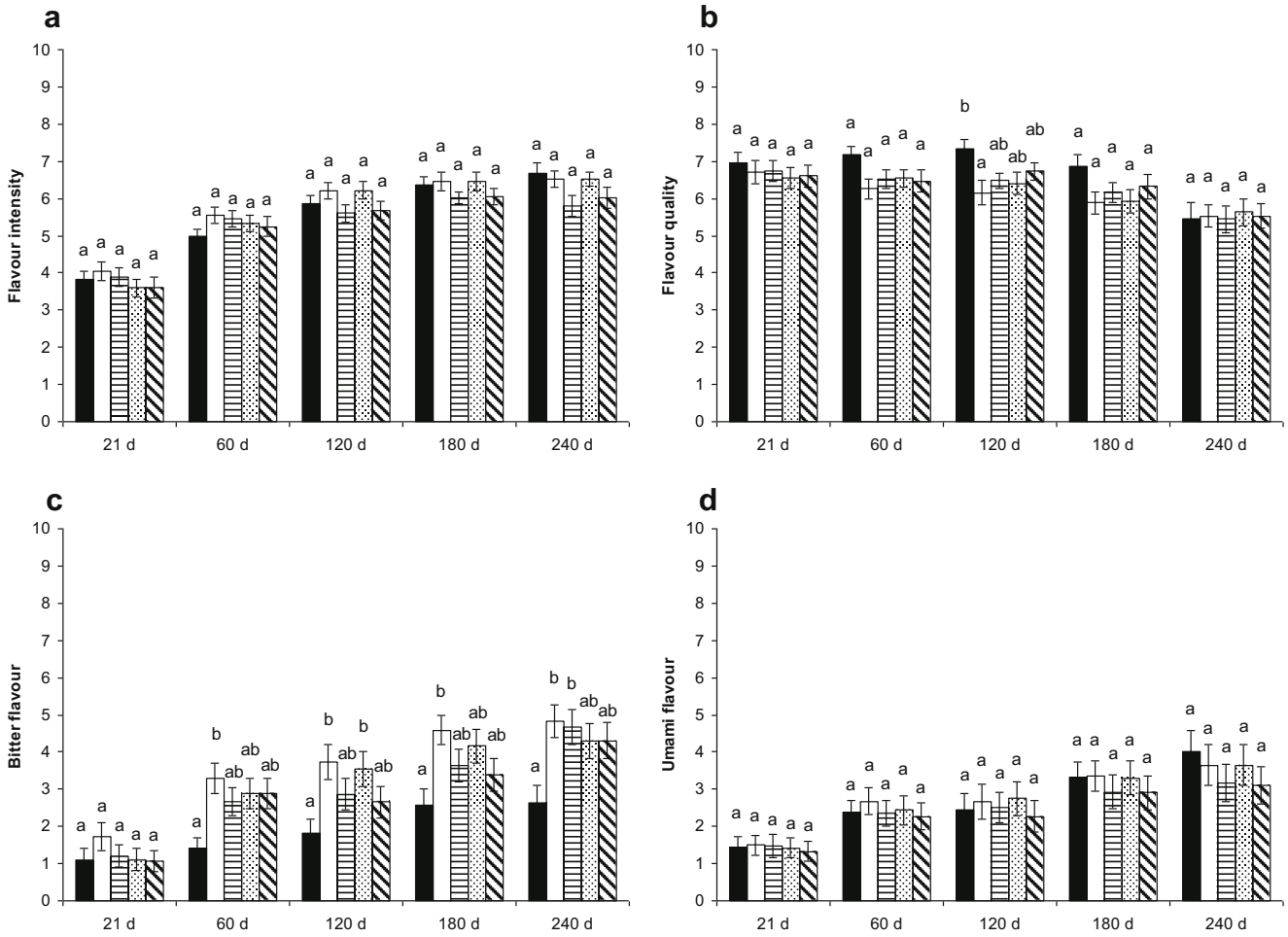


Fig. 8 Sensory characteristics (**a** flavour intensity, **b** flavour quality, **c** bitter flavour, **d** umami flavour) during ripening and refrigerated storage of control cheese (*black*) and cheeses HP-treated at 400 MPa on day 14 (*white*), 600 MPa on day 14 (*horizontally striped*), 400 MPa on day 21 (*dotted*) or 600 MPa on day 21 (*obliquely striped*). Means (bars with SEM) at the same sampling date *with the same letter* do not differ significantly ($p>0.05$)

the present study during cheese ripening, in spite of casein degradation. The firmer texture of HPP cheeses can be associated with the microstructural changes induced by pressurization. HPP cheeses had a more continuous protein matrix than control cheese (O'Reilly et al. 2003) and denser and more compact protein network (Picon et al. 2013b), as shown by confocal scanning laser microscopy.

Sensory Characteristics

Flavour intensity scores increased gradually from day 21 to day 240 in control and HPP cheeses, with no significant differences between cheeses at any stage of ripening (Fig. 8). Flavour quality of control cheese remained fairly unchanged from day 21 to day 180 and then declined from day 180 to day 240 (Fig. 8). Quality scores of HPP cheeses did not differ from those of control cheese throughout ripening, except for 400W2 cheese on day 120 which had a significantly ($p<0.05$) lower quality score than the respective control cheese. Increases in flavour intensity during ripening of control and HPP cheeses in the present study were more marked than those recorded for ovine milk cheeses (Calzada et al. 2014a), while flavour quality of control cheese hardly declined during ripening contrarily to the drastic decline observed for ovine milk cheese, probably caused by the abundance of undesirable microorganisms in the latter cheese (Calzada et al. 2013a) and the excessive proteolysis brought about by the cardoon coagulant (Calzada et al. 2014a). In the present study, acid scores (data not shown) did not vary significantly with HPP or time of ripening. Bitter scores were significantly ($p<0.05$) influenced by HPP and time of ripening, with a gradual increase during ripening in all cheeses and with higher values for HPP cheeses than for control cheese from day 60 onwards (Fig. 8), but the increase in bitterness did not suffice to affect flavour quality. Sweet and salty scores were not influenced by HPP or time of ripening (data not shown). Umami scores increased gradually in control and HPP cheeses from day 21 to day 240, with no significant

differences between cheeses (Fig. 8). HP-treated Cheddar cheese showed significantly less strong flavour than control cheese, and lower pungent, onion-like, salty, acidic, and bitter flavour scores (Rynne et al. 2008).

Significant correlations were found for flavour intensity scores with total FAA (r=0.821, p<0.001) and hydrophilic peptides (r=0.554, p<0.01), umami scores with total FAA (r=0.881, p<0.001) and hydrophilic peptides (r=0.465, p<0.05), and flavour intensity scores with umami scores (r=0.943, p<0.001), considering cheeses from day 21 to day 240. Negative correlations were found for flavour quality scores of those cheeses with total FAA (r=−0.505, p<0.01), hydrophilic peptides (r=−0.648, p<0.001), flavour intensity scores (r=−0.561, p<0.01), umami scores (r=−0.704, p<0.001) and bitterness scores (r=−0.796, p<0.001). Some of these correlations were to be expected since FAA and, at a considerably lesser degree, hydrophilic peptides contribute to the development of cheese flavour and particularly to the umami notes (Sousa et al. 2001). Also, the negative correlation between flavour quality and bitterness has been previously reported (Picon et al. 2013a). The negative correlations of flavour quality with total FAA and hydrophilic peptides cannot be considered as cause-effect relationships but are rather explainable by their opposite evolution during cheese ripening, with a decrease in flavour quality scores and increases in the levels of FAA and hydrophilic peptides. In fact, the correlations of flavour quality scores with total FAA at a particular stage of ripening (i.e. 120, 180 or 240 days) were all of positive sign although non-significant, with r values under 0.30, while the correlations of flavour intensity scores with total FAA at each of those stages of ripening showed r values above 0.75.

Conclusions

HPP of cheese made from unpasteurized cow milk inactivated microorganisms and enzymes. Death rates of microorganisms mostly depended on the pressure level applied, with reductions of lactic acid bacteria counts by 2.0–2.3 log units at 400 MPa and by 5.8–6.0 log units at 600 MPa. Populations of coagulase-positive staphylococci and Gram-negative bacteria were lowered to counts below the detection level in all HPP cheeses. Aminopeptidase inactivation was more dependent on the day cheeses were treated, with activity losses of 68–73 % when HPP was applied on day 14 and 87–89 % on day 21. The hydrolysis of α_s-casein was enhanced in cheeses treated at 400 MPa and that of β-, κ- and para-κ-caseins in 400 and 600 MPa cheeses. Concomitantly, a more pronounced increase in γ-caseins, hydrophilic peptides and hydrophobic peptides was recorded for all the HPP cheeses than for control cheese. However, the formation of FAA was retarded in HPP cheeses, with up to 64 % less total FAA in 600 MPa cheeses than in control cheese on day 240. Total biogenic amines were lowered even more, by up to 94 % in 600 MPa cheeses on day 240. A firmer texture, determined by instrumental methods, was recorded for HPP cheeses than for control cheese at the end of the storage period. In spite of the higher bitterness scores recorded for HPP cheeses from day 60 onwards, flavour intensity and flavour quality were not significantly influenced by treatments. Minor changes in cheese texture and flavour caused by HPP should not affect consumer acceptance. HPP is a feasible procedure to improve the safety of cheese made from unpasteurized cow milk, due to its favourable effect on the reduction of biogenic amines without compromising sensory characteristics during a prolonged ripening and storage period.

Acknowledgments This work was supported by project AGL2009-07801 from the Spanish Ministry of Science and Innovation (MICINN). J. Calzada was the recipient of a MICINN fellowship. The authors thank the valuable help of A. Vence with the manufacture and supply of cheeses and of Hiperbaric (Burgos, Spain) with high-pressure treatments.

References

Arqués, J. L., Rodriguez, E., Gaya, P., Medina, M., & Nuñez, M. (2005). Effect of combinations of high-pressure treatment and bacteriocin-producing lactic acid bacteria on the survival of *Listeria monocytogenes* in raw milk cheese. *International Dairy Journal, 15*, 893–900.

Ávila, M., Garde, S., Gaya, P., Medina, M., & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy Science, 89*, 2882–2893.

Calzada, J., del Olmo, A., Picón, A., Gaya, P., & Nuñez, M. (2013a). Reducing biogenic-amine-producing bacteria, decarboxylase activity, and biogenic amines in raw milk cheese by high-pressure treatments. *Applied and Environmental Microbiology, 79*, 1277–1283.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013b). Proteolysis and biogenic amine buildup in high-pressure treated ovine milk blue-veined cheese. *Journal of Dairy Science, 96*, 4816–4829.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014a). Using high-pressure processing for reduction of proteolysis and prevention of over-ripening of raw milk cheese. *Food and Bioprocess Technology, 7*, 1404–1413.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014b). High-pressure processing for the control of lipolysis, volatile compounds and off-odours in raw milk cheese. *Food and Bioprocess Technology, 7*, 2207–2217.

Curtin, A. C., & McSweeney, P. L. H. (2003). Catabolism of aromatic amino acids in cheese-related bacteria: aminotransferase and decarboxylase activities. *Journal of Dairy Research, 70*, 249–252.

Fernández-García, E., Tomillo, J., & Nuñez, M. (1999). Effect of added proteinases and level of starter culture on the formation of biogenic amines in raw milk Manchego cheese. *International Journal of Food Microbiology, 52*, 189–196.

García-Risco, M. R., Olano, A., Ramos, M., & López-Fandiño, R. (2000). Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *Journal of Dairy Science, 83*, 2184–2189.

Garde, S., Arqués, J. L., Gaya, P., Medina, M., & Nuñez, M. (2007). Effect of high-pressure treatments on proteolysis and texture of ewes' raw milk La Serena cheese. *International Dairy Journal, 17*, 1424–1433.

Garde, S., Tomillo, J., Gaya, P., Medina, M., & Nuñez, M. (2002). Proteolysis in Hispánico cheese manufactured using a mesophilic starter, a thermophilic starter and bacteriocin-producing *Lactococcus lactis* subsp. *lactis* INIA 415 adjunct culture. *Journal of Agricultural and Food Chemistry, 50*, 3479–3485.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71*, 496–499.

Joosten, H. M. L. J., & Northolt, M. D. (1987). Conditions allowing the formation of biogenic-amines in cheese. 2. Decarboxylase properties of some nonstarter bacteria. *Netherlands Milk and Dairy Journal, 41*, 259–280.

Juan, B., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. J. (2007). Effects of high pressure on proteolytic enzymes in cheese: relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science, 90*, 2113–2125.

Krause, I., Bockhardt, A., Neckermann, H., Henle, T., & Klostermeyer, H. (1995). Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. *Journal of Chromatography A, 715*, 67–79.

Ladero, V., Fernández, M., Cuesta, I., & Alvarez, M. A. (2010). Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. *Food Microbiology, 27*, 933–939.

Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martín, M. C., & Alvarez, M. A. (2012). Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiology, 30*, 132–138.

Lawrence, R. C., Creamer, L. K., & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science, 70*, 1748–1760.

Leuschner, R. G., Heidel, M., & Hammes, W. P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. *International Journal of Food Microbiology, 39*, 1–10.

Linares, D., Martín, M. C., Ladero, V., Alvarez, M. A., & Fernández, M. (2011). Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition, 51*, 691–703.

Malone, A. S., Wick, C., Shellhammer, T. H., & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science, 86*, 1139–1146.

Messens, W., Estepar-Garcia, J., Dewettinck, K., & Huyghebaert, A. (1999). Proteolysis of high-pressure-treated gouda cheese. *International Dairy Journal, 9*, 775–782.

Norton, T., & Sun, D.-W. (2008). Recent advances in the use of high pressure as an effective processing technique in the food industry. *Food and Bioprocess Technology, 1*, 2–34.

Oliveira, N. M., Dourado, F. Q., Peres, A. M., Silva, M. V., Maia, J. M., & Teixeira, J. A. (2011). Effect of guar gum on the physicochemical, thermal, rheological and textural properties of green edam cheese. *Food and Bioprocess Technology, 4*, 1414–1421.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L., & Beresford, T. P. (2002). Effects of high-pressure treatment on viability and autolysis of starter bacteria and proteolysis in cheddar cheese. *International Dairy Journal, 12*, 915–922.

O'Reilly, C. E., Kelly, A. L., Oliveira, J. C., Murphy, P. M., Auty, M. A. E., & Beresford, T. P. (2003). Effect of varying high-pressure treatment conditions on acceleration of ripening of cheddar cheese. *Innovative Food Science and Emerging Technologies, 4*, 277–284.

Picon, A., Alonso, R., Gaya, P., & Nuñez, M. (2013a). High-pressure treatment and freezing of raw goat milk curd for cheese manufacture: effects on cheese characteristics. *Food and Bioprocess Technology, 6*, 2820–2830.

Picon, A., Alonso, R., van Wely, K. H. M., & Nuñez, M. (2013b). Microstructural, textural and colour characteristics during ripening of Hispánico cheese made using high-pressure-treated ovine milk curd. *Food and Bioprocess Technology, 6*, 3056–3067.

Pircher, A., Bauer, F., & Paulsen, P. (2007). Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. *European Food Research and Technology, 226*, 225–231.

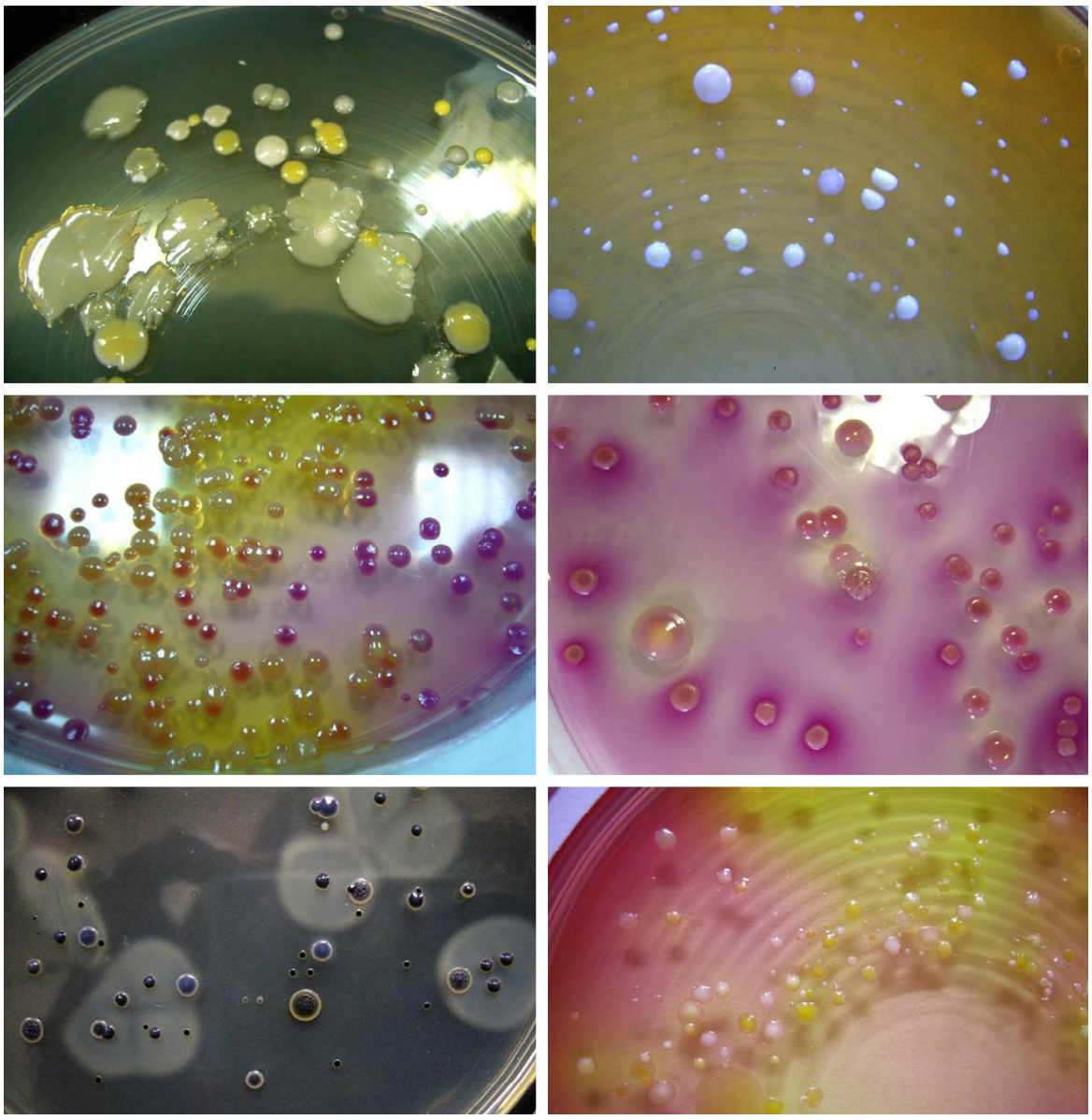
Rynne, N. M., Beresford, T. P., Guinee, T. P., Sheehan, E., Delahunty, C. M., & Kelly, A. L. (2008). Effect of high-pressure treatment of 1 day-old full-fat Cheddar cheese on subsequent quality and ripening. *Innovative Food Science and Emerging Technologies, 9*, 429–440.

Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal, 11*, 327–345.

Vassal, L., Monnet, V., Le Bars, D., Roux, C., & Gripon, J. C. (1986). Relation entre le pH, la composition chimique et la texture des fromages de type camembert. *Le Lait, 66*, 341–351.

Wick, C., Nienaber, U., Anggraeni, O., Shellhammer, T. H., & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial cheddar cheeses treated with high pressure. *Journal of Dairy Research, 71*, 107–115.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11*, 185–201.



Capítulo 10.

Effect of high-pressure-processing on the lipolysis, volatile compounds, odour and colour of cheese made from unpasteurized milk.

Fotografía: colonias de bacterias aerobias totales en agar PC (superior, izquierda), bacterias lácticas en agar MRS (superior, derecha), bacterias Gram negativas en agar MacConkey (centro, izquierda), coliformes y enterobacterias lactosa negativo en agar VRB (centro, derecha), estafilococos coagulasa positivo y negativo en agar BP (inferior, izquierda), y *Micrococcaceae* en agar MS (inferior, derecha), obtenidas a partir de siembras de queso Arzúa-Ulloa.

Food and Bioprocess Technology: An International Journal

Effect of High-Pressure-Processing on the Lipolysis, Volatile Compounds, Odour and Colour of Cheese Made from Unpasteurized Milk

--Manuscript Draft--

Manuscript Number:	
Full Title:	Effect of High-Pressure-Processing on the Lipolysis, Volatile Compounds, Odour and Colour of Cheese Made from Unpasteurized Milk
Article Type:	Original Research
Keywords:	High-pressure-processing; Lipolysis; Volatile compounds; Odour; Cheese
Corresponding Author:	Manuel Nunez INIA Madrid, SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	INIA
Corresponding Author's Secondary Institution:	
First Author:	Javier Calzada, MSc
First Author Secondary Information:	
Order of Authors:	Javier Calzada, MSc
	del Olmo Ana, PhD
	Antonia Picon, PhD
	Manuel Nunez
Order of Authors Secondary Information:	
Abstract:	Cheeses made from unpasteurized milk were high-pressure-processed (HPP) 14 or 21 days after manufacture at 400 or 600 MPa, in order to prevent excessive accumulation of flavour compounds, in particular free fatty acids (FFA) and volatile compounds (VC). They were compared with untreated control cheese throughout a 240-day period. On day 60, cheeses treated at 400 MPa had esterase activity values 57.1-58.0% lower than control cheese while cheeses treated at 600 MPa had values 82.3-82.8% lower, with no significant differences between total FFA concentrations of cheeses. However, on day 240 total FFA concentrations were 6.5-9.0% lower in 400 MPa cheeses than in control cheese, and 15.0-16.9% lower in 600 MPa cheeses, with more marked differences for short-chain FFA and less marked for hexadecanoic and octadecanoic acids. Fifty-two of the 70 VC found in cheese were significantly influenced by HPP. On day 240, total alcohols reached the highest levels in control and 400 MPa cheeses, total aldehydes, ketones and hydrocarbons in 600 MPa cheeses, and total sulphur compounds in 400 MPa and 600 MPa cheeses. Levels of total volatile acids, esters, ethers, benzenic compounds and terpenoids in 240-day cheese were not affected by HPP. Odour quality and intensity were not significantly influenced by HPP. Higher L* values were recorded for 600 MPa cheeses, and higher a* values for control and 400 MPa cheeses. Principal component analysis on the groups of volatile compounds highlighted the different evolvement of volatiles in control, 400 MPa and 600 MPa cheeses during ripening.

Effect of High-Pressure-Processing on the Lipolysis, Volatile Compounds, Odour and Colour of Cheese Made from Unpasteurized Milk

Javier Calzada · Ana del Olmo · Antonia Picon · Manuel Nuñez

Departamento de Tecnología de Alimentos, INIA, Carretera de La Coruña Km 7, Madrid, 28040

Spain

Short running head: Lipolysis and volatiles in HPP cheese

Corresponding author:

M. Nuñez. Phone # 34-913476799; Fax # 34-913572293. E-mail: nunez@inia.es

Abstract Cheeses made from unpasteurized milk were high-pressure-processed (HPP) 14 or 21 days after manufacture at 400 or 600 MPa, in order to prevent excessive accumulation of flavour compounds, in particular free fatty acids (FFA) and volatile compounds (VC). They were compared with untreated control cheese throughout a 240-day period. On day 60, cheeses treated at 400 MPa had esterase activity values 57.1-58.0% lower than control cheese while cheeses treated at 600 MPa had values 82.3-82.8% lower, with no significant differences between total FFA concentrations of cheeses. However, on day 240 total FFA concentrations were 6.5-9.0% lower in 400 MPa cheeses than in control cheese, and 15.0-16.9% lower in 600 MPa cheeses, with more marked differences for short-chain FFA and less marked for hexadecanoic and octadecanoic acids. Fifty-two of the 70 VC found in cheese were significantly influenced by HPP. On day 240, total alcohols reached the highest levels in control and 400 MPa cheeses, total aldehydes, ketones and hydrocarbons in 600 MPa cheeses, and total sulphur compounds in 400 MPa and 600 MPa cheeses. Levels of total volatile acids, esters, ethers, benzenic compounds and terpenoids in 240-day cheese were not affected by HPP. Odour quality and intensity were not significantly influenced by HPP. Higher L* values were recorded for 600 MPa cheeses, and higher a* values for control and 400 MPa cheeses. Principal component analysis on the groups of volatile compounds highlighted the different evolvement of volatiles in control, 400 MPa and 600 MPa cheeses during ripening.

Keywords High-pressure-processing · Lipolysis · Volatile compounds · Odour · Cheese

Introduction

During the manufacture and ripening of cheese, a myriad of compounds responsible for cheese flavour are formed by the microorganisms and enzymes involved in glycolysis, lipolysis, proteolysis and the subsequent secondary reactions (McSweeney and Sousa 2000; Yvon and Rijnen 2001; Collins et al. 2003). The numerous products resulting from carbohydrate metabolism, triglyceride hydrolysis and protein breakdown are further degraded, giving rise to non-volatile flavour compounds and volatile potent odorants responsible for the typical flavour, aroma and odour notes of each cheese variety. More than 600 volatile compounds have been identified in different cheese varieties and many of those compounds have been related to particular odour and aroma notes (Curioni and Bosset 2002).

Besides the coagulant enzymes and starter cultures used as a rule in cheese manufacture, the enzymes and microorganisms coming from milk are main agents in the ripening process of cheeses made from unpasteurized milk (Fernández-García et al. 2002; Gaya et al. 2005). Long ripening and refrigerated storage periods are characteristic of some cheese varieties, forced by regulatory issues or market conditions. The action of enzymes and microorganisms, which persists even at refrigeration temperatures, may result in cheese over-ripening before consumption. Unpasteurized milk cheeses are more prone to over-ripening, because of their more marked enzyme activity and their complex microbial metabolic reactions. Although freezing of ripe cheese considerably retards biochemical changes and maintains flavour characteristics, the texture and the visual appearance of cheese may become negatively affected (Tejada et al. 2000; Van Hekken et al. 2005).

High-pressure-processing (HPP) meets the consumer demand for minimally processed fresher-tasting foods, since its effect on flavour characteristics is negligible (Norton and Sun 2008). HPP reduces the populations of pathogenic and spoilage microorganisms in milk and cheese (O'Reilly et al. 2000; Shao and Ramaswamy 2011) and inactivates proteinases, peptidases and esterases present

in cheese when sufficiently high pressure levels are applied (Malone et al. 2003; Huppertz et al. 2004; Juan et al. 2007; Ávila et al. 2007). Free fatty acids (FFA) and volatile compounds (VC) of cheese are also affected by HPP (Ávila et al. 2006; Ávila et al. 2007).

The application of HPP to prevent the excessive accumulation of flavour compounds in cheese made from unpasteurized milk seems therefore a feasible strategy, on the basis of the results of previous studies. The effects of HPP are dependent on the pressure level applied and the length of treatment. In the particular case of cheese, the stage of ripening at which HPP is applied is also a crucial parameter for the retention of sensory characteristics. Garde et al. (2007) reported the lowest flavour quality scores for ripe cheese treated at 400 MPa on day 2 after manufacture while HPP did not impair the sensory characteristics of cheese treated at 400 MPa on day 50.

In a previous study, the proteolysis of unpasteurized milk cheese was decelerated and the levels of biogenic amines lowered by applying HPP at 400 or 600 MPa, on days 14 and 21 after manufacture (Calzada et al. 2014b). The objective of the present work was to investigate the suitability of HPP at 400 or 600 MPa, applied on days 14 or 21 after manufacture, in order to control the excessive build-up of flavour compounds in unpasteurized milk cheese. Lipolysis, volatile compounds, odour and colour of HPP-treated cheeses were compared with those of untreated control cheese throughout a 240-day period.

Materials and Methods

Cheese Manufacture and High-pressure-processing

Cheese was manufactured from 750 L of unpasteurized cow milk in each of two trials, carried out on consecutive days as previously described (Calzada et al. 2014b). Cheeses (1.15 kg average weight out of the press) were ripened at 8 ºC and 72% RH until day 60 and at 5 ºC and 75% RH

afterwards. Part of the cheeses, coded as 400W2, 600W2, 400W3 and 600W3, were HPP-treated at 400 or 600 MPa after ripening for 14 or 21 days, respectively, as previously described (Calzada et al. 2014b). After treatments, HPP cheeses were unpackaged and ripened under the same conditions of control cheese.

From each of the two cheese-making trials, one cheese was analyzed on day 1, three cheeses (control, 400W2, 600W2) on day 14 and five cheeses (control, 400W2, 600W2, 400W3, 600W3) on each of the sampling dates day 21, day 60, day 120, day 180 and day 240. At each of these sampling dates, two 100 g pieces per cheese were wrapped in aluminum foil, vacuum-packaged, and frozen at -40 ºC until chemical analyses.

Enzymatic and Chemical Determinations

Esterase activity was determined in duplicate as described by Ávila et al. (2007), with some modifications. Ten grams of cheese were homogenized with 20 mL of 0.1 M, pH 7.0 phosphate buffer in an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany), centrifuged at 10,000 g for 20 min at 4 ºC and filtered through Whatman No. 2 paper (Whatman Int. Ltd., Maidstone, UK). The chromogenic substrate was α-naphthylbutyrate (Sigma-Aldrich, Steinhem, Germany). The assay mixture (30 μL of chromogenic substrate, 600 μL of distilled water, 100, 200 or 400 μL of cheese homogenate and 0.1 M, pH 7.5 phosphate buffer up to a final volume of 1230 μL) was incubated for 1 h at 37 ºC in a water bath and centrifuged at 12,000 g for 5 min at room temperature. Finally, 900 μL of supernatant were mixed with 150 μL of 2.7 mg/mL Fast Red TR salt (Sigma-Aldrich) aqueous solution. After 5 min at room temperature, the absorbance was measured at 537 nm using a DU650 spectrophotometer (Beckman Coulter Inc., Brea, CA). Esterase activity was calculated from absorbance values in the range of 0.1 to 0.9 by means of a α-naphthol

standard curve. One unit of enzymatic activity was defined as the amount of enzyme that liberated 1 pmol of α-naphthol per min and g of cheese at 37 ºC and pH 7.5.

Free fatty acids from butyric acid ($C_{4:0}$) to linolenic acid ($C_{18:3}$), together with acetic, propionic and benzoic acids, were determined in duplicate by gas chromatography (GC) as described by Fernández-García et al. (2006). Prior to analysis, frozen cheese pieces were thawed overnight at 4 ºC. A solid-phase extraction technique was used for the extraction of carboxylic acids from cheese, with pentanoic, nonanoic and heptadecanoic acids added as internal standards. An HP 6890 gas chromatograph (Agilent Technologies, Las Rozas, Spain) equipped with an automatic sampler (HP 7683), a split/splitless injector, a FFAP column (Agilent Technologies, 30 m x 0.32 mm i.d. x 0.25 μm film thickness) and a flame ionization detector, was used for the analysis. Injection (1 μL of sample) was performed in split mode at 1:20 split ratio, at 260 ºC. Helium was the carrier gas, with the flow set for maintaining a constant pressure of 0.80 kg/cm^2. For chromatographic separation the temperature was increased from 65 to 240 ºC, at a rate of 10 ºC/min, and held at 240 ºC for 12.5 min. Standard solutions of carboxylic acids were used for the calculation of calibration curves. Individual acids were separated, identified and quantified, and their concentrations expressed in mg per gram of cheese dry matter (DM).

Volatile compounds were determined in triplicate by gas chromatography-mass spectrometry (GC-MS) as described by Calzada et al. (2014a). They were extracted from cheese using a solid-phase microextraction method (SPME). Ten grams of cheese were homogenized with 20 g of Na_2SO_4 and 25μL of an aqueous solution of 1,058 mg/L cyclohexanone in a mechanical grinder. Five grams of the mixture were weighed in a 15 mL headspace glass vial sealed with a PTFE faced silicone septum (Supelco, Bellefonte, PA, USA) and the vials were submerged in a thermostatic bath at 30 °C. A SPME manual holder equipped with a 2 cm x 50/30 μm StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fibre (Supelco) was inserted through the PTFE septum for headspace extraction, after which it was inserted into the GC

injection port for desorption (270 °C/10 min in splitless mode). An HP 6890-MSD HP 5973 apparatus (Agilent, Palo Alto, CA, USA) with a capillary column (60 m long; 0.25 mm i.d.; 0.5 μm film thickness; Innowax, Agilent Technologies) was used for GC-MS, with helium flow at 1.4 mL/min for 1 min followed by 1 mL/min. The temperature program was 7 min at 40 ºC, first ramp 2 ºC/min to 90 ºC, second ramp 3 ºC/min to 150 ºC, final ramp 9 ºC/min to 240 ºC, and 8 min at 240 ºC. Detection was performed with electron impact ionization, with 70 eV ionization energy operating in the full-scan mode at 1.74 scans/s. Source and quadrupole temperatures were 230 and 150 ºC, respectively. Compound identification was carried out by injection of commercial standards and by spectra comparison using the Wiley7Nist05 Library (Wiley & Sons Inc., Germany). The sum of abundances of characteristic ions was used for the semi-quantitation of compounds. Relative abundances of compounds were calculated by dividing the peak area of each compounds by the peak area of the internal standard and multiplying the quotient by 10^2.

Odour Evaluation

Seventeen trained panellists carried out the evaluation of odour intensity and quality (preference) of 21-, 60-, 120- and 240-day cheeses, scoring on a 0- to 10-point scale. Five cheeses per session (one control and four HPP cheeses, manufactured on the same day), coded with 3-digit numbers, were randomly presented to panellists. Odour was defined as the olfactory sensation felt directly by smelling the cheese (Fernández-García et al. 2002). In addition, panellists were asked to evaluate putrid, rancid and acid odour notes, also scoring on a 0- to 10-point scale.

Colour Determination

Colour parameters in the CIE-LAB colour space L* (lightness), a* (redness) and b* (yellowness) were determined at six points of the cheese interior, as previously described (Picon et al. 2013). A CM-2600d spectrocolorimeter equipped with a SpectraMagic 3.6 software (Minolta Camera Co., Osaka, Japan), with iluminant D65 (standard daylight) and a 10º observer angle, with specular component included (SCI), was used.

Statistical Analysis

Two-way analyses of variance (ANOVA) were carried out on the levels of FFA and VC, with HPP treatment and time after manufacture as main effects. Means were compared using Tukey's test, with p assigned at 0.05. Principal component analyses (PCA) were carried out on individual FFA concentrations, total levels of VC groups and individual VC levels, for the discrimination of samples according to HPP treatment and time after manufacture. The SPSS Win 14.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of data.

Results and Discussion

Acetic, Propionic and Benzoic Acids

The concentrations of acetic, propionic and benzoic acids in unpasteurized milk cheese were all significantly (p<0.001) influenced by HPP treatment and time after manufacture, according to the ANOVA. Acetic acid increased steadily from 0.707 mg/g DM on day 1 (data not shown) to a maximum level of 2.102 mg/g DM in 240-day control cheese (Table 1). It is produced by lactic acid bacteria and other microorganisms coming from raw milk, principally through the metabolism of lactose, lactate and citrate. HPP influenced negatively the formation of acetic acid, in particular

when 600 MPa were applied to cheese. Thus, the concentrations of acetic acid on day 240 were 35.9-39.5% lower in 400 MPa cheeses, and 56.4-56.6% lower in 600 MPa cheeses, than in control cheese (Table 1). Acetic acid, with a typical vinegar odour note, is a major odorant of cheese varieties such as Cheddar, Gruyère and Emmental (Curioni and Bosset 2002). It contributes to cheese flavour and aroma by itself and by forming esters with fruity notes.

Propionic acid followed a different accumulation pattern. It sharply increased from 0.031 mg/g DM on day 1 (data not shown) to a maximum level of 0.395 mg/g DM in 14-day control cheese (Table 1). Thereafter, its concentration generally declined, with slight differences between HPP and control cheeses. Propionic acid, which also shows a vinegar odour note, mostly derives from the microbial metabolism of lactate. It is found in Swiss-type cheeses, Camembert and some ewe milk cheeses (Molimard and Spinnler 1996; Fernández-García et al. 2004). Similarly to acetic acid, propionic acid has a direct role in cheese odour and aroma and also contributes to ester formation.

Benzoic acid increased from 0.027 mg/g DM on day 1 (data not shown) to 0.234 mg/g DM on day 14 (Table 1), similarly to propionic acid. Following this initial increase, its accumulation persisted in control cheese, in which it reached 0.398 mg/g DM on day 180, and in most of the HPP cheeses (Table 1). Concentrations found from day 14 onwards (Table 1) clearly exceeded the maximum concentrations of benzoic acid reported for pasteurized cow milk cheeses (0.0245 mg/g) or raw cow milk cheeses (0.0287 mg/g) by Iammarino et al. (2011), although up to 0.137 mg/g were found in Vacherin cheeses from different manufacturers (Sieber et al. 1995). According to these authors, benzoic acid present in cheese may derive from the microbial enzymatic conversion of hippuric acid, from phenylalanine breakdown or from benzaldehyde auto-oxidation.

Branched-chain carboxylic acids such as 2-methylpropanoic, 2-methylbutanoic and 3-methylbutanoic acids, respectively derived from the catabolism of valine, isoleucine and leucine (Yvon and Rijnen 2001), were not detected in any of the analyzed samples of control and HPP cheeses, probably due to the absence or low levels of bacteria with the required enzymatic activity.

Esterase Activity and Free Fatty Acids

Esterase activity of control cheese increased from 4.63 pmol α-naphthol min^{-1} g^{-1} on day 1 to 10.75 pmol α-naphthol min^{-1} g^{-1} on day 120 (Table 2), a result which can be associated with the lysis of starter lactic acid bacteria during ripening followed by the release of their intracellular esterases to the medium. HPP was responsible for a decline in esterase activity immediately after treatments, by 30.0, 49.1, 45.4 and 70.8% in 400W2, 600W2, 400W3 and 600W3 cheeses, respectively (Table 2). The more marked declines in esterase activity recorded for 600W2 than for 400W2 cheese and for 600W3 than for 400W3 cheese can be ascribed to the effect of the higher pressure level applied. The more marked declines in 400W3 than in 400W2 cheese and in 600W3 than in 600W2 cheese may be explained by the fact that a higher proportion of intracellular esterase from lactic acid bacteria and other microorganisms had been spontaneously released outside the cells on day 21 than on day 14, as shown by the increase in the esterase activity values of control cheese. Enzymes outside the bacterial cells would presumably be more susceptible to inactivation by HPP than in the interior of the cells. During the rest of the ripening period, a gradual decrease in esterase activity values occurred in all the HPP cheeses, in particular in those treated at 600 MPa (Table 2).

The concentration of total short-chain (SC, $C_{4:0}$ to $C_{8:0}$) FFA increased in control cheese from 0.017 mg/g DM on day 1 (data not shown) to 0.190 mg/g DM on day 240 (Table 3). SC FFA, mostly produced through the esterase-mediated hydrolysis of triacylglycerides, also derive from the fermentation of lactose and lactate, from the degradation of amino acids and from the oxidation of some ketones, esters and aldehydes (Collins et al. 2003). Concentrations of SC FFA were significantly (p<0.05) lower in 400 MPa cheeses than in control cheese only on day 240 while in 600 MPa cheeses they were lower from day 60 onwards. In the present work, butanoic acid was the major SC FFA throughout ripening in control and HPP cheeses, followed by hexanoic and octanoic acids, all three with the same accumulation pattern as total SC FFA. Butanoic acid has a rancid

cheese-like odour and is a key odorant of many cheese varieties although at high levels, usually due to butyric fermentation, may cause flavour defects. Hexanoic and octanoic acids are characteristic flavour compounds of hard cheese varieties such as Grana Padano and Roncal (Curioni and Bosset 2002).

The concentration of total medium chain (MC, $C_{10:0}$ to $C_{14:0}$) FFA increased from 0.178 mg/g DM on day 1 (data not shown) to 0.400 mg/g DM on day 240 (Table 3), just a 2.25-fold increase compared to the 11.24-fold increase of SC FFA during the same period. HPP had a lower influence on MC FFA than on SC FFA. Significant (p<0.05) differences between cheeses were not recorded until day 240, when control cheese showed the highest MC FFA levels. Similar results on the non-significant effect of cheese HPP on total MC FFA concentrations were reported for goat milk cheese (Delgado et al. 2012). MC FFA have relatively low perception thresholds, what makes them important contributors to the aroma of cheese varieties such as Camembert, Cheddar, Grana Padano and Roncal (Curioni and Bosset 2002).

The concentration of total long chain (LC, $C_{16:0}$ to $C_{18:3}$) FFA increased from 0.669 mg/g DM on day 1 (data not shown) to 1.307 mg/g DM on day 240 (Table 3). This 1.95-fold increase was even lower than the increase of MC FFA during the same period. As in the case of MC FFA, significant (p<0.05) differences between cheeses were not recorded until day 240, time at which control cheese had higher LC FFA levels. Formation of LC FFA in control and HPP cheeses can be ascribed to the action of milk lipoprotein lipase, which does not seem to lose activity in cheeses made from HPP-treated goat milk (Buffa et al. 2001) and in cheese made from goat raw milk HPP-treated at 400 or 600 MPa (Delgado et al. 2012), as proven by the unaltered accumulation of LC FFA during ripening. LC FFA usually reach high concentrations in many cheese varieties, particularly in blue-veined cheeses in which the levels frequently exceed 30 mg/g (Calzada et al. 2013), but their contribution to cheese aroma is lowered by the high perception thresholds (Curioni and Bosset 2002).

Volatile Compounds

Seventy individual VC were found in control and HPP cheeses analyzed by SPME followed by GC-MS. According to the analysis of variance, 52 VC were significantly (p<0.05) influenced by the HPP treatment and 63 VC by the time elapsed after manufacture. The 70 VC were grouped into 19 alcohols, 9 esters, 9 hydrocarbons, 7 ketones, 6 acids, 6 ethers, 4 aldehydes, 3 benzenic compounds, 2 terpenoids, 2 sulphur compounds and 3 miscellaneous compounds. During early ripening of control cheese, ketones, acids and alcohols were the predominant VC groups, accounting for 52.6%, 25.6% and 18.3%, respectively, of the overall abundance of VC (pooled data from control cheese on days 14 and 21), followed by benzenic compounds with 1.16% and esters with 1.12%, while ethers, terpenoids, hydrocarbons, aldehydes and sulphur compounds accounted for 0.48%, 0.30%, 0.20%, 0.12% and 0.11%, respectively, of the overall abundance (Table 4). The proportions of VC groups varied with HPP treatment and time of ripening (Tables 5 and 6). During late ripening the predominant VC groups were ketones, alcohols and acids, accounting for 38.6%, 31.1% and 25.4% of the overall abundance, respectively (pooled data of control and HPP cheeses on days 60, 120 and 240), followed by benzenic compounds with 1.68% and esters with 1.13%, while ethers, terpenoids, hydrocarbons, aldehydes and sulphur compounds accounted for 0.89%, 0.58%, 0.42%, 0.16% and 0.06% of the overall abundance, respectively. It must be taken into account that SPME is more effective in extracting VC of medium and high boiling point in comparison with the purge and trap method which extracts better the VC of low boiling point (Mallia et al. 2005). According to these authors, there are exceptions such as acetaldehyde which is better extracted by SPME, in spite of its low boiling point.

Total ketones of control cheese reached their maximum concentration on day 14 and gradually declined afterwards (Tables 4 and 5). In contrast, total ketones of HPP cheeses increased markedly

from day 21 to day 60, time at which they reached significantly (p<0.05) higher levels than in control cheese. Afterwards, their levels fell sharply in 400 MPa cheeses while they remained in 600 MPa cheeses at significantly (p<0.05) higher levels than in control cheese (Table 5). Total ketones represented only 16.5% of the overall abundance of VC in control cheeses while they accounted for 44.5% of the overall abundance in HPP cheeses (pooled data of cheeses on days 60, 120 and 240). The main ketones (data not shown) were 3-hydroxy-2-butanone (acetoin), 2-butanone, and 2,3-butanedione (diacetyl), which accounted for 67.7%, 22.4% and 7.0% of the overall abundance of total ketones, respectively. 3-Hydroxy-2-butanone has a sour milk odour note, 2,3-butanedione a buttery odour note and 2-butanone a butterscotch odour note (Curioni and Bosset 2002). The accumulation pattern of 3-hydroxy-2-butanone and 2,3-butanedione in control and HPP cheeses was similar to that of total ketones. The pattern of 2-butanone differed, with a strong increase in control and 400 MPA cheeses from day 14 to day 21 which continued until day 120, and a less marked increase in 600 MPa cheeses which ceased on day 60. Microbial metabolism of lactose, the main agent responsible for the formation of 2,3-butanedione and 3-hydroxy-2-butanone (Fox and Wallace 1997), is responsible for the accumulation of these ketones until day 14 in control cheese but does not explain the increase in the levels of these ketones in HPP cheeses afterwards, once lactose is exhausted. The increase in 2-butanone levels from day 14 to day 60 can be ascribed to the microbial reduction of 2,3-butanedione and 3-hydroxy-2-butanone. Minor ketones were 2-propanone, with wood pulp and hay notes, 2-heptanone, with animal and blue cheese notes, and 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone, with herbaceous and truffle notes (Curioni and Bosset 2002).

Total volatile acids of control cheese increased until day 21, then kept stable until day 120 and afterwards declined (Tables 4 and 5). In 400 MPa cheeses their increase persisted until day 60 and declined afterwards, while in 600 MPa cheeses their accumulation pattern was similar to that of control cheese, although differences between HPP and control cheeses were not significant. In fact,

total acids accounted for 21.1% of the overall abundance of VC in control cheeses and 26.5% in HPP cheeses (pooled data of days 60, 120 and 240). The major volatile acids (data not shown) were ethanoic, propanoic and butanoic, accounting for 62.7%, 25.8% and 11.2%, respectively, of the overall abundance of this chemical group. The first two acids show vinegar odour notes, and the latter a rancid cheesy note, all being mostly formed through microbial metabolism. Ethanoic and propanoic acids increased in control cheese until day 21, remained fairly constant until day 120 and then declined, probably due to ester formation, while butanoic acid increased until day 120 and did not vary afterwards, what can be associated with the different mechanisms of formation (Yvon and Rijnen 2001; Collins et al. 2003). Branched-chain carboxylic acids were not detected by SPME followed by GC-MS, what can be ascribed to the absence or the low levels of bacteria with the ability to catabolise branched-chain amino acids. Minor volatile acids, at levels 100 times lower or less than the major volatile acids, were hexanoic, 4-hexenoic and octanoic, presumably derived from the hydrolysis of triglycerides.

Total alcohols of control cheese increased consistently until day 120 and declined afterwards (Tables 4 and 5). Their pattern of accumulation in HPP cheeses differed depending on the pressure level applied, with maximum levels on days 120 or 240 in 400 MPa cheeses and decreases starting on day 21 in 600 MPa cheeses (Tables 4 and 5). Total alcohols represented 58.2% of the overall abundance of VC in control cheeses and only 23.9% in HPP cheeses (pooled data of days 60, 120 and 240). The major alcohols (data not shown) were 2-butanol, ethanol and 1,3-butanediol, accounting for 54.7%, 21.2% and 15.2% of the overall abundance of alcohols, respectively. 2-Butanol shows chemical and floral notes, ethanol chemical and dry notes, and 1,3-butanediol milk creamy buttery notes (Curioni and Bosset 2002; Mallia et al. 2005). Biosynthesis of alcohols may take place in cheese through different mechanisms (Molimard and Spinnler 1996). 2-Butanol is mostly formed by the enzymatic reduction of 2-butanone and ethanol is derived from lactose metabolism, while 1,3-butanediol comes from the metabolism of lactose and citrate. Other alcohols

found were 2,3-butanediol, 1-propanol, 2-propanol, 2-propen-1-ol, 2-methyl-1-propanol, 1,3-propanediol, 1-butanol, 1-pentanol, 2-pentanol, 3-methyl-2-buten-1-ol, 3-methyl-3-buten-1-ol, 1-hexanol, 2,3-hexanediol, 2-ethyl-1-hexanol, cyclohexanol and 2-heptanol, all at levels considerably lower than those of the major alcohols. A branched-chain alcohol, 2-methyl-1-propanol, was detected in cheese in spite of the absence of the precursor branched-chain acid and aldehyde.

Total benzenic compounds followed the same accumulation pattern in control and HPP cheeses, with a slight increase until day 60 and a slight decrease afterwards (Tables 4 and 5). Benzenic compounds accounted for 1.44% and 1.75% of the overall abundance of VC in control cheeses and HPP cheeses, respectively, with no significant differences due to treatments. The principal benzenic compound was naphthalene, accounting for 89.8% of the overall abundance of this group of VC compounds, followed by benzenethanol and toluene which represented 5.7% and 4.5%, respectively. Naphtalene, with moth-ball tar-like odour, has been found in cow, ewe and goat milk smoked cheeses (Guillén and Sopelana 2004). Benzenethanol, with floral, rose notes, has been detected in raw milk cheeses (Sulejmani et al. 2014). Toluene, with nutty, bitter almond notes, contributes to Cheddar cheese odour (Arora et al. 1995). Benzenic compounds probably derive from the catabolism of aromatic amino acids by cheese microbiota other than lactic acid bacteria.

Total esters also followed the same accumulation pattern in control and HPP cheeses, with a slight increase until day 60 and a slight decrease afterwards (Tables 4 and 5). Esters accounted for 1.16% of the overall abundance of VC in control cheeses and for 1.12% of the overall abundance in HPP cheeses, with no significant differences due to treatments. The predominant ester was a cyclic ester, γ-butyrolactone, which accounted for 66.8% of the overall abundance of esters, followed by ethyl acetate and ethyl butyrate, accounting for 13.0% and 6.4% of the overall abundance. Lactones are formed from hydroxylated fatty acids by lactonization, with the closing of the ring occurring by the action of pH, microorganisms or both (Molimard and Spinnler 1996); they have peach, apricot and coconut fruity notes and low perception thresholds. Linear esters are formed in dairy systems

through two enzymatic mechanisms, esterification, a reaction in which esters are formed from alcohols and carboxylic acids, and alcoholysis, a transferase reaction in which fatty acids from acylglycerols and acyl-CoA derivatives are directly transferred to alcohols (Liu et al. 2004). In the present work, the increase in esters levels was not accompanied by a decline in the levels of acids or alcohols in control or HPP cheeses. Esters generally show sweet, fruity and floral notes, have a low perception threshold and mask the sharpness and bitterness imparted by fatty acids and amines, thus contributing to a pleasant cheese flavour. Ethyl acetate, with fruity pineapple notes, and ethyl butyrate, with green fruit and apple notes, have been identified as potent odorants in cow and ewe milk cheese varieties (Curioni and Bosset 2002).

Total ethers increased in control cheese and 400 MPa cheeses until day 60 and declined afterwards, while in 600 MPa cheeses the increase persisted until days 120 or 240 (Tables 4 and 6). Higher values were reached in HPP cheeses than in control cheese from day 60 onwards, although the differences were not significant in most cases. Ethers accounted for 0.53% of the overall abundance of VC in control cheeses and 0.99% in HPP cheeses (pooled data of days 60, 120 and 240). Major ethers (data not shown) were 1-methoxy-2-propanol, 2-(2-ethoxyethoxy)-ethanol and 1,1'-oxybis-2-propanol, accounting for 80.4%, 7.7% and 4.2% respectively of the overall abundance of total ethers. No odour active ethers were found in cow or ewe raw milk cheeses by Mallia et al. (2005), although the presence of ethers in smoked goat raw milk cheese was reported by Guillén et al. (2004). Ethers are not considered to be key odorants in cheese and their origin is not well known (Curioni and Bosset 2002).

Total terpenoids increased gradually in control and HPP cheeses until day 240, with few significant differences between cheeses (Tables 4 and 6). Terpenoids accounted for 0.63% of the overall abundance of VC in control cheeses and 0.56% in HPP cheeses (pooled data of days 60, 120 and 240). The two terpenoids found, pristine and borneol, accounted for 94.0% and 6.0% of the overall abundance of total terpenoids, respectively (data not shown). Terpenoids, commonly present

in the volatile fraction of cheeses (Carbonell et al. 2002; Fernández-García et al. 2004), are considered to come from the animal diet (Toso et al. 2002).

Total hydrocarbons hardly varied from day 60 to day 120 in control and HPP cheeses, with slightly higher levels in HPP cheeses, and declined considerably from day 120 to day 240, time at which 600 MPa cheeses showed significantly (p<0.05) higher levels than control cheese (Tables 4 and 6). Linear hydrocarbons are generally formed through the oxidation of FFA. Total hydrocarbons accounted for 0.26% of the overall abundance of VC in control cheeses and 0.46% in HPP cheeses (pooled data of days 60, 120 and 240). The most abundant hydrocarbons were 2,2,4,6,6-pentamethyl heptane, hexane and 3,3-dimethylhexane which accounted for 50.5%, 19.5% and 13.0%, respectively, of the overall abundance of this chemical group (data not shown). Hydrocarbons, in spite of being commonly found in the volatile fraction of raw milk cheese varieties (Carbonell et al. 2002; Fernández-García et al. 2002; Fernández-García et al. 2004), are not considered as main key odorants (Thierry et al. 1999).

Total aldehydes did not vary significantly with time of ripening and showed slight differences between control and HPP cheeses (Tables 4 and 6). Total aldehydes accounted for 0.09% of the overall abundance of VC in control cheeses and 0.18% in HPP cheeses (pooled data of days 60, 120 and 240). The principal aldehydes were acetaldehyde, 3-methylbutanal and benzaldehyde which accounted for 38.5%, 32.6% and 19.1%, respectively, of the overall abundance of total aldehydes (data not shown). Acetaldehyde is mostly formed through the metabolism of lactose and may also derive from glycine, while 3-methylbutanal is derived from leucine and benzaldehyde from the catabolism of aromatic amino acids (Yvon and Rijnen 2001), although it also may be formed through the oxidation of toluene. Acetaldehyde shows green odour notes while 3-methylbutanal has pleasant fruity odour notes al low concentrations which turn into green malty at high levels and is a potent odorant in Camembert, Cheddar, Emmental and Gruyère cheeses (Curioni and Bosset 2002).

Total sulphur compounds suffered a slight decline from day 60 to day 120 followed by a drastic decline from day 120 to day 240, with no significant differences between cheese until day 240, time at which HPP cheeses had higher (p<0.05) levels than control cheese (Tables 4 and 6). Total aldehydes accounted for 0.09% of the overall abundance of VC in control cheeses and 0.18% in HPP cheeses (pooled data of days 60, 120 and 240). The two sulphur compounds found, dimethylsulphide and dimethylsulfoxide, accounted for 80.2% and 19.8%, respectively, of the overall abundance of this chemical group (data not shown). Most sulphur compounds derive from methanethiol, a compound of putrid and faecal-like aroma which is formed from methionine by the action of cystathionine or methionine lyases (Weimer et al. 1999). *Lactococcus, Lactobacillus, Brevibacterium, Micrococcus, Corynebacterium, Pseudomonas* and probably other genera of Gram-negative bacteria produce sulphur compounds in cheese. At low concentrations, sulphur compounds contribute with their cowy, feedy, garlic, onion, cooked cabbage, cauliflower, mashed potato odour notes of to the aroma of Limburger, Camembert, Cheddar, Blue and ewes milk cheeses (Bonnarme et al. 2000; Fernández-García et al. 2004). However, high concentrations of sulphur compounds may result unpleasant because of their very low perception thresholds (Weimer et al. 1999).

The three miscellaneous compounds found in the present work, chloroform, propanamine and methoxy-phenyl-oxime, were minor volatiles which accounted for only 0.058%, 0.039%, and 0.008%, respectively, of the overall abundance of VC in control and HPP cheeses (data not shown). Chloroform, a compound with sweet hay odour notes found in different cheese varieties (Curioni and Bosset 2002), declined from day 60 onwards and was generally present at higher levels in HPP cheeses. Propanamine, a compound with ammonia and fish-like odour notes found in Camembert cheese (Molimard and Spinnler 1996), was not detected until day 60 and was only present in control and 400 MPa cheeses, at levels which increased until day 120 and decreased afterwards. Methoxy-phenyl-oxime, a compound without characteristic odour notes which has been detected in Gouda cheese (Jung et al. 2013), followed a similar trend to that of chloroform.

Principal Component Analysis

Principal component analysis (PCA) was firstly carried out on the concentrations of individual carboxylic acids. The objective was to discriminate control and HPP cheeses on days 14 to 240 after manufacture, and to ascertain their evolution during the time elapsed after manufacture, on the basis of their concentrations of carboxylic acids. Function 1, formed by C4:0, C6:0, C8:0, C10:0, C12:0, C14:0; C16:0, C18:0, C18:1; C18:2 and C18:3 acids, explained 73.8% of the variance, while function 2, formed by C3:0 and benzoic acids, explained 11.6% and function 3, formed by C2:0 acid, explained 7.1%. The distribution of control and HPP cheeses on the plane defined by functions 1 and 2 of the PCA of carboxylic acids is shown on Fig. 1. Function 1 evolved from negative values (at 14, 21 and 60 days) to positive values (at 120, 180 and 240 days). Function 2 followed a very particular trend, evolving from negative to positive values on the left hemiplane (from day 14 to day 60) and from positive to negative values on the right hemiplane (from day 120 to day 240). PCA of carboxylic acids did not achieve a clear discrimination between sampling times or treatments.

PCA was also performed on the levels of individual volatile compounds. The objective was to discriminate control and HPP cheeses on days 60, 120 and 240 after manufacture, and to ascertain their evolution during the time elapsed after manufacture, on the basis of the individual compounds present in the volatile fraction. Function 1, formed by 4 hydrocarbons, 2 acids, 2 sulfur compounds, 6 ketones, 2 aromatic compounds, 1 aldehyde, 1 ester, 4 alcohols, 1 terpenoid, 1 ether, chloroform and methoxy-phenyl-oxime, and with a negative coefficient by 1 acid, 3 esters and 5 alcohols, explained 37.7% of the variance, while function 2, formed by 2 esters, 1 ketone, 7 alcohols, 1 aldehyde, 1 acid, 1 ether, 1 terpenoid, and propanamine, explained 21.3%, and function 3, formed by 4-hexenoic acid, explained 6.5%. The distribution of control and HPP cheeses on the plane

defined by functions 1 and 2 of the PCA of the 70 individual volatile compounds (not shown) was similar to that obtained with the PCA of the 13 groups of volatile compounds (Fig. 2).

A third PCA was carried out on the levels of the 13 groups of volatile compounds. The objective was to discriminate control and HPP cheeses on days 60, 120 and 240 after manufacture, and to ascertain their evolution during the time elapsed after manufacture, on the basis of the levels of the main groups of compounds present in the volatile fraction. Function 1 (PC1), mainly formed by the groups of volatile acids, aldehydes, esters, ketones, hydrocarbons, benzenic compounds and sulphur compounds, explained 41.9% of the variance, while function 2 (PC2), formed by the groups of alcohols, terpenoids and miscellaneous compounds, explained 23.5% and function 3, mainly formed by the group of ethers, explained 12.8%. The distribution of control and HPP cheeses on the plane defined by functions 1 and 2 of the PCA of the groups of volatile compounds is shown on Fig. 2. Until day 120 of storage, control cheeses remained located on the right side of the plane, with positive PC1 values characterized by high amounts of acids, aldehydes, esters, ketones, hydrocarbons, benzenic and sulphur compounds, associated with cheesy, fruity, green, sweet, creamy and pleasant flavour notes. Simultaneously, they evolved from negative PC2 values on days 14 and 21 to positive PC2 values from day 60 onwards, with high levels of alcohols, terpenoids and miscellaneous compounds, associated with herbal, pungent, sweet and alcoholic flavour notes. On day 240, control cheese was located at the upper-left quadrant with negative PC1 and positive PC2 values, characterized by herbal, pungent, sweet and alcoholic notes but deficient in cheesy, creamy, fruity and pleasant notes. HPP induced lower PC2 values, in particular when applied at the higher pressure level. The 400 MPa cheeses showed on day 60 a volatile profile similar to that of 21-day control cheese, with cheesy, creamy and pleasant flavour notes. From day 120 onwards, they were located at the upper-left quadrant, together with the 240-day control cheese. The 600 MPa cheeses maintained the cheesy, creamy and pleasant flavour notes profile until day 120. From day 60 to day 240, they evolved from positive to negative PC1 values, the latter associated with mild flavour

notes. They remained until day 240 in the lower hemiplane, with negative PC2 values, without development of pungent and alcoholic notes.

Odour Characteristics

Odour intensity of control and HPP cheeses at each of the sampling times did not differ significantly, but there was an increase in odour intensity with the time elapsed after manufacture with scores ranging from 4.56 to 5.02 on day 60 and from 5.78 to 6.34 on day 240 (data not shown). Odour quality showed no significant differences between HPP and control cheeses at each of the sampling times and did not vary significantly with the time elapsed after manufacture, with scores ranging from 5.47 to 5.98 on day 60 and from 5.62 to 6.01 on day 240 (data not shown). Similarly, the scores of putrid, rancid and acid odour attributes did not vary significantly with HPP treatments or with the time elapsed after manufacture. Contrarily to these results, beneficial effects of HPP at 400 or 600 MPa on the odour characteristics of cheese made from raw ewe milk were observed during the refrigerated storage of cheeses until day 240 (Calzada et al. 2014a). In the present work, no accumulation of undesirable volatile compounds occurred in control cheese, in contrast to what happened in the control cheese made from raw ewe milk, a fact which would explain why there were no beneficial effects on odour characteristics due to HPP treatment.

Colour Parameters

Colour parameters of control and HPP cheeses are shown on Table 7. Lightness (L*) generally reached the highest values in 600 MPa cheeses, in particular in 600W3 cheese. It tended to decrease with time in control and HPP cheeses. Redness (a*) showed higher levels in control cheese than in HPP cheeses, in particular in 600 MPa cheeses, which exhibited the lowest values at all sampling

times. It declined sharply from day 180 to day 240 in control and HPP cheeses. Yellowness (b*) showed few significant differences between cheeses at each of the sampling times. It increased in control and HPP cheeses along the 240-day period.

Previous works on the effect of HPP on cheese colour generally dealt with cheeses ripened for shorter periods. Ávila et al. (2008) reported few differences attributable to HPP at 400 MPa in the colour parameters of Hispánico cheese ripened for 50 days; during this period, L* hardly varied, while a* tended to decrease and b* to increase, with higher values in the HPP cheese. Voigt et al. (2012) obtained higher L* and b* values, and similar a* values, for Cheddar cheese made from milk treated at 600 MPa than for control cheese. Minor differences between the colour parameters of control and HPP Brie cheeses were recorded from day 30 to day 90, while on day 120 significantly higher L* values, and lower a* and b* values, were recorded for HPP cheeses (Calzada et al. 2014c). Picon et al. (2013) recorded the lowest L* and a* values in cheeses made from curds treated at 500 MPa while the highest b* values were found for control cheese and cheese made from curd treated at 200 MPa. According to these authors, cheese colour parameters are influenced by the microstructural changes caused by HPP, the lower amount of available water in HPP-treated cheese and the concentration of cheese components associated with moisture loss during ripening.

Conclusions

High-pressure-processing of unpasteurized milk cheese lowered esterase activity, in particular when a pressure level of 600 MPa was applied. The concentration of total FFA on day 60 was not affected by HPP, but lower concentrations of total FFA were recorded on day 240 for HPP cheeses than for control cheese, with more marked differences for short-chain FFA. The levels of most of the individual volatile compounds found in cheese were significantly influenced by HPP. The level of total alcohols in 240-day cheeses was lowered by HPP at 600 MPa, which increased the levels of

total aldehydes, ketones and hydrocarbons. Total sulphur compounds were lowered by HPP at 400 and 600 MPa while total acids, esters, ethers, benzenic compounds and terpenoids were not affected by HPP. The odour characteristics of cheeses (odour quality and intensity) were hardly influenced by HPP. Colour parameters were affected by HPP, with higher L* values and lower a* values recorded in 600 MPa cheeses.

Acknowledgements This research was funded by AGL 2009-07801 project (Ministry of Science and Innovation, Spain). The authors thank the PDO dairy for providing the cheeses and Hiperbaric for HPP treatments. J. Calzada was the recipient of a FPI grant (Ministry of Science and Innovation, Spain).

References

Arora, G., Cormier, F., & Lee, B. (1995). Analysis of odor-active volátiles in Cheddar cheese headspace by multidimensional GC/MS/sniffing. *Journal of Agricultural and Food Chemistry, 43,* 748-752.

Ávila, M., Garde, S., Fernández-García, E., Medina, M., & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the odor and aroma of Hispánico cheese: correlation of volatile compounds and sensory analysis. *Journal of Agricultural and Food Chemistry, 54,* 382-389.

Ávila, M., Calzada, J., Garde, S., & Nuñez, M. (2007). Effect of a bacteriocin-producing *Lactococcus lactis* strain and high-pressure treatment on the esterase activity and free fatty acids in Hispánico cheese. *International Dairy Journal, 17,* 1415-1423.

Ávila, M., Garde, S., & Nuñez, M. (2008). Effect of a bacteriocin-producing lactic culture and high pressure treatment on the color of Hispánico cheese. *Milchwissenschaft, 63*, 406-409.

Bonnarme, P., Psoni, L., & Spinnler, H. E. (2000). Diversity of L-methionine catabolism pathways in cheese-ripening bacteria. *Applied and Environmental Microbiology, 66,* 5514-5517.

Buffa, M., Guamis, B., Pavia, M., Trujillo, A. J. (2001). Lipolysis in cheese made from raw, pasteurized or high-pressure-treated goats' milk. *International Dairy Journal, 11,* 175-179.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2013). High-pressure-processing decelerates lipolysis and formation of volatile compounds in ovine milk blue-veined cheese. *Journal of Dairy Science, 96*, 7500-7510.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014a). High-pressure-processing for the control of lipolysis, volatile compounds and off-odours in raw milk cheese. *Food and Bioprocess Technology, 7*, 2207-2217.

Calzada, J., del Olmo, A., Picon, A., Gaya, P., & Nuñez, M. (2014b). Effect of high-pressure processing on the microbiology, proteolysis, biogenic amines and flavour of cheese made from unpasteurized milk. *Food and Bioprocess Technology* DOI 10.1007/s11947-014-1406-7

Calzada, J., del Olmo, A., Picon, A., & Nuñez, M. (2014c). Effect of high-pressure-processing on the lipolysis and volatile compounds of Brie cheese during ripening and refrigerated storage. *International Dairy Journal, 39*, 232-239.

Carbonell, M., Nuñez, M., & Fernández-García, E. (2002). Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavor characteristics. *Lait, 82,* 683-698.

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13,* 841-866.

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal, 12,* 959-984.

Delgado, F. J., González-Crespo, J., Cava, R., & Ramírez, R. (2012). High-pressure treatment applied throughout ripening of a goat cheese caused minimal changes on free fatty acids content and oxidation in mature cheese. *Dairy Science and Technology, 92,* 237-248.

Fernández-García, E., Carbonell, M., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. *Journal of Dairy Research 69,* 579-593.

Fernández-García, E., Carbonell, M., Gaya, P., & Nuñez, M. (2004). Evolution of the volatile components of ewes raw milk Zamorano cheese. Seasonal variation. *International Dairy Journal, 14,* 701-711.

Fernández-García, E., Carbonell, M., Calzada, J., & Nuñez, M. (2006). Seasonal variation of the free fatty acids contents of Spanish ovine milk cheeses protected by a designation of origin: A comparative study. *International Dairy Journal, 16,* 252-261.

Fox, P. F., & Wallace, J. M. (1997). Formation of flavor compounds in cheese. *Advances in Applied Microbiology, 45,* 17-85.

Garde, S., Arqués, J. L., Gaya, P., Medina, M., & Nuñez, M. (2007). Effect of high pressure treatments on the proteolysis and texture of ewes' raw milk La Serena cheese. *International Dairy Journal, 17*, 1424-1433.

Gaya, P., Sánchez, C., Nuñez, M., Fernández-García, E. (2005). Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation. *Journal of Dairy Research, 72,* 287-295.

Guillén, M. D., Ibargoitia, M. L., Sopelana, P., Palencia, G., & Fresno, M. (2004). Components detected by means of solid-phase microextraction and gas chromatography/mass spectrometry in the headspace of artisan fresh goat cheese smoked by traditional methods. *Journal of Dairy Science, 87*, 284-299.

Guillén, M. D., & Sopelana, P. (2004). Occurrence of polycyclic aromatic hydrocarbons in smoked cheese. *Journal of Dairy Science, 87*, 556-564.

Huppertz, T., Fox, P. F. , & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research, 71,* 496-499.

Iammarino, M., Di Taranto, A., Palermo, C., & Muscarella, M. (2011). Survey of benzoic acid in cheeses: contribution to the estimation of an admissible maximum limit. *Food Additives and Contaminants, Part B, 4*, 231-237.

Juan, B., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. (2007). Effects of high pressure on proteolytic enzymes in cheese: relationship with the proteolysis of ewe milk cheese. *Journal of Dairy Science, 90,* 2113-2125.

Jung, H. J., Ganesan, P., Lee, S. J., & Kwak, H. S. (2013). Comparative study of flavor in cholesterol-removed Gouda cheese and Gouda cheese during ripening. *Journal of Dairy Science, 96*, 1972-1983.

Liu, S.-Q., Holland, R., & Crow, V. L. (2004). Esters and their biosynthesis in fermented dairy products: a review. *International Dairy Journal, 14*, 923-945.

Mallia, S., Fernández-García, E., & Bosset, J. O. (2005). Comparison of purge and trap and solid phase microextraction techniques for studying the volatile aroma compounds of three European PDO hard cheeses. *International Dairy Journal, 15,* 741-758.

Malone, A. S., Wick, C., Shellhammer, T. H., & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science, 86*, 1139-1146.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheese during ripening: A review. *Lait, 80,* 293-324.

Molimard, P., & Spinnler, H. E. (1996). Compounds involved in the flavour of surface mold-ripened cheeses: origins and properties. *Journal of Dairy Science, 79,* 169-184.

Norton, T., & Sun, D.-W. (2008). Recent advances in the use of high pressure as an effective processing technique in the food industry. *Food and Bioprocess Technology, 1,* 2-34.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., & Murphy, P. M. (2000). Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Applied and Environmental Microbiology, 66*, 4890-4896.

Picon, A., Alonso, R., van Wely, K. H. M., & Nuñez, M. (2013). Microstructural, textural and colour characteristics during ripening of Hispánico cheese made using high-pressure-treated ovine milk curd. *Food and Bioprocess Technology, 6,* 3056-3067.

Shao, Y. W., & Ramaswamy, H. S. (2011). *Clostridium sporogenes*-ATCC 7955 spore destruction kinetics in milk under high pressure and elevated temperature treatment conditions. *Food and Bioprocess Technology, 4,* 458-468.

Sieber, R., Bütikofer, U., & Bosset, J. O. (1995). Benzoic acid as a natural compound in cultured dairy products and cheese. *International Dairy Journal, 5*, 227-246.

Sulejmani, E., Rafajlovska, V., & Guneser, O. (2014). Characterization of volatiles in Beaten cheeses "Bieno sirenje" by SPME/GC-MS: influence of geographical origin. *Journal of the Serbian Chemical Society, 79*, 927-939.

Tejada, L., Gómez, R., Vioque, M., Sánchez, E., Mata, C., & Fernández-Salguero, J. (2000). Effect of freezing and frozen storage on the sensorial characteristics of Los Pedroches, a Spanish ewe milk cheese. *Journal of Sensory Studies, 15,* 251-262.

Thierry, A., Maillard, M.-B., & Le Quéré, J.-L. (1999). Dynamic headspace analysis of Emmental aqueous phase as a method to quantify changes in volatile flavor compounds during ripening. *International Dairy Journal, 9*, 453-463.

Toso, B., Procida, G., & Stefanoni, B. (2002). Determination of volatile compounds in cows' milk using headspace GC-MS. *Journal of Dairy Research, 69*, 569-577.

Van Hekken, D. L., Tunick, M. H., & Park, Y. W. (2005). Effect of frozen storage on the proteolytic and rheological properties of soft caprine milk cheese. *Journal of Dairy Science, 88,* 1966-1972.

Voigt, D. D., Chevalier, F., Donaghy, J. A., Patterson, M. F., Qian, M. C., & Kelly, A. L. (2012). Effect of high-pressure treatment of milk for cheese manufacture on proteolysis, lipolysis, texture and functionality of Cheddar cheese during ripening. *Innovative Food Science and Emerging Technologies, 13,* 23-30.

Weimer, B., Seefeldt, K., & Dias, B. (1999). Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek, 76,* 247-261.

Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11,* 185-201.

Table 1 Concentrations of acetic, propionic and benzoic acids during ripening of control and HPP cheeses made from unpasteurized milk

Acid	Days	Control cheese	HPP cheeses[1]			
			400W2	600W2	400W3	600W3
Acetic[2]	21	1.121 ± 0.051^{a}	1.071 ± 0.014^{a}	0.991 ± 0.021^{a}	1.051 ± 0.033^{a}	1.064 ± 0.017^{a}
	60	1.436 ± 0.024^{c}	1.064 ± 0.027^{a}	1.083 ± 0.047^{a}	1.268 ± 0.031^{b}	1.122 ± 0.037^{ab}
	120	1.785 ± 0.038^{c}	1.316 ± 0.063^{b}	1.004 ± 0.023^{a}	1.367 ± 0.029^{b}	1.003 ± 0.032^{a}
	180	1.868 ± 0.105^{c}	1.154 ± 0.080^{ab}	0.893 ± 0.039^{a}	1.416 ± 0.045^{b}	0.995 ± 0.040^{a}
	240	2.102 ± 0.034^{c}	1.272 ± 0.028^{b}	0.912 ± 0.021^{a}	1.348 ± 0.032^{b}	0.917 ± 0.028^{a}
Propionic[2]	21	0.349 ± 0.029^{a}	0.398 ± 0.008^{a}	0.311 ± 0.029^{a}	0.340 ± 0.014^{a}	0.350 ± 0.007^{a}
	60	0.358 ± 0.020^{a}	0.349 ± 0.007^{a}	0.378 ± 0.055^{a}	0.398 ± 0.018^{a}	0.359 ± 0.018^{a}
	120	0.378 ± 0.016^{a}	0.343 ± 0.011^{a}	0.355 ± 0.015^{a}	0.381 ± 0.016^{a}	0.364 ± 0.002^{a}
	180	0.377 ± 0.005^{a}	0.306 ± 0.010^{a}	0.348 ± 0.020^{a}	0.354 ± 0.051^{a}	0.379 ± 0.030^{a}
	240	0.304 ± 0.010^{a}	0.287 ± 0.027^{a}	0.317 ± 0.010^{a}	0.299 ± 0.014^{a}	0.366 ± 0.043^{a}
Benzoic[2]	21	0.244 ± 0.041^{a}	0.283 ± 0.018^{a}	0.213 ± 0.033^{a}	0.237 ± 0.027^{a}	0.224 ± 0.010^{a}
	60	0.302 ± 0.004^{a}	0.290 ± 0.015^{a}	0.335 ± 0.041^{a}	0.316 ± 0.036^{a}	0.294 ± 0.009^{a}
	120	0.345 ± 0.026^{a}	0.341 ± 0.017^{a}	0.328 ± 0.012^{a}	0.358 ± 0.015^{a}	0.343 ± 0.014^{a}
	180	0.398 ± 0.010^{a}	0.300 ± 0.008^{a}	0.337 ± 0.015^{a}	0.362 ± 0.060^{a}	0.356 ± 0.032^{a}
	240	0.314 ± 0.018^{a}	0.285 ± 0.028^{a}	0.303 ± 0.002^{a}	0.279 ± 0.016^{a}	0.340 ± 0.043^{a}

[1]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

[2]Concentrations are expressed in mg/g cheese DM, as mean ± SEM of duplicate determinations on two cheese-making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

Table 2 Esterase activity[1] during ripening of control and HPP cheeses made from unpasteurized milk

Days	Control cheese	HPP cheeses[2]			
		400W2	600W2	400W3	600W3
1	4.63 ± 0.18				
14	6.32 ± 0.19^{b}	4.42 ± 0.28^{a}	3.22 ± 0.50^{a}		
21	7.71 ± 0.06^{d}	4.63 ± 0.14^{c}	2.73 ± 0.12^{b}	4.21 ± 0.06^{c}	2.25 ± 0.10^{a}
60	7.90 ± 0.09^{c}	3.32 ± 0.09^{b}	1.36 ± 0.09^{a}	3.39 ± 0.11^{b}	1.40 ± 0.16^{a}
120	10.75 ± 0.19^{c}	4.26 ± 0.30^{b}	1.80 ± 0.04^{a}	3.91 ± 0.06^{b}	1.79 ± 0.02^{a}
180	11.21 ± 0.23^{c}	3.50 ± 0.24^{b}	0.74 ± 0.13^{a}	2.82 ± 0.24^{b}	0.66 ± 0.05^{a}
240	10.72 ± 0.15^{c}	3.18 ± 0.08^{b}	0.97 ± 0.10^{a}	3.51 ± 0.09^{b}	1.20 ± 0.03^{a}

[1]Esterase activity is expressed in pmol of α-naphthol per min and g, as mean ± SEM of duplicate determinations on two cheese-making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

[2]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

Table 3 Concentrations of short-chain (SC), medium-chain (MC) and long-chain (LC) free fatty acids (FFA) during ripening of control and HPP cheeses made from unpasteurized milk

Acids	Days	Control cheese	HPP cheeses[1]			
			400W2	600W2	400W3	600W3
SC FFA[2]	21	0.038 ± 0.004^{a}	0.032 ± 0.002^{a}	0.030 ± 0.002^{a}	0.035 ± 0.003^{a}	0.034 ± 0.002^{a}
	60	0.070 ± 0.003^{b}	0.050 ± 0.002^{a}	0.045 ± 0.004^{a}	0.058 ± 0.005^{ab}	0.050 ± 0.004^{a}
	120	0.113 ± 0.005^{b}	0.098 ± 0.008^{b}	0.057 ± 0.003^{a}	0.107 ± 0.002^{b}	0.059 ± 0.002^{a}
	180	0.143 ± 0.009^{b}	0.117 ± 0.014^{b}	0.069 ± 0.007^{a}	0.120 ± 0.009^{b}	0.075 ± 0.004^{a}
	240	0.190 ± 0.009^{c}	0.147 ± 0.010^{b}	0.087 ± 0.003^{a}	0.148 ± 0.006^{b}	0.086 ± 0.003^{a}
MC FFA[2]	21	0.190 ± 0.007^{a}	0.198 ± 0.005^{a}	0.188 ± 0.006^{a}	0.181 ± 0.010^{a}	0.185 ± 0.006^{a}
	60	0.228 ± 0.005^{a}	0.234 ± 0.010^{a}	0.257 ± 0.010^{a}	0.239 ± 0.011^{a}	0.240 ± 0.006^{a}
	120	0.296 ± 0.010^{a}	0.303 ± 0.008^{a}	0.282 ± 0.002^{a}	0.295 ± 0.007^{a}	0.280 ± 0.002^{a}
	180	0.356 ± 0.010^{a}	0.333 ± 0.013^{a}	0.325 ± 0.012^{a}	0.335 ± 0.012^{a}	0.316 ± 0.002^{a}
	240	0.400 ± 0.005^{c}	0.384 ± 0.006^{bc}	0.350 ± 0.005^{a}	0.370 ± 0.010^{ab}	0.361 ± 0.006^{ab}
LC FFA[2]	21	0.657 ± 0.035^{a}	0.699 ± 0.012^{a}	0.671 ± 0.020^{a}	0.634 ± 0.026^{a}	0.632 ± 0.018^{a}
	60	0.745 ± 0.010^{a}	0.746 ± 0.023^{a}	0.871 ± 0.025^{b}	0.810 ± 0.030^{ab}	0.800 ± 0.014^{ab}
	120	0.982 ± 0.021^{a}	1.006 ± 0.029^{a}	0.969 ± 0.006^{a}	0.996 ± 0.034^{a}	0.938 ± 0.015^{a}
	180	1.174 ± 0.033^{a}	1.120 ± 0.044^{a}	1.089 ± 0.046^{a}	1.112 ± 0.037^{a}	1.078 ± 0.010^{a}
	240	1.307 ± 0.020^{c}	1.244 ± 0.017^{bc}	1.141 ± 0.025^{a}	1.210 ± 0.026^{ab}	1.165 ± 0.008^{ab}

[1]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

[2]Concentrations are expressed in mg/g cheese DM, as mean ± SEM of duplicate determinations on two cheese-making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

Table 4 Levels of the main groups of volatile compounds in control cheese on days 14 and 21 of ripening.

Chemical group[1]	Time of ripening	
	Day 14	Day 21
Ketones	10593 ± 1180	8792 ± 1213
Acids	4298 ± 600	5127 ± 624
Alcohols	2518 ± 403	4209 ± 525
Benzenic compounds	203.6 ± 24.1	223.9 ± 37.2
Esters	175.2 ± 25.2	238.8 ± 27.1
Ethers	69.7 ± 9.6	105.5 ± 13.6
Terpenoids	50.52 ± 5.53	59.91 ± 8.13
Hydrocarbons	24.23 ± 5.01	50.72 ± 8.22
Aldehydes	17.60 ± 2.08	26.30 ± 3.53
Sulphur compounds	17.92 ± 1.82	22.66 ± 3.79

[1]Levels of VC groups (sums of individual VC) are expressed in relative abundance (compound peak area x 10^2 / internal standard peak area), as mean ± SEM of triplicate determinations on two cheese making trials.

Table 5 Levels of total ketones, acids, alcohols, benzenic compounds and esters during ripening of control and HPP cheeses made from unpasteurized milk

Chemical group	Days	Control cheese	HPP cheeses[1]			
			400W2	600W2	400W3	600W3
Ketones	60	5211 ± 829 a	21057 ± 2433 c	14344 ± 1893 bc	18262 ± 1555 c	10874 ± 1758 ab
	120	4049 ± 543 a	2858 ± 530 a	10428 ± 1327 b	3669 ± 605 a	10622 ± 937 b
	240	1381 ± 175 a	759 ± 62 a	7432 ± 740 b	980 ± 116 a	6990 ± 992 b
Acids	60	5392 ± 840 a	7334 ± 1201 a	5494 ± 733 a	7595 ± 1122 a	5625 ± 738 a
	120	5197 ± 524 a	5255 ± 811 a	5070 ± 482 a	5515 ± 724 a	5247 ± 618 a
	240	3018 ± 369 a	4520 ± 539 a	4588 ± 675 a	3475 ± 272 a	4718 ± 516 a
Alcohols	60	11663 ± 1109 b	3432 ± 463 a	2522 ± 360 a	4052 ± 436 a	3402 ± 474 a
	120	16181 ± 1448 d	7395 ± 627 b	1983 ± 176 a	12055 ± 1442 c	2399 ± 124 a
	240	9582 ± 1026 b	8955 ± 1219 b	1508 ± 198 a	8547 ± 751 b	1998 ± 267 a
Benzenic compounds	60	388.8 ± 45.0 a	506.0 ± 59.3 a	417.6 ± 57.3 a	469.2 ± 51.5 a	402.0 ± 40.9 a
	120	334.8 ± 31.8 a	307.4 ± 33.2 a	350.6 ± 57.5 a	389.0 ± 43.9 a	341.7 ± 38.3 a
	240	206.2 ± 31.3 a	286.4 ± 52.5 a	285.7 ± 54.9 a	211.1 ± 19.5 a	280.8 ± 29.0 a
Esters	60	276.2 ± 41.4 a	349.3 ± 55.5 a	295.4 ± 47.1 a	314.4 ± 38.1 a	280.3 ± 34.7 a
	120	267.2 ± 24.2 a	228.7 ± 27.5 a	188.9 ± 15.9 a	232.0 ± 25.9 a	214.1 ± 16.4 a
	240	204.7 ± 25.0 a	167.2 ± 22.4 a	154.7 ± 26.9 a	133.7 ± 13.2 a	160.6 ± 21.8 a

[1]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

[2]Levels are expressed in relative abundance (compound peak area x 10^2 / internal standard peak area), as mean ± SEM of triplicate determinations on two cheese making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

Table 6 Levels of total ethers, terpenoids, hydrocarbons, aldehydes and sulphur compounds during ripening and refrigerated storage of control and HPP cheeses made from unpasteurized milk

Chemical group	Days	Control cheese	HPP cheeses[1]			
			400W2	600W2	400W3	600W3
Ethers	60	148.3 ± 12.9 a	266.4 ± 66.9 a	208.0 ± 39.1 a	279.3 ± 44.2 a	131.0 ± 16.2 a
	120	103.3 ± 14.2 a	245.1 ± 37.1 a	224.1 ± 27.2 a	176.1 ± 41.6 a	159.9 ± 13.7 a
	240	91.3 ± 11.1 a	214.7 ± 65.4 a	133.3 ± 18.5 a	155.1 ± 36.2 a	205.5 ± 75.4 a
Terpenoids	60	113.4 ± 8.4 a	115.8 ± 14.0 a	83.32 ± 6.62 a	119.4 ± 15.0 a	84.31 ± 7.15 a
	120	143.8 ± 9.6 b	126.0 ± 13.3 ab	90.46 ± 8.03 a	126.7 ± 12.9 ab	93.08 ± 5.91 a
	240	151.2 ± 14.7 a	152.0 ± 13.8 a	120.3 ± 10.6 a	135.3 ± 9.9 a	118.0 ± 15.7 a
Hydrocarbons	60	74.65 ± 5.37 a	97.59 ± 9.97 ab	98.68 ± 17.43ab	146.25 ±19.26b	113.61 ±6.37ab
	120	72.51 ± 10.37 a	138.13 ±18.61b	105.66 ±14.71ab	104.29 ±10.49ab	131.98 ±14.62b
	240	22.69 ± 2.20 a	30.10 ± 3.67 a	53.59 ± 2.77 b	39.22 ± 5.13 ab	54.26 ± 6.27 b
Aldehydes	60	20.46 ± 2.66 a	31.79 ± 3.53 ab	42.16 ± 5.57 bc	32.70 ± 2.84 ab	51.16 ± 4.82 c
	120	19.44 ± 2.25 a	30.54 ± 5.73 ab	38.94 ± 1.96 b	27.93 ± 3.97 ab	39.43 ± 4.18 b
	240	19.60 ± 2.65 a	29.33 ± 3.75 ab	41.39 ± 5.15 bc	17.35 ± 1.52 a	51.97 ± 6.91 c
Sulphur compounds	60	12.08 ± 2.22 a	22.74 ± 4.31 a	18.36 ± 2.29 a	17.78 ± 3.11 a	18.59 ± 3.61 a
	120	8.41 ± 0.96 a	12.06 ± 2.08 a	12.12 ± 0.80 a	9.97 ± 1.29 a	13.55 ± 2.64 a
	240	3.69 ± 0.59 a	8.16 ± 1.28 b	8.79 ± 1.21 b	5.56 ± 0.76 ab	8.90 ± 1.29 b

[1]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

[2]Levels are expressed in relative abundance (compound peak area x 10^2 / internal standard peak area), as mean ± SEM of triplicate determinations on two cheese making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

Table 7 CIE-LAB colour parameters during ripening of control and HPP cheeses made from unpasteurized milk

Parameter	Days	Control cheese	HPP cheeses[1]			
			400W3	600W3	400W5	600W5
L* (lightness)[2]	21	85.13 ± 0.35[a]	85.24 ± 0.22[ab]	86.57 ± 0.39[b]	85.29± 0.47[ab]	86.10 ± 0.32[ab]
	60	83.31 ± 0.21[a]	84.44 ± 0.40[bc]	84.92 ± 0.26[c]	84.13± 0.21[abc]	83.76 ± 0.27[ab]
	120	82.84 ± 0.37[abc]	82.16 ± 0.35[ab]	83.66 ± 0.28[c]	82.07± 0.31[a]	83.33 ± 0.25[bc]
	180	80.60 ± 0.49[a]	80.57 ± 0.63[a]	82.87 ± 0.52[b]	80.72± 0.40[a]	82.80 ± 0.46[b]
	240	76.58 ± 0.47[a]	77.99 ± 0.68[a]	78.94 ± 0.70[a]	77.24 ± 0.67[a]	77.99 ± 0.56[a]
a* (redness)[2]	21	1.99 ± 0.04[c]	1.67 ± 0.02[b]	1.25 ± 0.33[a]	1.75 ± 0.04[b]	1.35 ± 0.03[a]
	60	2.13 ± 0.03[c]	1.85 ± 0.05[b]	1.46 ± 0.03[a]	1.88 ± 0.03[b]	1.42 ± 0.04[a]
	120	2.00 ± 0.07[c]	1.72 ± 0.03[b]	1.09 ± 0.05[a]	1.59 ± 0.02[b]	1.06 ± 0.04[a]
	180	2.12 ± 0.03[c]	2.06 ± 0.07[c]	1.16 ± 0.04[a]	1.75 ± 0.04[b]	1.12 ± 0.03[a]
	240	1.63 ± 0.06[c]	1.36 ± 0.09[bc]	0.72 ± 0.10[a]	1.17 ± 0.07[b]	0.56 ± 0.10[a]
b* (yellowness)[2]	21	19.01 ± 0.44[a]	18.17 ± 0.36[a]	18.77 ± 0.49[a]	18.22 ± 0.47[a]	18.75 ± 0.40[a]
	60	19.31 ± 0.24[ab]	18.68 ± 0.16[a]	19.06 ± 0.23[ab]	19.27 ± 0.29[ab]	19.68 ± 0.24[b]
	120	22.12 ± 0.27[a]	22.14 ± 0.37[a]	21.58 ± 0.26[a]	22.16 ± 0.27[a]	22.11 ± 0.37[a]
	180	22.42 ± 0.25[a]	22.53 ± 0.42[a]	22.32 ± 0.36[a]	22.15 ± 0.23[a]	21.46 ± 0.29[a]
	240	24.13 ± 0.17[a]	24.14 ± 0.16[a]	23.65 ± 0.19[a]	23.97 ± 0.16[a]	23.70 ± 0.27[a]

[1]Codes for HPP cheeses are 400W2, HPP at 400 MPa on day 14; 600W2, HPP at 600 MPa on day 14; 400W3, HPP at 400 MPa on day 21; 600W3, HPP at 600 MPa on day 21.

[2]Results are expressed as mean ± SEM of six determinations on the cheese interior in two cheese making trials. Means in the same row at the same sampling date bearing the same superscript do not differ significantly.

Figure legends

Fig. 1. Distribution of HPP and control cheeses made from unpasteurized milk on the plane defined by functions 1 and 2 of the principal component analysis performed on the concentrations of individual carboxylic acids. Each symbol represents the averaged value of the two cheese-making trials. Treatments are as follows: control, **C**; 400W2, black circle; 600W2, black triangle; 400W3, open circle; 600W3, open triangle. Days after manufacture are indicated on each of the symbols.

Fig. 2. Distribution of HPP and control cheeses made from unpasteurized milk on the plane defined by functions 1 and 2 of the principal component analysis performed on the total levels of the groups of volatile compounds. Each symbol represents the averaged value of the two cheese-making trials. Treatments are as follows: control, **C**; 400W2, black circle; 600W2, black triangle; 400W3, open circle; 600W3, open triangle. Days after manufacture are indicated on each of the symbols.

Figure 1

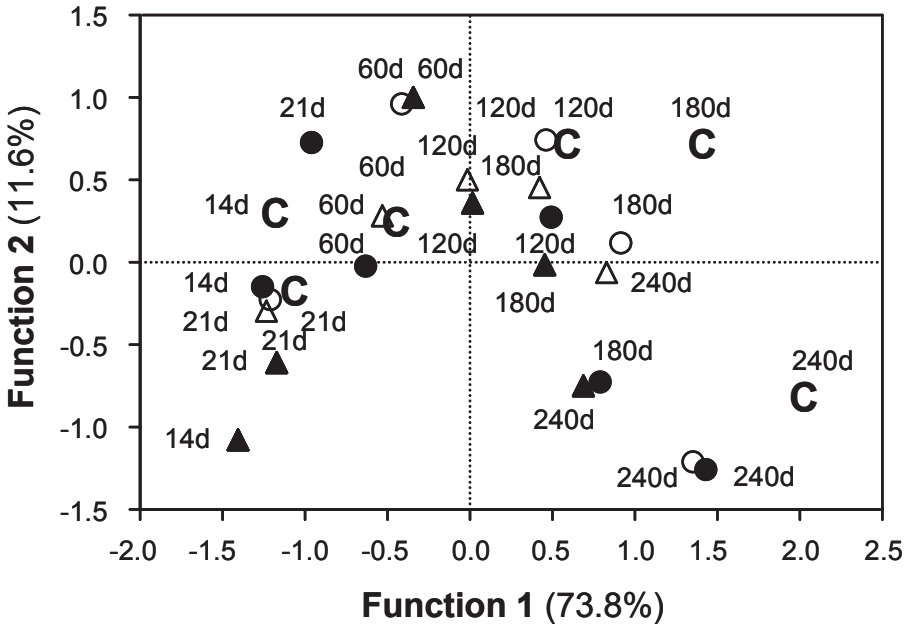
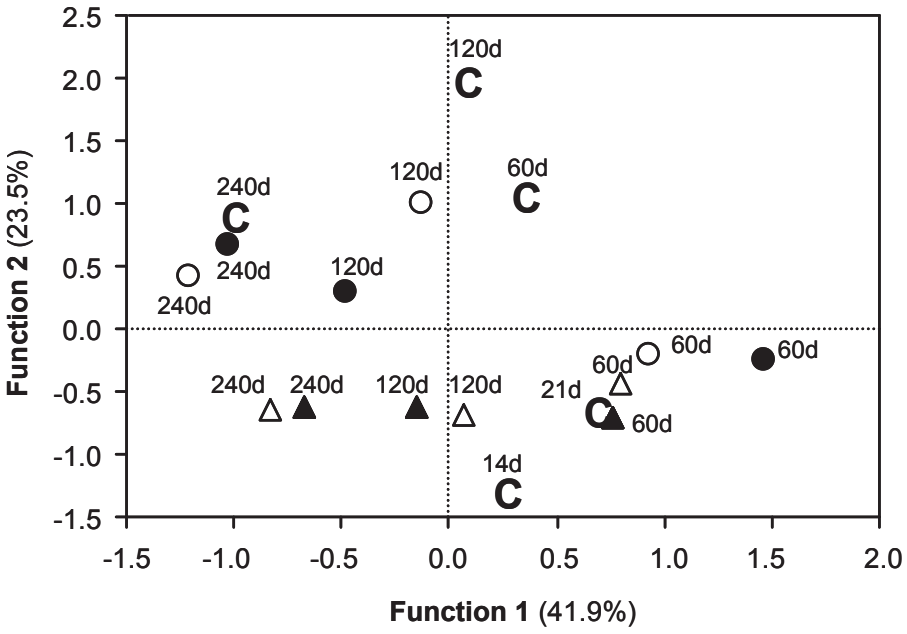

Function 2 (11.6%)
Function 1 (73.8%)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
60d
60d
21d
120d
120d
180d
C
120d
C
60d
180d
14d
C
60d
C
180d
60d
120d
120d
14d
C
240d
180d
21d
21d
21d
180d
240d
21d
C
14d
240d
240d
240d

Figure 2


2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Function 2 (23.5%)
Function 1 (41.9%)
120d
C
120d
60d
C
240d
C
240d
240d
120d
60d
60d
60d
240d
240d
120d
120d
21d
C
60d
14d
C

Capítulo 11. Discusión general.

Fotografía: cabina de cata para la evaluación organoléptica de los quesos (superior) y aspecto de las muestras preparadas para cata de queso azul Roncari-blue (centro izquierda), Torta del Casar (centro derecha), queso Brie (inferior izquierda) y queso Arzúa-Ulloa (inferior derecha).

La maduración es una de las fases más importantes en la elaboración del queso, ya que durante este periodo se desarrolla el sabor, el aroma, la textura y la apariencia de las distintas variedades de queso (McSweeney, 2004). El correcto equilibrio de compuestos responsables del aroma y sabor determina el momento óptimo para el consumo de cada variedad (Mulder, 1952). Este equilibrio se alcanza durante la maduración mediante diversos procesos bioquímicos que afectan a los componentes químicos del queso como se describe en la Introducción. Una vez alcanzado el equilibrio óptimo, estos procesos continúan, pudiendo provocar un desequilibrio de compuestos responsables del aroma y sabor y consecuentemente una pérdida de calidad del producto, limitando la vida útil de este. El empleo de temperaturas de refrigeración ralentiza aunque no llega a impedir este fenómeno conocido como sobremaduración. Apenas existen estudios enfocados a evitar este fenómeno, aunque sí se ha conseguido alargar la vida útil del queso fresco mediante el empleo de altas presiones (Daryaei *et al.*, 2006, Evert-Arriagada *et al.*, 2014). Además del problema de la sobremaduración, la abundancia de aminoácidos libres junto a la presencia de microorganismos con actividad descarboxilasa, puede desembocar en la formación y acumulación de aminas biógenas, con los consecuentes problemas para la salud del consumidor (Linares *et al.*, 2011).

En el presente trabajo de investigación se ha estudiado el efecto de las altas presiones hidrostáticas sobre distintas variedades de queso a lo largo de un prolongado periodo de tiempo post-tratamiento, con el fin de evitar la sobremaduración y aumentar la vida útil de las variedades estudiadas. Se examinaron diferentes parámetros al objeto de evaluar la proteolisis, lipolisis y catabolismo de aminoácidos y ácidos grasos libres (AGL), además de parámetros microbiológicos, texturométricos, colorimétricos y organolépticos así como el pH y extracto seco (ES). Se seleccionaron cuatro variedades de quesos, que se describen a continuación. El queso azul (Roncari-blue) fabricado con leche pasteurizada de oveja y madurado con mohos (*Penicillium roqueforti*) en su interior, cuyos resultados se recogen en los capítulos 2 y 3. La Torta del Casar, fabricado con leche cruda de oveja y cuajo vegetal, cuyos resultados se recogen en los capítulos 4, 5 y 6. El queso Brie, fabricado con leche pasteurizada de vaca y madurado con mohos (*Penicillium camemberti*) en su superficie, cuyos

resultados se recogen en los capítulos 7 y 8. Y el queso Arzúa-Ulloa, fabricado con leche cruda de vaca, cuyos resultados se recogen en los capítulos 9 y 10.

Los quesos estudiados fueron tratados con dos niveles de presión (400 y 600 MPa) aplicados en distintos momentos de la maduración, seleccionados en función de la variedad. Los quesos tratados por alta presión, así como sus respectivos controles no tratados, fueron evaluados en distintos momentos de la maduración y del almacenamiento en refrigeración, elegidos también en función de la variedad de queso.

- **Queso azul (Roncari-blue)**

El queso azul se caracteriza por la presencia de *Penicillium roqueforti* en su interior. En el queso azul investigado (Roncari-blue), elaborado con leche pasteurizada de oveja, se estudió la evolución de las variables microbiológicas, químicas y sensoriales durante la maduración y el almacenamiento en refrigeración hasta el día 360. Mediante los análisis microbiológicos, se comprobó una disminución de los niveles de bacterias lácticas y bacterias mesófilas totales durante la maduración y el almacenamiento en refrigeración, desde valores de 9,54 y 8,76 log ufc/g, respectivamente, a día 1, hasta 8,02 y 7,99 log ufc/g a día 180, y 6,57 y 5,75 log ufc/g a día 360. Los niveles de *P. roqueforti* sufrieron también un descenso gradual, desde 7,73 log ufc/g a día 21 hasta 7,10 log ufc/g a día 180 y 5,66 log ufc/g a día 360. El pH aumentó desde 4,76 a día 1 hasta 6,24 a día 60, al igual que se ha observado en queso Gorgonzola (Gobbetti *et al.*, 1997), y posteriormente descendió hasta 5,34 a día 360. El extracto seco aumentó desde 46,63 % a día 1 hasta 53,95 % a día 90 y posteriormente se mantuvo en estos mismos valores hasta el día 360.

Las α_S-, β-, κ- y *p*-κ-caseínas sufrieron una fuerte reducción del 93, 87, 78 y 46 % respectivamente, entre los días 1 y 21. Esta elevada hidrólisis de caseínas es debida a la presencia de plasmina residual, que mantiene parte de su actividad tras los tratamientos térmicos de pasteurización (Ismail & Nielsen, 2010), junto a la presencia de cuajo, enzimas de las bacterias lácticas y principalmente las enzimas de *P. roqueforti* (Cantor *et al.*, 2004). La α_S- y la κ-caseína no volvieron a detectarse a partir del día 90, mientras que la β- y la *p*-κ-caseína continuaron disminuyendo a lo largo del periodo de almacenamiento en refrigeración hasta el día 360. Los péptidos hidrófilos aumentaron ligeramente durante todo el almacenamiento en refrigeración hasta alcanzar un valor

de 29,81 UA/mg ES a día 360, mientras que los péptidos hidrófobos descendieron de forma gradual hasta el día 360, alcanzando un valor de 4,54 UA/mg ES. El ratio de péptidos hidrófobos/hidrófilos descendió gradualmente durante todo el almacenamiento en refrigeración. La actividad aminopeptidasa alcanzó valores máximos de 24,83 nmoles de *p*-nitroanilina/min·g a día 42 con el sustrato Leu-*p*-NA y de 24,58 nmoles de *p*-nitroanilina/min·g a día 63 con el sustrato Lys-*p*-NA. Posteriormente la actividad aminopeptidasa fue descendiendo hasta valores de 3,26 y 3,21 nmoles de *p*-nitroanilina/min·g a día 360 con los sustratos Leu-*p*-NA y Lys-*p*-NA, respectivamente. La alta actividad de las exopeptidasas de *P. roqueforti* hicieron que la concentración de aminoácidos libres aumentase desde 1,49 mg/g ES en el día 1 hasta 61,20 mg/g ES a día 90, siendo estas concentraciones más altas que las encontradas en queso Gorgonzola (Gobbetti *et al.*, 1997) pero inferiores a las encontradas en otros quesos azules como Stilton (Madkor *et al.*, 1987b) o Picón Bejes-Tresviso (Prieto *et al.*, 2000). Posteriormente los niveles de aminoácidos libres continuaron aumentando hasta el día 360, llegando a alcanzar una concentración de 116,76 mg/g ES. De igual manera, la proteolisis global aumentó durante la maduración y el almacenamiento en refrigeración.

Las aminas biógenas, procedentes de la descarboxilación de los aminoácidos, aumentaron desde 0,007 mg/g ES de aminas biógenas totales a día 90 hasta 0,218 mg/g ES a día 360, siendo estos valores más bajos que la media encontrada en otros quesos azules elaborados con diferentes tipos de leche (Flórez *et al.*, 2006, Fernández *et al.*, 2007). A día 90 únicamente se detectó β-feniletilamina, que aumentó hasta una concentración de 0,06 mg/g ES a día 360, mientras que la triptamina, tiramina y putrescina únicamente se encontraron a partir del día 180, alcanzando valores a día 360 de 0,07, 0,05 y 0,03 mg/g ES, respectivamente. La espermidina sólo se detectó en los días 270 y 360, alcanzando una concentración de 0,01 mg/g ES a día 360.

La actividad esterasa alcanzó un máximo de 38,53 pmoles de α-naftol/min·g a día 42, disminuyendo posteriormente y volviendo a aumentar hasta alcanzar 33,74 pmoles de α-naftol/min·g a día 180. Los niveles de ácidos grasos libres totales aumentaron hasta 83,82 mg/g ES a día 360, con valores durante la maduración similares a los encontrados en Gorgonzola y Stilton (Madkor *et al.*, 1987a, Gobbetti *et al.*, 1997). Los

ácidos grasos libres de cadena corta ($C_{4:0}$ - $C_{8:0}$) aumentaron hasta 8,88 mg/g ES a día 360, mientras que los ácidos grasos libres de cadena media ($C_{10:0}$ - $C_{14:0}$) y de cadena larga ($C_{16:0}$ - $C_{18:3}$) aumentaron hasta 18,31 y 56,63 mg/g ES respectivamente a día 360, siendo estos valores muy superiores a los encontrados en variedades de queso no maduradas por mohos (Ávila *et al.*, 2007, Delgado *et al.*, 2011), debido a la elevada actividad que presentan las lipasas de *P. roqueforti* (Cantor *et al.*, 2004), aunque menores a los encontrados en otros quesos azules (Prieto *et al.*, 2000). El ácido oleico fue el mayoritario durante la maduración y el almacenamiento en refrigeración, alcanzando valores de 27,03 mg/g ES a día 360. El ácido etanoico, procedente principalmente del metabolismo de lactosa, lactato y citrato (McSweeney & Sousa, 2000), aumentó hasta el día 63 alcanzando una concentración de 1,72 mg/g ES y posteriormente descendió hasta 1,39 mg/g ES a día 360.

El grupo mayoritario de compuestos volátiles fue el de los ácidos volátiles, seguido de alcoholes y cetonas. Los grupos de compuestos más característicos de los quesos azules son el de las cetonas, que aportan aromas florales y frutales, seguido del grupo de los alcoholes, que aportan aromas a fresco, frutales y hierba (Curioni & Bosset, 2002, Flórez *et al.*, 2006, Voigt *et al.*, 2010). La menor concentración de cetonas encontradas en este estudio puede deberse a la toxicidad que presentan las altas concentraciones de ácidos grasos, especialmente los de cadena larga, sobre el micelio del moho, lo que puede reducir la formación de cetonas (Kinsella & Hwang, 1976). Los niveles de ácidos volátiles, ésteres, terpenos, compuestos nitrogenados y azufrados aumentaron entre los días 180 y 360. Los niveles de alcoholes y cetonas también aumentaron, aunque más ligeramente. Por otro lado, los aldehídos y los compuestos bencénicos descendieron y los hidrocarburos se mantuvieron en los mismos niveles.

En el análisis sensorial se determinó que únicamente la intensidad de sabor y el parámetro de sabor umami aumentaron durante el almacenamiento en refrigeración. La calidad de sabor, así como el resto de los parámetros sensoriales, se mantuvieron en valores similares hasta el día 360.

El almacenamiento en refrigeración indujo leves cambios químicos en el queso azul, que no se vieron reflejados en una pérdida de calidad de sabor del queso y únicamente provocaron un aumento de la intensidad de sabor. A pesar de no observarse efectos

negativos de sobremaduración, sí se produjo una acumulación de aminas biógenas que, a pesar de encontrarse en niveles bajos, podrían plantear problemas de salud en individuos con un sistema de detoxificación deficiente o en individuos que se encuentren bajo tratamiento con fármacos inhibidores de este sistema.

En queso azul se aplicaron tratamientos de altas presiones de 400 y 600 MPa a las 3 semanas (400-3S y 600-3S), a las 6 semanas (400-6S y 600-6S) y a las 9 semanas (400-9S y 600-9S) de maduración. La aplicación de estos tratamientos indujo una serie de cambios respecto al control en los diferentes tiempos (Tablas 5 y 6). Comparando los quesos tratados a lo largo del almacenamiento en refrigeración con el queso control en su momento óptimo (180 días), se pueden apreciar los cambios debidos al efecto de las altas presiones (Tabla 7).

Las reducciones de los niveles de microorganismos provocadas por las altas presiones, en especial con los tratamientos de 600 MPa, que redujeron los niveles de *P. roqueforti* por debajo del límite de detección, no lograron atenuar la fuerte hidrólisis de α_S-caseína, que ya a día 90 no se detectó en el queso control. En los quesos tratados con 400 MPa a las 9 semanas y los tratados con 600 MPa a las 3 y 6 semanas se detectaron pequeñas cantidades de α_S-caseína a día 180. Los quesos tratados con 600 MPa a las 3 semanas presentaron niveles de β-caseína similares a los del control de 180 días, pero únicamente hasta el día 270. Los tratamientos de altas presiones atenuaron levemente la hidrólisis de *p*-κ-caseína, pero en ningún caso consiguieron mantener niveles similares a los del queso control de 180 días. Por lo que respecta a los péptidos, únicamente los quesos tratados con 600 MPa a las 3 y 6 semanas consiguieron mantener hasta el día 360 niveles de péptidos hidrófilos similares o inferiores a los del control de 180 días, mientras que a día 360 los niveles de péptidos hidrófobos de todos los quesos tratados fueron superiores a los del control de 180 días, haciendo que el ratio de péptidos hidrófobos/hidrófilos también fuese mayor. La reducción de la actividad aminopeptidasa provocada por las altas presiones se vio reflejada en los niveles de aminoácidos libres de los quesos tratados a las 3 semanas y de los tratados con 600 MPa a las 6 y 9 semanas, que mantuvieron niveles inferiores o similares a los del queso control de 180 días hasta el final del almacenamiento en refrigeración, al igual que sucedió con la proteolisis global.

Tabla 5. Tendencias de las características microbiológicas y químicas del queso azul tratado por alta presión con respecto al queso control.

Parámetro	400-3S	600-3S	400-6S	600-6S	400-9S	600-9S
ES	NS	NS	NS	NS	NS	NS
pH	↓ 90 a 360 d	↓ 90 a 360 d	NS	NS	↓ 180 d	↓ 180 d
Aerobios totales	↓ 90 a 270 d	↓↓ hasta 360 d	↓ 180 a 360 d	↓↓ hasta 360 d	↓ 180 a 360 d	↓↓ hasta 360 d
Bacterias lácticas	↓ 90 a 270 d	↓↓ hasta 360 d	↓ 180 a 360 d	↓↓ hasta 360 d	↓ 180 a 360 d	↓↓ hasta 360 d
P. roqueforti	↓ hasta 360 d	↓↓ hasta 360 d	↓ hasta 360 d	↓↓ hasta 360 d	↓ hasta 360 d	↓↓ hasta 360 d
α-caseína	↑ 90 d	↑ 90 d	↑ 90 d	↑ 90 d	NS	NS
β-caseína	↑ 90 d	↑ 180 y 270 d	NS	↑ 360 d	NS	NS
κ-caseína	NS	NS	NS	NS	NS	NS
p-κ-caseína	NS	↑ 270 d	NS	NS	NS	NS
Péptidos hidrófilos	↑ 90 d	NS	↑ 90 d	NS	↑ 360 d	↑ 90 d
Péptidos hidrófobos	↑ 90, 180 y 360 d	↑ 90 a 360 d	↑ 360 d	↑ 90, 180 y 360 d	↑ 270 y 360 d	↑ 270 y 360 d
Ratio hidrófobos/filos	↑ 90, 180 y 360 d	↑ 90 a 360 d	↑ 360 d	↑ 90, 180 y 360 d	↑ 270 d	↑ 270 y 360 d
Act. aminopeptidasa (Lys)	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d
Act. aminopeptidasa (Leu)	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d	↓ hasta 360 d
Aminoácidos libres totales	↓ 90 a 360 d	↓ 90 a 360 d	NS	↓ 270 y 360 d	↑ 180 d	↓ 360 d
Proteolisis global (OPA)	↓ 180 a 360 d	↓ 90 a 360 d	NS	↓ 180 y 360 d	NS	↓ 180 y 360 d
Tiramina	↓ 360 d	↓ 360 d	↓ 360 d	↓ 360 d	↓ 360 d	↓ 360 d
Putrescina	NS	NS	NS	↑ 270 y 360 d	NS	↑ 270 y 360 d
Triptamina	↑ 360 d	NS	NS	↑ 360 d	NS	↑ 360 d
Feniletilamina	↓ 180 d, ↑ 360 d	↑ 360 d	↓ 180 d	↓ 180 d	↓ 180 d, ↑ 270 d	↓ 180 d, ↑ 270 y 360 d
Aminas totales	NS	↓ 180 d	↓ 180 a 360 d	↓ 180 d	NS	↑ 360 d
Actividad esterasa	↓ 21, 42, 180 a 360 d	↓ hasta 360 d	↓ 42 d	↓ 42, 270 y 360 d	↓ 360 d	↓ 63, 180 a 360 d
Etanoico + propanoico	↓ 42 a 360 d	↓ hasta 360 d	↓ 42 y 63 d	↓ 42, 63, 180 a 360 d	↓ 180 d	↓ 90 a 360 d
AGL cadena corta	↓ 42, 63, 180 a 360 d	↓ 21 a 63, 180 a 360 d	↑ 63 y 90 d	↑ 90 d	↑ 63 d	↑ 90 d
AGL cadena media	↓ 42, 180 y 270 d	↓ 21, 42, 180 a 360 d	NS	NS	NS	NS
AGL cadena larga	↓ 42, 180 y 270 d	↓ 21, 42, 180 a 360 d	NS	NS	NS	NS
Ácidos volátiles	↓ 180 y 360 d	↓ 180 y 360 d	NS	NS	NS	NS
Alcoholes volátiles	↓ 180 y 360 d	↓↓ 180 y 360 d	↓ 180 d	↓↓ 180 y 360 d	↓ 180 y 360 d	↓↓ 180 y 360 d
Aldehídos volátiles	NS	↓ 180 d	NS	↓ 180 d	NS	↓ 180 d
Cetonas volátiles	NS	↓ 180 y 360 d	↓ 180 d	NS	↓ 180 d	↓ 360 d
Ésteres volátiles	↓ 180 y 360 d	↓↓ 180 y 360 d	↓ 360 d	↓ 180 y 360 d	↓ 180 y 360 d	↓ 180 y 360 d
Hidrocarburos volátiles	NS	↓ 180 d	NS	NS	↓ 180 d	↓ 180 y 360 d
Bencénicos volátiles	↓↓ 180 y 360 d	↓↓ 180 y 360 d	NS	NS	↓ 180 y 360 d	↓ 360 d
Azufrados volátiles	NS	NS	NS	NS	NS	NS
Nitrogenados volátiles	↓ 360 d	↓ 360 d	NS	NS	NS	NS
Terpenos volátiles	NS	↓ 180 y 360 d	NS	NS	NS	NS

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos. OPA; *o*-ftalaldehído (técnica espectrofotométrica).

La acumulación de aminas biógenas totales únicamente se vio atenuada por los tratamientos de 400 MPa aplicados a las 6 semanas de maduración, mientras que los tratamientos de 600 MPa aplicados a las 9 semanas aumentaron los niveles de aminas biógenas totales. En ninguno de los quesos tratados con altas presiones se lograron mantener las aminas biógenas totales a día 360 en niveles similares a los del control de 180 días. Las altas presiones lograron reducir la acumulación de tiramina a día 360, aunque sus niveles fueron superiores a los del queso control de 180 días. Los quesos tratados con 400 MPa a las 3 semanas y los tratados con 600 MPa a las 6 y 9 semanas alcanzaron a día 360 niveles de triptamina superiores a los del queso control, mientras que los quesos tratados con 400 MPa a las 3 semanas y los tratados con 600 MPa a las 3 y 9 semanas alcanzaron niveles superiores de β-feniletilamina a día 360 y los quesos tratados con 600 MPa a las 6 y 9 semanas alcanzaron a día 360 niveles superiores de putrescina. La liberación de enzimas al medio, debida a la lisis de microorganismos con actividad descarboxilasa, pudo causar el aumento de los niveles de aminas biógenas en algunos de los quesos tratados.

Tabla 6. Tendencias de las características sensoriales del queso azul tratado por alta presión con respecto al queso control.

Parámetro	400-3S	600-3S	400-6S	600-6S	400-9S	600-9S
Calidad sabor	↓ 90 d	↓ 90 a 360 d	NS	NS	NS	NS
Intensidad sabor	NS	↓ 180 a 360 d	NS	NS	NS	NS
Sabor umami	NS	NS	NS	NS	NS	NS
Sabor ácido	NS	NS	NS	NS	NS	NS
Sabor amargo	NS	NS	NS	NS	NS	NS
Sabor dulce	NS	NS	NS	NS	NS	NS
Sabor salado	NS	NS	NS	NS	NS	NS

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos.

La reducción de la actividad esterasa causada por los tratamientos de 600 MPa y el tratamiento de 400 MPa aplicado a las 3 semanas atenuó la formación de ácidos grasos de cadena corta, pero únicamente los quesos tratados a las 3 semanas mantuvieron a día 360 niveles de ácidos grasos de cadena corta similares o inferiores a los del queso control de 180 días. Los niveles de ácidos grasos libres de cadena media de los quesos tratados con 400 MPa a las 3 semanas y los tratados con 600 MPa se mantuvieron hasta

el día 360 en niveles similares o inferiores a los del queso control de 180 días, mientras que los niveles de ácidos grasos libres de cadena larga se mantuvieron en niveles ligeramente superiores a los del queso control de 180 días en los quesos tratados con 400 MPa a las 3 semanas y los tratados con 600 MPa a las 6 y 9 semanas, y en niveles inferiores en los quesos tratados con 600 MPa a las 3 semanas. Esta reducción de los niveles de ácidos grasos libres se ha observado en otras variedades de queso al ser tratados por alta presión (Saldo *et al.*, 2003, Rynne *et al.*, 2008). Los ácidos etanoico y propanoico se mantuvieron en niveles más bajos que los del queso control de 180 días en todos los quesos tratados, especialmente en los quesos tratados a las 3 semanas y los tratados con 600 MPa a las 6 y 9 semanas.

Los distintos grupos de compuestos volátiles se vieron afectados de diferente manera por los tratamientos de altas presiones. Así, los quesos tratados con 400 MPa a las 3 y 6 semanas tuvieron niveles de cetonas más altos que los del queso control de 180 días, mientras que los tratados con 600 MPa se mantuvieron en niveles más bajos, y únicamente en los quesos tratados con 400 MPa a las 9 semanas se mantuvieron en niveles similares a los del control de 180 días. Todos los quesos tratados, especialmente los tratados con 600 MPa, mantuvieron niveles de alcoholes inferiores a los del queso control de 180 días. Únicamente los tratamientos aplicados a las 3 semanas consiguieron atenuar el aumento de ácidos volátiles manteniendo niveles similares a los del control de 180 días. Todos los tratamientos atenuaron la formación de ésteres, manteniéndose los quesos tratados con 400 MPa a las 6 y 9 semanas en niveles ligeramente inferiores a los del queso control de 180 días y el resto de quesos tratados en niveles muy inferiores. Los tratamientos de 600 MPa y el tratamiento de 400 MPa aplicado a las 3 semanas mantuvieron hasta el día 360 niveles de compuestos nitrogenados y azufrados similares o inferiores a los del queso control de 180 días. El aumento de los niveles de terpenos se consiguió atenuar con los tratamientos de altas presiones, manteniéndose los niveles de los quesos tratados a las 6 y 9 semanas similares a los del control de 180 días. El descenso de los aldehídos que se dio en el queso control se vio atenuado por las altas presiones, aunque todos los quesos tratados se mantuvieron en niveles más bajos que los del control de 180 días. Los quesos

tratados mantuvieron los niveles de hidrocarburos y compuestos bencénicos por debajo de los del queso control de 180 días.

Tabla 7. Valores de las características microbiológicas, químicas y sensoriales en queso azul control de 180 días, quesos tratados por alta presión de 180-360 días y queso control de 360 días.

Parámetro	Control 180 d	400-3S 6 a 12 m (rango)	600-3S 6 a 12 m (rango)	400-6S 6 a 12 m (rango)	600-6S 6 a 12 m (rango)	400-9S 6 a 12 m (rango)	600-9S 6 a 12 m (rango)	Control 360 d
pH	**5,5**	5,1 - 5,2	4,9 - 5,1	5,2 - 5,4	5,2 - 5,4	5,3 - 5,4	5,3 - 5,4	5,3
Aerobios totales[a]	**8,0**	5,4 - 6,1	3,9 - 4,3	4,6 - 6,7	3,5 - 3,8	4,1 - 5,9	3,8 - 4,1	5,6
Bacterias lácticas[a]	**8,0**	6,4 - 6,7	3,2 - 4,0	5,6 - 7,5	3,2 - 3,5	5,5 - 7,1	3,3 - 3,9	6,6
Lactobacilos[a]	**4,9**	3,4 - 3,7	1,0 - 1,8	2,6 - 4,5	1,2 - 1,5	2,5 - 4,1	1,3 - 1,9	3,6
P. roqueforti[a]	**7,1**	2,8 - 4,4	nd	3,4 - 5,9	nd	2,6 - 5,8	nd	5,7
α-caseína[b]	**0,0**	0,0 - 0,5	0,0 - 0,2	0,0 - 0,07	0,0 - 0,3	0,0 - 0,06	0,0 - 0,0	0,0
β-caseína[b]	**0,80**	0,13 - 1,20	0,17 - 1,98	0,16 - 0,62	0,32 - 0,66	0,07 - 0,33	0,09 - 0,39	0,05
p-κ-caseína[b]	**3,5**	1,3 - 2,9	2,0 - 4,5	0,6 - 2,6	1,2 - 3,9	0,6 - 1,7	1,3 - 2,6	1,2
Péptidos hidrófilos[c]	**28,1**	27,8 - 31,8	25,9 - 29,4	26,7 - 33,1	27,6 - 29,2	27,9 - 36,1	27,1 - 31,9	29,8
Péptidos hidrófobos[c]	**4,8**	4,1 - 7,6	6,5 - 9,1	4,8 - 6,8	5,4 - 7,1	5,9 - 6,9	5,2 - 7,9	4,5
Ratio hidrófobos/filos	**0,17**	0,15 - 0,24	0,25 - 0,31	0,16 - 0,23	0,19 - 0,24	0,19 - 0,22	0,19 - 0,25	0,15
Act. aminopeptidasa (Lys)[d]	**6,3**	1,0 - 3,7	1,0 - 3,2	1,3 - 3,3	0,9 - 2,4	1,7 - 2,9	0,8 - 2,2	3,2
Act. aminopeptidasa (Leu)[d]	**6,7**	1,1 - 4,4	1,4 - 4,8	1,4 - 3,3	0,9 - 2,6	1,8 - 2,9	0,9 - 2,7	3,3
Aminoácidos libres totales[b]	**88,6**	60,1 - 81,5	36,5 - 54,3	91,0 - 116,7	81,0 - 95,8	92,9 - 123,5	79,0 - 94,8	116,8
Proteolisis global (OPA)[e]	**8,3**	6,5 - 8,1	4,5 - 5,5	8,6 - 11,4	7,3 - 8,8	8,9 - 10,4	7,0 - 9,0	10,4
Tiramina[b]	**0,007**	0,008 - 0,032	0,007 - 0,033	0,005 - 0,021	0,006 - 0,032	0,006 - 0,031	0,005 - 0,027	0,052
Putrescina[b]	**0,017**	0,015 - 0,034	0,016 - 0,040	0,013 - 0,033	0,020 - 0,047	0,013 - 0,038	0,019 - 0,050	0,033
Triptamina[b]	**0,062**	0,067 - 0,103	0,046 - 0,077	0,054 - 0,075	0,043 - 0,104	0,069 - 0,094	0,058 - 0,120	0,071
Feniletilamina[b]	**0,013**	0,008 - 0,074	0,012 - 0,078	0,005 - 0,066	0,008 - 0,063	0,004 - 0,080	0,007 - 0,092	0,061
Aminas biógenas totales[b]	**0,10**	0,10 - 0,24	0,08 - 0,23	0,08 - 0,18	0,08 - 0,24	0,09 - 0,23	0,09 - 0,29	0,22
Actividad esterasa[f]	**33,6**	8,4 - 12,5	4,3 - 7,7	26,2 - 34,5	6,0 - 28,4	11,6 - 32,0	8,5 - 19,8	28,2
Etanoico + propanoico[b]	**1,7**	0,5 - 0,6	0,3 - 0,4	1,1 - 1,4	0,7 - 0,9	0,9 - 1,4	0,6 - 0,9	1,4
AGL cadena corta[b]	**5,1**	1,9 - 5,2	1,6 - 2,2	6,4 - 11,8	4,4 - 7,2	6,0 - 10,0	4,6 - 7,6	8,9
AGL cadena media[b]	**12,2**	2,7 - 11,8	3,8 - 4,5	14,3 - 25,7	9,3 - 13,6	12,3 - 18,6	8,0 - 14,4	18,3
AGL cadena larga[b]	**36,1**	10,6 - 38,3	12,2 - 15,1	41,1 - 66,9	31,2 - 44,2	39,8 - 54,7	32,9 - 45,0	56,6
Ácidos volátiles[g]	**2465**	1517 - 2955	1441 -2090	2631 - 4455	2506 -3611	2804 - 4390	2844 - 3971	4211
Alcoholes volátiles[g]	**1452**	744 - 806	209 - 239	481 - 512	1070 - 1281	775 - 881	242 - 332	1496
Aldehídos volátiles[g]	**15**	11 - 15	5 - 9	7 - 15	7 - 12	9 - 10	5 - 11	11
Cetonas volátiles[g]	**1285**	809 - 1717	397 - 670	355 - 1837	847 - 1005	649 - 1280	534 - 726	1364
Ésteres volátiles[g]	**868**	200 - 420	47 - 61	698 - 760	284 - 311	506 - 841	173 - 190	1493
Bencénicos volátiles[g]	**17**	8 - 9	5 - 6	12 - 14	13 - 14	10 - 11	11 - 14	16
Hidrocarburos volátiles[g]	**10**	8 - 8	6 - 6	6 - 7	7 - 7	6 - 6	5 - 6	10
Terpenos volátiles[g]	**1,5**	1,0 - 1,1	0,6 - 0,9	1,4 - 1,7	1,6 - 1,7	1,4 - 1,5	1,4 - 1,5	1,9
Nitrogenados volátiles[g]	**3,4**	2,3 - 2,9	2,4 - 3,1	3,9 - 4,1	3,3 - 3,7	3,8 - 4,4	3,3 - 3,5	5,4
Calidad de sabor	**7,2**	6,2 - 6,5	5,2 - 5,5	7,1 - 7,3	6,5 - 6,6	6,6 - 7,2	6,6 - 7,5	7,0
Intensidad de sabor	**7,3**	7,1 - 7,3	6,3 - 6,5	7,0 - 7,9	7,1 - 7,4	7,3 - 7,4	6,8 - 7,5	7,6

nd: no detectado, OPA: *o*-ftalaldehído, [a]: (log ufc/g), [b]: (mg/g ES), [c]: (UA/mg ES), [d]:(nmol de *p*-nitroanilina/min g), [e]: (unidades de absorbancia), [f]: (pmol α-naftol/min g), [g]: (abundancia relativa).

Las altas presiones atenuaron levemente el aumento de la intensidad de sabor, excepto en los quesos tratados con 400 MPa a las 6 semanas que presentaron valores más altos a día 360. Los quesos tratados con 600 MPa a las 3 semanas fueron los que obtuvieron menor puntuación de intensidad de sabor. La calidad de sabor de los quesos tratados se mantuvo en valores similares o ligeramente inferiores a los del queso control de 180 días, excepto en los quesos tratados con 600 MPa a las 3 semanas que recibieron la puntuación de calidad de sabor más baja.

Las altas presiones provocaron el hundimiento de la parte central del queso, que llegó a ser de un 36 % respecto al borde en los quesos tratados con 400 MPa a las 3 semanas.

Considerando estos resultados junto con los del análisis de componentes principales (recogido en el capítulo 3), que discrimina única y escasamente entre tiempos, se puede concluir que los tratamientos de altas presiones afectaron muy levemente al queso azul. El queso control no experimentó apenas variaciones entre su momento óptimo de consumo (6 meses) y el final del periodo de almacenamiento en refrigeración (12 meses). Los cambios bioquímicos durante el almacenamiento en refrigeración no afectaron a la calidad global del queso, aunque sí se produjo un cierto aumento de aminas biógenas. Las altas presiones no consiguieron evitar la formación de aminas biógenas y sin embargo se produjo cierta pérdida de calidad de sabor en los quesos tratados con 600 MPa a las 3 semanas. Se puede así concluir que la aplicación de altas presiones no representa una mejora adicional en la conservación del queso azul, al menos en lo que respecta a la variedad estudiada y en las condiciones empleadas en el presente trabajo.

- **Torta del Casar**

La Torta del Casar se caracteriza por ser un queso elaborado con leche cruda de oveja coagulada con cuajo vegetal obtenido de las flores del cardo (*Cynara cardunculus*). En la Torta del Casar se estudió la evolución de las variables microbiológicas, químicas, de textura y sensoriales durante la maduración y el almacenamiento en refrigeración hasta los 240 días. Mediante los análisis microbiológicos, se observó un aumento en los niveles de bacterias mesófilas totales y bacterias lácticas entre los días 1 y 21, desde 7,74 y 7,56 log ufc/g respectivamente

hasta 9,51 y 9,46 log ufc/g. Posteriormente los niveles fueron reduciéndose lentamente hasta 8,61 y 8,42 log ufc/g a día 240. De forma similar, los niveles de enterococos aumentaron entre el día 1 y 21 desde 6,50 log ufc/g hasta 7,32 log ufc/g, reduciéndose hasta 6,80 log ufc/g a día 240, y los niveles de lactobacilos aumentaron entre el día 1 y el día 35 de 5,14 log ufc/g hasta 8,13 log ufc/g, manteniéndose en los mismos niveles hasta el día 240. Los niveles de *Micrococcaceae*, coliformes y bacterias gram negativas descendieron desde 6,39, 5,52 y 6,01 log ufc/g respectivamente a día 1 hasta 5,75, 2,87 y 5,37 log ufc/g a día 240. Los niveles de estafilococos coagulasa positivos descendieron desde 5,23 log ufc/g a día 1 hasta 2,30 log ufc/g a día 180, manteniéndose por debajo del límite de detección a día 240. Los valores de pH descendieron de 6,49 a día 1 hasta 5,19 a día 21, y aumentaron posteriormente hasta valores de 6,13 al final del periodo de almacenamiento en refrigeración, mientras que el extracto seco aumentó desde 45,56 % a día 1 hasta 54,67 % a día 240.

El empleo de cuajo vegetal en la elaboración de este queso hizo que la concentración de la κ-CN disminuyese un 96 % entre los días 1 y 21, no volviéndose a detectar a partir del día 35. La α_S- y la β-CN sufrieron reducciones del 79 y 62 % respectivamente entre los días 1 y 35. Descensos similares se han registrado en la misma variedad de queso (Delgado *et al.*, 2010b) y en queso Serra elaborado con cuajo vegetal (Macedo & Malcata, 1997). La *p*-κ-caseína sufrió reducciones menores durante la maduración y el posterior almacenamiento en refrigeración. Los péptidos hidrófilos aumentaron con el tiempo, alcanzando un valor de 41,99 UA/mg ES a día 240, mientras que los hidrófobos se mantuvieron en niveles en torno a 25 UA/mg ES, haciendo que el ratio de péptidos hidrófobos/hidrófilos se redujese a lo largo del tiempo hasta un valor de 0,64 a día 240. La actividad aminopeptidasa, con Leu–*p*-nitroanilida y Lys-*p*-nitroanilida como sustratos, alcanzó valores máximos de 80,67 y 38,10 nmoles de *p*-nitroanilina/min·g respectivamente a día 60, siendo valores muy superiores a los obtenidos en queso de la Serena durante la maduración (Garde *et al.*, 2007), y posteriormente se fue reduciendo hasta el final del periodo de refrigeración alcanzando valores de 6,42 y 4,67 nmoles de *p*-nitroanilina/min·g con Leu–*p*-nitroanilida y Lys-*p*-nitroanilida respectivamente a día 240. La concentración de aminoácidos libres aumentó desde 4,82 mg/g ES a día 60, valor similar a los obtenidos en Torta del Casar y queso de

la Serena durante la maduración (Garde *et al.*, 2007, Delgado *et al.*, 2010b) hasta 23,30 mg/g ES a los 240 días. La proteolisis global (estimada por el método OPA) aumentó igualmente a lo largo del tiempo.

La actividad tirosina descarboxilasa se triplicó entre los días 60 y 180, provocando un aumento de la concentración de tiramina desde 0,37 mg/g ES a día 60 hasta 1,09 mg/g ES a día 240. El resto de aminas biógenas detectadas aumentaron igualmente durante el almacenamiento en refrigeración. La putrescina, triptamina, β-feniletilamina, cadaverina e histamina aumentaron desde 0,29, 0,07, 0,07, 0,16 y 0,14 mg/g ES, respectivamente, a día 60 hasta 0,95, 0,83, 0,29, 0,69 y 0,30 mg/g ES a día 240. Las aminas biógenas totales aumentaron desde 1,09 mg/g ES a día 60 hasta 3,69 mg/g ES a día 240, siendo niveles muy superiores a los encontrados en diferentes variedades de quesos de leche cruda (Ordóñez *et al.*, 1997, Fernández-García *et al.*, 1999, Fernández-García *et al.*, 2000).

La actividad esterasa aumentó hasta 9,64 pmoles de α-naftol/min·g a día 60 y posteriormente se redujo hasta 5,28 pmoles de α-naftol/min·g a día 120, manteniéndose en estos niveles hasta el día 240. Los niveles de ácidos grasos libres aumentaron a lo largo de la maduración y del almacenamiento en refrigeración. En esta variedad de queso la hidrólisis de triglicéridos es debida, principalmente, a la acción de la lipoproteína lipasa presente en la leche, la cual tiene preferencia por hidrolizar los ácidos grasos situados en la posición sn-3 del triglicérido, donde se sitúan preferentemente ácidos grasos de menos de 10 átomos de carbono (Collins *et al.*, 2003, Deeth, 2006), los cuales contribuyen a su sabor característico. Los ácidos grasos libres de cadena corta fueron los más abundantes durante la maduración y el almacenamiento en refrigeración, al igual que se observó en otro estudio sobre esta variedad de queso (Delgado *et al.*, 2009) en el que aumentaron hasta 8,69 mg/g ES a día 240. Los ácidos grasos libres de cadena media y larga llegaron a alcanzar 0,88 y 3,02 mg/g ES, respectivamente, al final del almacenamiento en refrigeración. El ácido etanoico, procedente del metabolismo de lactosa, lactato y citrato (McSweeney & Sousa, 2000), fue el más abundante hasta el día 180, aportando su típico aroma a vinagre, mientras que a día 240 el más abundante fue el ácido butírico, aportando aroma típico de queso rancio (Curioni & Bosset, 2002). Los ácidos de cadena ramificada,

2-metilpropanoico y 3-metilbutanoico, procedentes del catabolismo de la valina y leucina respectivamente (Yvon & Rijnen, 2001), aumentaron a lo largo de la maduración y el periodo de almacenamiento en refrigeración aportando aromas a sudor, rancio y pútrido.

El grupo de compuestos volátiles más abundante al final de la maduración fue el de los ácidos volátiles, mientras que el más abundante durante el almacenamiento en refrigeración fue el grupo de los alcoholes, siendo el 2-butanol, formado por reducción de la 2-butanona (Molimard & Spinnler, 1996), el alcohol mayoritario. Todos los grupos aumentaron durante el almacenamiento en refrigeración, excepto el de ácidos volátiles, que disminuyó. Cabe destacar el drástico aumento del grupo de compuestos azufrados, provocado principalmente por el aumento de dimetildisulfuro, que no se había descrito en esta variedad de queso (Delgado *et al.*, 2010a), pero sí en queso de la Serena, aunque en menor concentración (Carbonell *et al.*, 2002). Este compuesto, que aporta al queso aromas de azufre y pútrido, tiene un bajo umbral de percepción (Curioni & Bosset, 2002). La gran acumulación de dimetildisulfuro durante el almacenamiento en refrigeración fue una de las causas de la pérdida de calidad de sabor del queso control durante este periodo.

Durante el almacenamiento en refrigeración la firmeza y la elasticidad descendieron a lo largo del tiempo, al igual que en otro estudio sobre la Torta del Casar (Delgado *et al.*, 2010b), debido a la fuerte hidrólisis de la matriz de caseínas, especialmente α_{S1}-caseína, y al aumento de pH, según la teoría de Creamer & Olson (1982). La calidad de sabor y de olor disminuyeron significativamente, mientras que la intensidad de sabor y de olor, el sabor amargo y el olor a pútrido y rancio aumentaron durante el almacenamiento en refrigeración.

El almacenamiento en condiciones de refrigeración no fue suficiente para frenar los procesos bioquímicos responsables de la formación de los compuestos implicados en el aroma y sabor de la Torta del Casar. Esto provocó el desequilibrio de algunos de estos compuestos, produciéndose una pérdida de la calidad del sabor y del olor a partir de los 120 días. Junto a esta pérdida de calidad, también se encontró que los niveles de aminas biógenas presentes en el queso control a partir de los 120 días fueron excesivos, pudiendo provocar efectos nocivos para la salud del consumidor.

Tabla 8. Tendencias de las características microbiológicas y químicas de la Torta del Casar tratada por alta presión con respecto al queso control.

Parámetro	400-3S	600-3S	400-5S	600-5S
ES	NS	↑ 120 a 240 d	↑ 120 a 240 d	↑ 120 a 240 d
pH	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
Aerobios totales	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Bacterias lácticas	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Lactobacilos	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Micrococos	↓ 120 a 240 d	↓ 120 a 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Gram negativos	↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
Coliformes	↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
Estafilococos coagulasa +	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
Enterococos	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Actividad de cardosinas	NS	NS	↑ 240 d	NS
Actividad peptidolítica	NS	↓ 60 d	NS	↓ 60 d
α-caseína	NS	↑ 35 y 60 d	NS	↑ 60 d
β-caseína	NS	↑ 120 a 240 d	NS	↑ 180 y 240 d
κ-caseína	NS	NS	NS	NS
p-κ-caseína	NS	↑ 180 d	NS	↑ 180 y 240 d
Péptidos hidrófilos	↑ 60 d, ↓ 240 d	↓ 240 d	↓ 60 y 240 d	↓ 240 d
Péptidos hidrófobos	↑ 180 d	NS	↓ 60 y 120 d	↓ 120 d
Ratio hidrófobos/filos	↓ 60 d	↓ 60 d	NS	NS
Act. aminopeptidasa (Lys)	↓ 60 d	↓ 35, 60 y 120 d	↓ 35 y 60 d	↓ 35 y 60 d
Act. aminopeptidasa (Leu)	NS	↓ 35, 60 y 120 d	↓ 35 y 60 d	↓ 35 y 60 d
Aminoácidos libres totales	↑ 35 y 60 d	↓ 240 d	↑ 180 d	NS
Proteolisis global (OPA)	NS	↓ 35 a 240 d	NS	↓ 60 a 240 d
Actividad TDC	↓ 60 y 180 d	↓ 60 y 180 d	↓ 60 y 180 d	↓ 60 y 180 d
Tiramina	NS	↓ 60 a 240 d	NS	↓ 60 a 240 d
Putrescina	↓ 35 a 120 d	↓↓ 35 a 240 d	↓ 60 a 240 d	↓↓ 60 a 240 d
Triptamina	↓ 35 y 60 d	↓ 35, 60, 120 y 240 d	↓ 35, 60 y 240 d	↓↓ 35 a 240 d
Feniletilamina	↓ 35 a 120 d	↓ 35 a 240 d	↓ 35 a 240 d	↓ 35 a 240 d
Cadaverina	↓ 35 a 240 d	↓ 35 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d
Histamina	↓ 35 y 60 d	↓↓ 35 a 240 d	↓ 60 d	↓↓ 60 a 240 d
Aminas totales	↓ 60 a 240 d	↓↓ 60 a 240 d	↓ 60 a 240 d	↓↓ 60 a 240 d
Actividad esterasa	↓ 35, 180 y 240 d	↓↓ 35 a 240 d	↓ 35 y 240 d	↓ 35 a 240 d
Etanoico + propanoico	↓↓ 35 a 240 d	↓↓ 35 a 240 d	↓ 120 y 240 d	↓ 120 y 240 d
AGL cadena ramificada	↓↓ 35 a 240 d	↓↓ 35 a 240 d	↓ 120 y 240 d	↓ 120 y 240 d
AGL cadena corta	↓ 35 a 240 d	↓ 35 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
AGL cadena media	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
AGL cadena larga	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
Ácidos volátiles	↓ 60 a 240 d	↓ 60 a 240 d	NS	NS
Alcoholes volátiles	↓ 60 y 120 d	↓ 60 a 240 d	↓ 120 d	↓ 120 y 240 d
Aldehídos volátiles	↓ 120 y 240 d	↓ 120 y 240 d	↓ 120 y 240 d	↓ 120 y 240 d
Cetonas volátiles	↓↓ 60 d	NS	↓ 60 y 120 d	↓ 120 d
Ésteres volátiles	↓↓ 60 a 240 d	↓↓ 60 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d
Éteres volátiles	↓ 120 d	↓ 120 d	↓ 120 d	NS
Hidrocarburos volátiles	NS	NS	NS	NS
Bencénicos volátiles	NS	NS	NS	NS
Azufrados volátiles	↓↓ 120 y 240 d	↓↓ 120 y 240 d	↓↓ 120 y 240 d	↓↓ 120 y 240 d

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos. OPA; *o*-ftalaldehído (técnica espectrofotométrica), TDC; tirosina descarboxilasa.

Con el objetivo de reducir la pérdida de calidad y la acumulación de aminas biógenas en la Torta del Casar se aplicaron tratamientos de 400 y 600 MPa a las 3 semanas (400-3S y 600-3S) y a las 5 semanas (400-5S y 600-5S) de maduración. Estos tratamientos ocasionaron una serie de cambios significativos respecto al control en los diferentes tiempos (Tablas 8 y 9). Por otro lado, comparando los quesos tratados a lo largo del almacenamiento en refrigeración con el queso control en su momento óptimo (60 días), se puede apreciar que en la mayoría de los casos se logró evitar o atenuar los efectos negativos asociados a la sobremaduración (Tablas 10 y 11).

Tabla 9. Tendencias de las características de textura y sensoriales de la Torta del Casar tratada por alta presión con respecto al queso control.

Parámetro	400-3S	600-3S	400-5S	600-5S
Fracturabilidad	NS	NS	NS	NS
Firmeza	↓↓ 35 y 180 d	↑ 120 d	NS	NS
Elasticidad	↓↓ 35 a 180 d	↑ 120 d	NS	NS
Calidad sabor	↑ 120 a 240 d	↑ 120 a 240 d	↑ 120 a 240 d	↑ 120 a 240 d
Intensidad sabor	NS	↓ 60 a 240 d	NS	↓ 180 y 240 d
Sabor umami	NS	↓ 120 d	NS	NS
Sabor ácido	NS	NS	NS	NS
Sabor amargo	NS	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
Sabor dulce	NS	NS	NS	↑ 180 d
Sabor salado	NS	NS	NS	NS
Calidad de olor	↑ 240 d	↑ 240 d	↑ 120 a 240 d	↑ 240 d
Intensidad de olor	↓ 240 d	↓ 240 d	↓ 240 d	↓ 240 d
Olor pútrido	↓ 240 d	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d
Olor rancio	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d	↓ 120 a 240 d

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos.

La aplicación de tratamientos de altas presiones provocó una reducción de los niveles de microorganismos, que en el caso de los tratamientos de 600 MPa fue acompañado de la inactivación de las enzimas proteolíticas, provocando una reducción de la hidrólisis de caseínas. Los niveles de α_S-, β- y *p*-κ-caseínas en los quesos tratados con 600 MPa se mantuvieron durante todo el periodo de almacenamiento en refrigeración en niveles similares o superiores a los del queso control de 60 días. La hidrólisis de caseínas, tanto en el queso control como en los tratados por alta presión, es debida a la acción de la plasmina, que mantiene parte de su actividad incluso después de ser tratada a 800 MPa (Malone *et al.*, 2003), y a las cardosinas, que no

perdieron actividad por las altas presiones sino que, por el contrario, aumentó a día 240 con el tratamiento de 400 MPa aplicado a las 5 semanas. Sin embargo, en queso de la Serena tratado a 300 y 400 MPa, sí que se redujo la hidrólisis de caseínas (Garde *et al.*, 2007).

Los péptidos hidrófobos se mantuvieron en los quesos tratados a las 3 semanas en niveles similares a los del queso control a los 60 días durante el almacenamiento en refrigeración, mientras que en los quesos tratados a las 5 semanas se mantuvieron en niveles inferiores. Por el contrario, los niveles de péptidos hidrófilos fueron superiores a los del queso control de 60 días durante el almacenamiento de todos los quesos tratados, de igual forma que sucedió en queso de la Serena con los tratamientos aplicados a día 2 (Garde *et al.*, 2007). La reducción de la actividad aminopeptidasa provocada por los tratamientos de altas presiones no consiguió que durante el almacenamiento en refrigeración los niveles de aminoácidos libres de los quesos tratados se mantuviesen por debajo de los niveles del queso control de 60 días, al igual que ocurrió en queso de la Serena con los tratamientos aplicados a día 2 (Garde *et al.*, 2007). La inactivación de algunas de las enzimas responsables del catabolismo de los aminoácidos fue posiblemente la causa de su acumulación en los quesos tratados. El efecto de las altas presiones sobre los aminoácidos se vio igualmente reflejado en la proteolisis global.

La reducción de la actividad de la tirosina descarboxilasa debida a las altas presiones hizo que los niveles de tiramina durante el almacenamiento de los quesos tratados con 600 MPa fuesen similares a los del queso control de 60 días. Todos los tratamientos consiguieron mantener niveles de cadaverina hasta los 240 días similares a los del queso control de 60 días. La triptamina y la β-feniletilamina se mantuvieron en niveles similares o ligeramente superiores a los del queso control a día 60 durante el almacenamiento de los quesos tratados con 600 MPa y de los tratados con 400 MPa a las 5 semanas. Excepto el tratamiento de 400 MPa aplicado a las 3 semanas, las altas presiones redujeron los niveles de putrescina, aunque únicamente los tratamientos de 600 MPa mantuvieron niveles inferiores a los del queso control de 60 días. Los tratamientos de 600 MPa redujeron además los niveles de histamina, manteniendo niveles inferiores a los del queso control de 60 días.

Tabla 10. Valores de las características microbiológicas y químicas en Torta del Casar control de 60 días, quesos tratados por alta presión de 60-240 días y queso control de 120-240 días (sobremadurado).

Parámetro	Control 60 días	400-3S 60 a 240 d (rango)	600-3S 60 a 240 d (rango)	400-5S 60 a 240 d (rango)	600-5S 60 a 240 d (rango)	Control 120 a 240 d (rango)
ES (%)	**51,2**	51,6 - 54,9	51,7 - 56,3	53,0 - 57,0	52,4 - 57,3	52,2 - 54,7
pH	**5,4**	5,4 - 5,7	5,4 - 5,6	5,3 - 5,7	5,4 - 5,6	5,8 - 6,1
Aerobios totales[a]	**9,4**	8,0 - 8,5	5,4 - 6,3	7,1 - 7,9	4,3 - 5,0	8,6 - 9,2
Bacterias lácticas[a]	**9,2**	8,0 - 8,1	5,0 - 5,7	6,8 - 7,7	3,7 - 4,0	8,4 - 8,9
Lactobacilos[a]	**8,3**	7,7 - 8,0	3,3 - 4,4	6,6 - 6,9	2,3 - 3,1	8,0 - 8,4
Enterococos[a]	**7,2**	5,8 - 6,5	2,4 - 3,4	5,2 - 6,4	nd - 2,5	6,8 - 7,1
Micrococos[a]	**6,6**	5,2 - 6,3	3,7 - 6,3	3,1 - 4,7	2,4 - 3,6	5,8 - 6,0
Gram negativos[a]	**6,9**	nd - 3,2	nd	nd	nd	5,4 - 6,5
Coliformes[a]	**4,4**	nd - 1,0	nd	nd	nd	2,4 - 3,4
Estafilococos coagulasa +[a]	**2,6**	nd	nd	nd	nd	nd - 2,2
α-caseína [b]	**5,8**	3,2 - 6,4	5,0 - 8,6	3,1 - 5,9	5,1 - 8,8	3,8 - 5,5
β-caseína [b]	**81,9**	39,3 - 80,9	84,8 - 103,2	40,2 - 87,5	81,2 - 102,4	41,8 - 58,1
p-κ-caseína [b]	**16,1**	10,3 - 15,4	13,8 - 15,1	10,3 - 15,5	14,7 - 16,9	11,3 - 13,5
Péptidos hidrófilos[c]	**13,2**	17,2 - 33,2	15,4 - 27,7	12,8 - 32,8	14,4 - 31,0	24,1 - 42,0
Péptidos hidrófobos[c]	**24,7**	23,9 - 26,4	24,2 - 24,7	20,0 - 22,7	19,9 - 24,4	22,9 - 26,7
Ratio hidrófobos/filos	**1,9**	0,8 - 1,6	0,9 - 1,6	0,6 - 1,7	0,8 - 1,6	0,6 - 1,1
Act. aminopeptidasa (Lys)[d]	**38,1**	5,0 - 25,7	2,8 - 7,2	4,3 - 9,3	3,8 - 14,1	4,7 - 11,3
Act. aminopeptidasa (Leu)[d]	**80,7**	5,5 - 67,5	4,5 - 19,1	4,4 - 41,3	5,8 - 35,2	6,4 - 31,0
Aminoácidos libres totales[b]	**4,8**	7,4 - 25,4	5,0 - 16,5	6,5 - 26,0	5,5 - 17,5	13,6 - 23,3
Proteolisis global (OPA)[e]	**0,8**	0,9 - 3,4	0,7 - 2,0	0,9 - 3,1	0,8 - 2,0	1,7 - 3,5
TDC [f]	**0,7**	0,03 - 0,2	0,05 - 0,1	0,1 - 0,7	0,00 - 0,06	3,0
Tiramina [b]	**0,4**	0,4 - 1,0	0,2 - 0,4	0,3 - 0,8	0,3 - 0,5	0,6 - 1,1
Putrescina [b]	**0,3**	0,2 - 1,2	0,1 - 0,1	0,2 - 0,5	0,2 - 0,2	0,6 - 1,0
Triptamina [b]	**0,07**	0,01 - 0,35	0,00 - 0,13	0,03 - 0,13	0,03 - 0,09	0,20 - 0,38
Feniletilamina [b]	**0,07**	0,02 - 0,25	0,00 - 0,12	0,04 - 0,12	0,03 - 0,12	0,18 - 0,29
Cadaverina [b]	**0,16**	0,05 - 0,09	0,04 - 0,13	0,09 - 0,13	0,09 - 0,15	0,49 - 0,69
Histamina [b]	**0,14**	0,10 - 0,41	0,00 - 0,02	0,09 - 0,38	0,04 - 0,08	0,20 - 0,30
Aminas totales [b]	**1,1**	0,7 - 3,3	0,4 - 0,9	0,8 - 2,0	0,7 - 1,0	2,3 - 3,7
Actividad esterasa[g]	**9,6**	2,1 - 8,5	0,8 - 1,7	2,9 - 7,6	1,1 - 5,8	5,3 - 5,6
Etanoico + propanoico [b]	**6,7**	4,1 - 5,5	3,7 - 3,9	6,3 - 7,2	5,7 - 7,1	7,7 - 8,8
AGL cadena ramificada [b]	**0,8**	0,3 - 0,4	0,3 - 0,3	0,6 - 0,8	0,6 - 0,8	0,8 - 1,2
AGL cadena corta [b]	**1,9**	0,6 - 1,1	0,7 - 2,3	1,0 - 2,0	1,0 - 2,6	3,4 - 8,7
AGL cadena media [b]	**0,4**	0,4 - 0,6	0,4 - 0,6	0,3 - 0,6	0,4 - 0,5	0,7 - 0,9
AGL cadena larga [b]	**1,4**	1,4 - 2,1	1,2 - 1,9	1,3 - 1,9	1,4 - 1,7	2,6 - 3,0
Ácidos volátiles[h]	**12275**	5239 - 6680	5246 - 7201	7571 - 9878	8537 - 10962	9483 - 10444
Alcoholes volátiles[h]	**9065**	5191 - 14701	4866 - 7450	9071 - 14065	7879 - 10067	13490 - 15914
Aldehídos volátiles[h]	**10,7**	9,3 - 12,6	10,8 - 13,3	9,7 - 13,3	9,3 - 12,3	15,8 - 17,1
Cetonas volátiles[h]	**1429**	152 - 1836	1411 - 2475	387 - 2117	1095 - 1625	1778 - 2147
Ésteres volátiles[h]	**3651**	1411 - 1533	862 - 1488	2753 - 3522	1502 - 2403	3984 - 5536
Éteres volátiles[h]	**14,4**	11,3 - 18,1	11,9 - 16,2	16,1 - 24,3	17,4 - 24,6	20,1 - 23,7
Azufrados volátiles[h]	**14,1**	7,3 - 66,6	7,5 - 78,1	3,1 - 10,2	8,0 - 14,6	1158 - 6583

nd: no detectado, OPA: *o*-ftalaldehído, TDC: actividad tirosina descarboxilasa, [a]: (log ufc/g), [b]: (mg/g ES), [c]: (UA/mg ES), [d]:(nmol de *p*-nitroanilina/min g), [e]: (unidades de absorbancia), [f]: (mUA/g ES) [g]: (pmol α-naftol/min g), [h]: (abundancia relativa).

La reducción de la actividad esterasa provocada por los tratamientos de altas presiones consiguió que los niveles de ácidos grasos libres de cadena corta, mayoritariamente ácido butanoico, fueran similares o inferiores a los del queso control de 60 días. Las altas presiones consiguieron atenuar la formación de ácidos grasos de cadena media y larga, aunque sus niveles no fueron inferiores a los del queso control de 60 días. La reducción de los niveles de ácidos grasos libres por las altas presiones se ha observado previamente en otras variedades de queso (Saldo *et al.*, 2003, Ávila *et al.*, 2007, Voigt *et al.*, 2010, Delgado *et al.*, 2012). En los quesos tratados, los niveles de ácido etanoico, propanoico y ácidos de cadena ramificada fueron inferiores o similares a los del queso control de 60 días. Debido a la reducción de los niveles de ácidos grasos de cadena corta, ácidos ramificados y ácido etanoico y propanoico, se consiguió reducir la aparición de los sabores no deseados que estos compuestos aportan al queso cuando se encuentran en grandes cantidades (Curioni & Bosset, 2002).

Tabla 11. Valores de las características de textura y sensoriales en Torta del Casar control de 60 días, quesos tratados por alta presión de 60-240 días y queso control de 120-240 días (sobremadurado).

Parámetro	Control 60 días	400-3S 60 a 240 d (rango)	600-3S 60 a 240 d (rango)	400-5S 60 a 240 d (rango)	600-5S 60 a 240 d (rango)	Control 120 a 240 d (rango)
Firmeza (J)	**0,014**	0,002 - 0,006	0,017 - 0,048	0,009 - 0,020	0,008 - 0,028	0,009 - 0,021
Elasticidad (N/mm^2)	**0,021**	0,001 - 0,009	0,016 - 0,044	0,005 - 0,021	0,010 - 0,029	0,011 - 0,025
Calidad de sabor	**6,4**	5,0 - 7,0	5,1 - 6,0	5,7 - 6,7	5,8 - 6,7	0,9 - 3,7
Intensidad de sabor	**6,4**	6,2 - 7,2	5,1 - 6,2	6,2 - 7,2	5,8 - 6,3	7,0 - 7,3
Sabor umami	**3,3**	3,0 - 4,6	2,5 - 3,8	3,3 - 4,1	3,1 - 3,9	3,9 - 4,4
Sabor amargo	**2,5**	2,6 - 3,9	1,5 - 3,0	2,0 - 3,9	2,0 - 2,6	3,9 - 4,6
Sabor dulce	**0,4**	0,3 - 0,6	0,3 - 0,8	0,2 - 0,5	0,4 - 0,5	0,1 - 0,4
Calidad de olor	**6,4**	4,8 - 6,2	4,9 - 6,0	5,3 - 6,5	5,6 - 6,2	1,9 - 4,6
Intensidad de olor	**5,9**	5,7 - 6,4	5,6 - 6,1	5,9 - 6,7	5,7 - 6,5	6,7 - 7,9
Olor pútrido	**1,0**	1,5 - 2,9	0,9 - 2,4	1,0 - 2,0	1,2 - 2,3	2,6 - 6,8
Olor rancio	**0,5**	0,8 – 1,6	0,5 - 1,8	0,4 - 1,5	0,6 - 1,4	1,9 - 3,3

Los tratamientos por altas presiones afectaron de forma diferente a los distintos grupos de compuestos volátiles. Las altas presiones provocaron que los ácidos volátiles se mantuvieran durante el almacenamiento en refrigeración en niveles inferiores a los del queso control de 60 días. Los tratamientos evitaron el drástico aumento de compuestos azufrados registrado en el queso control, manteniendo los niveles de los quesos tratados en valores similares a los del queso control de 60 días. Las altas

presiones atenuaron la formación de ésteres, manteniendo en niveles inferiores a los del queso control de 60 días todos los quesos tratados hasta el final del almacenamiento en refrigeración. También consiguieron mantener los niveles de aldehídos en valores similares o ligeramente superiores a los del queso control de 60 días. Los tratamientos aplicados a las 3 semanas consiguieron controlar el incremento de éteres, manteniendo niveles similares a los del queso control de 60 días, en especial con el tratamiento de 600 MPa, mientras que los alcoholes se mantuvieron en niveles inferiores a los del queso control de 60 días en los quesos tratados con 600 MPa. Los quesos tratados con 600 MPa a las 5 semanas mantuvieron niveles de cetonas similares o ligeramente superiores a los del queso control de 60 días hasta el día 240. Sin embargo, los tratamientos de altas presiones no consiguieron mantener en valores similares a los del control de 60 días los niveles de hidrocarburos y compuestos bencénicos.

En lo que respecta a las características de textura, las altas presiones no lograron estabilizar ni mantener en valores similares a los del queso control de 60 días los parámetros de firmeza y elasticidad. Las altas presiones consiguieron evitar el aumento de la intensidad de olor, y de olor pútrido y rancio, así como el desarrollo de sabor amargo. Únicamente los tratamientos de 600 MPa consiguieron reducir el aumento de la intensidad de sabor, gracias a sus efectos sobre los parámetros microbiológicos y químicos de la Torta del Casar. De modo que se logró controlar la considerable pérdida de calidad de sabor y olor, manteniendo a los quesos tratados con valoraciones de sus características sensoriales similares a las del queso control en su momento óptimo.

Por otra parte, el análisis de componentes principales (PCA) de ácidos grasos y de grupos de compuestos volátiles (recogido en el capítulo 6) indicó que la Torta del Casar tratada a las 5 semanas de maduración mantuvo un perfil de ácidos grasos y de compuestos volátiles muy similar al del queso control de 60 días. Por consiguiente, se puede concluir que la Torta del Casar tratada por altas presiones a las 5 semanas, especialmente con 600 MPa, mantiene en gran medida las características de esta variedad de queso en su momento óptimo de consumo (60 días) hasta los 8 meses de almacenamiento en refrigeración, evitando además la formación y acumulación de aminas biógenas.

- **Queso Brie**

El queso Brie se caracteriza por la presencia de *P. camemberti* en su superficie. Tiene un periodo de maduración de unos 21 días y sufre una rápida evolución del sabor así como un aumento de intensidad de sabor durante su vida útil. En esta variedad de queso se estudió la evolución de las variables microbiológicas, químicas, de textura, color y sensoriales durante la maduración y el almacenamiento en refrigeración hasta el día 120. Mediante los análisis microbiológicos, se observó una disminución gradual de los niveles de bacterias lácticas y bacterias mesófilas totales durante la maduración y el almacenamiento en refrigeración, desde 8,99 y 8,75 log ufc/g respectivamente a día 1 hasta 8,43 y 8,46 log ufc/g a día 21 y 7,88 y 7,90 log ufc/g a día 120. Los niveles de *P. camemberti* se mantuvieron prácticamente estables a lo largo de todo el periodo de estudio, con niveles máximos de 6,95 log ufc/g a los 60 días, mientras que las levaduras no se detectaron hasta el día 90, encontrándose entonces en niveles de 6,52 log ufc/g. El pH del queso sufrió un rápido aumento desde 5,55 a día 21 hasta 7,55 a día 60 y posteriormente aumentó hasta 7,87 a día 120. El extracto seco del queso se redujo de 45,48 a día 21 a 42,27 a día 60 y se mantuvo en estos valores hasta el día 120.

La presencia de plasmina, que mantiene parte de su actividad tras los tratamientos térmicos de pasteurización (Ismail & Nielsen, 2010), junto a la presencia de cuajo residual y de *P. camemberti* en la superficie, provocaron descensos de α_S-, β- y κ-caseínas del 41, 37 y 36 %, respectivamente, entre los días 1 y 14 de maduración. Posteriormente sus niveles se fueron reduciendo gradualmente hasta el día 30, momento en el que el pH alcanzó un valor de 6,3 favorable para la actividad de la plasmina residual y de la metaloproteinasa de *P. camemberti* (Lenoir & Auberger, 1977), lo que ocasionó una mayor hidrólisis de α_S-, β- y κ-caseínas. Por otro lado, la *p*-κ-caseína se mantuvo en los mismos niveles entre los días 14 y 90, descendiendo posteriormente. Los niveles de péptidos aumentaron durante el almacenamiento en refrigeración del queso Brie. Los péptidos hidrófilos aumentaron gradualmente hasta alcanzar 47,35 UA/mg ES a día 120, mientras que los péptidos hidrófobos sufrieron un drástico aumento a partir del día 30, alcanzando una concentración de 72,41 UA/mg ES a día 90, coincidiendo con el aumento de pH que provocó una mayor hidrólisis de caseínas. El ratio de péptidos hidrófobos/hidrófilos aumentó también drásticamente

entre los días 30 y 90. La relación encontrada entre este ratio y el sabor amargo en queso (Gomez *et al.*, 1997) se vio reflejada en la valoración sensorial del amargor, que alcanzó a día 90 la mayor puntuación, de 6,41 sobre 10. La actividad aminopeptidasa aumentó hasta el día 60, alcanzando valores de 7,36 y 8,26 nmoles de *p*-nitroanilina/min·g con Leu-*p*-NA y Lys-*p*-NA como sustratos, respectivamente. La alta actividad de las exopeptidasas de los mohos del género *Penicillium* hizo que los niveles de aminoácidos liberados durante la proteolisis aumentasen en función del tiempo, desde valores de 1,94 mg/g ES a día 1 hasta 3,88 y 78,24 mg/g ES al final de la maduración (día 21) y del periodo de almacenamiento en refrigeración (día 120) respectivamente, siendo estos niveles más bajos a los encontrados en quesos madurados con mohos en su interior (Madkor *et al.*, 1987b, Prieto *et al.*, 2000). La proteolisis global se mantuvo en niveles similares hasta el día 30, y a partir del día 60 aumentó hasta el final del almacenamiento en refrigeración.

La actividad esterasa aumentó durante la maduración y el almacenamiento en refrigeración desde 0,83 pmoles de α-naftol/min·g a día 1 hasta 4,86 a día 21 y 18,14 a día 120. Esta variedad de queso se caracteriza por una elevada lipolisis, debida a la presencia de *P. camemberti*, aunque es menor que en los quesos azules al encontrarse el moho únicamente en la superficie (Spinnler & Gripon, 2004). Los niveles de los ácidos grasos libres aumentaron durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar concentraciones a los 120 días de 3,48, 5,03 y 21,58 mg/g ES de ácidos grasos de cadena corta, media y larga, respectivamente, siendo estos valores superiores a los encontrados en otras variedades de queso (Ávila *et al.*, 2007, Juan *et al.*, 2007, Voigt *et al.*, 2012) e inferiores a los encontrados en queso azul (Prieto *et al.*, 2000). Los ácidos grasos libres de cadena larga fueron el grupo mayoritario, con el oleico como el más abundante, el cual alcanzó niveles de 0,45 mg/g ES al final de la maduración, y de 13,10 mg/g ES a día 120. El ácido etanoico aumentó durante el almacenamiento en refrigeración, hasta 0,40 mg/g ES a día 120. También se produjo un aumento de los ácidos ramificados, que llegaron a alcanzar 1,03 mg/g ES al final del almacenamiento en refrigeración.

El grupo más abundante de compuestos volátiles a día 30 fue el de los ácidos volátiles, mientras que a día 60 y 120 fue el de las cetonas. El compuesto mayoritario

dentro las cetonas fue la acetoína a día 30, la acetona a día 60 y la 2-pentanona a día 120. Las cetonas, son compuestos característicos de esta variedad de queso y proceden principalmente de la β-oxidación parcial de los ácidos grasos libres, aportando al queso aromas frutales y florales (Molimard & Spinnler, 1996, Curioni & Bosset, 2002, Spinnler & Gripon, 2004). Los distintos grupos de compuestos volátiles evolucionaron de distinta manera durante el almacenamiento en refrigeración. Los niveles de ácidos e hidrocarburos descendieron, los ésteres y éteres se mantuvieron en niveles estables y los alcoholes, aldehídos, compuestos bencénicos, nitrogenados y azufrados aumentaron a lo largo del almacenamiento en refrigeración.

La migración de iones calcio (Ca^{2+}) del interior a la superficie, característica de esta variedad de quesos, provoca la desestabilización de la matriz de caseínas (Spinnler & Gripon, 2004), lo que unido a la disminución de los niveles de caseínas y del extracto seco, hizo que los tres parámetros de textura estudiados (fracturabilidad, firmeza y elasticidad) disminuyesen durante la maduración y alcanzasen valores muy bajos durante el posterior almacenamiento en refrigeración. El estudio del color mostró un ligero aumento del parámetro b* (tendencia al amarillo) tanto en la superficie como en el interior, mientras que el parámetro a* (tendencia al rojo) aumentó de forma drástica entre los días 90 y 120, y la L* (luminosidad) disminuyó durante el almacenamiento en refrigeración. La calidad de sabor y de olor disminuyeron significativamente durante el almacenamiento en refrigeración. La pérdida de calidad de sabor fue acompañada de un fuerte aumento de la intensidad de sabor y olor, y del sabor amargo y umami.

El almacenamiento en condiciones de refrigeración no impidió la sobremaduración del queso Brie, provocando un desequilibrio de los compuestos responsables del sabor y el aroma que se vio reflejado en la pérdida de calidad de olor y sabor ya a día 60. Los parámetros de sabor amargo y umami aumentaron en función del tiempo reduciendo la calidad de sabor del queso, al tiempo que aumentó la intensidad de sabor y olor.

Con el objetivo de reducir la pérdida de calidad de sabor y olor del queso Brie se aplicaron tratamientos de 400 y 600 MPa a las 2 semanas (400-2S y 600-2S) y a las 3 semanas (400-3S y 600-3S) de maduración. Estos tratamientos dieron lugar a una serie de cambios significativos respecto al control (Tablas 12 y 13). Por otro lado, al comparar los quesos tratados a lo largo del almacenamiento en refrigeración con el queso control

en su momento óptimo (30 días), se observó que en la mayoría de los casos fue posible evitar o atenuar los efectos negativos de la sobremaduración (Tablas 14 y 15).

Tabla 12. Tendencias de las características microbiológicas y químicas del queso Brie tratado por alta presión con respecto al queso control.

Parámetro	400-2S	600-2S	400-3S	600-3S
ES	↑ 60 a 120 d	↑ 60 a 120 d	↑ 60 a 120 d	↑ 90 y 120 d
pH	↓↓ 30 a 120 d	↓↓ 30 a 120 d	↓↓ 30 a 120 d	↓↓ 30 a 120 d
Aerobios totales	↓ hasta 120 d	↓↓ hasta 120 d	↓ hasta 120 d	↓↓ hasta 120 d
Bacterias lácticas	↓ hasta 120 d	↓↓ hasta 120 d	↓ hasta 120 d	↓↓ hasta 120 d
P. camemberti	↓ hasta 120 d	↓↓ hasta 120 d	↓ hasta 120 d	↓↓ hasta 120 d
Levaduras	↑ 60 d, ↓ 90 y 120 d	↓↓ 90 y 120 d	↓ 90 d	↓↓ 90 y 120 d
α-caseína	NS	↑↑ 90 y 120 d	NS	↑ 90 d
β-caseína	↑ 120 d	↑↑ 60 a 120 d	↑ 60 d	↑↑ 60 a 120 d
κ-caseína	↑ 120 d	NS	NS	NS
p-κ-caseína	NS	NS	NS	NS
Péptidos hidrófilos	↑ 14 a 60 d, ↓ 120 d	↑ 14 a 60 d	↑ 60 d, ↓ 120 d	↑ 60 d
Péptidos hidrófobos	↓↓ 60 a 120 d	↓↓ 60 a 120 d	↓↓ 60 a 120 d	↓↓ 60 a 120 d
Ratio hidrófobos/filos	↓ 14 a 30 d, ↓↓ 60 a 120 d	↓ 14 a 30 d, ↓↓ 60 a 120 d	↓ 21 y 30 d, ↓↓ 60 a 120 d	↓ 21 y 30 d, ↓↓ 60 a 120 d
Act. aminopeptidasa (Lys)	↓ hasta 120 d	↓ hasta 120 d	↓ 120 d	↓ 60 a 120 d
Act. aminopeptidasa (Leu)	↓ 21 a 120 d	↓ 21 a 120 d	↓ 120 d	↓ 30 a 120 d
Aminoácidos libres totales	↑↑ 21 a 120 d	↑ 21 a 90 d	↑↑ 21 a 120 d	↑ 30 a 120 d
Proteolisis global (OPA)	↑ 21 a 90 d	↑ 21 a 60 d	↑↑ 30 a 120 d	↑ 30 a 90 d
Actividad esterasa	↑ 60 a 120 d	↑ 90 a 120 d	↑↑ 21 a 120 d	NS
Etanoico + propanoico	↑↑ hasta 120 d	↑ hasta 30 d, ↓ 90 y 120 d	↑↑ hasta 120 d	↑ 30 d, ↓ 90 y 120 d
AGL cadena ramificada	↓↓ 60 a 120 d	↓↓ 60 a 120 d	↓↓ 60 a 120 d	↓↓ 90 y 120 d
AGL cadena corta	↑ 21 d, ↓ 30 a 120 d	↑ 21 d, ↓ 30 a 120 d	↑ 21 d, ↓ 60 a 120 d	↑ 21 d, ↓ 30 a 120 d
AGL cadena media	↓ 30, 90 y 120 d	↓ 30 a 120 d	↑ 21 d, ↓ 90 y 120 d	↓ 30, 90 y 120 d
AGL cadena larga	↓↓ 30 a 120 d	↓↓ 30 a 120 d	↑ 21 a 60 d, ↓ 120 d	↑ 21 d, ↓ 90 y 120 d
Ácidos volátiles	↑ 30 a 120 d	↑ 30 a 120 d	↑ 60 y 120 d	↑ 60 y 120 d
Alcoholes volátiles	↑↑ 30 a 120 d	↑ 30 a 120 d	↑↑ 30 a 120 d	↑ 30 a 120 d
Aldehídos volátiles	↑ 30 y 60 d	↑ 30 y 60 d	↑ 60 d	↑↑ 60 y 120 d
Cetonas volátiles	↑ 30 d, ↓↓ 120 d	↑ 60 d, ↓↓ 120 d	↓ 30 d, ↓↓ 120 d	↑ 60 d, ↓↓ 120 d
Ésteres volátiles	↑ 30 a 120 d	↑↑ 30 a 120 d	↑ 60 a 120 d	↑ 30 a 120 d
Éteres volátiles	↑↑ 30 a 120 d	↑↑ 30 a 120 d	↑↑ 30 a 120 d	↑↑ 30 a 120 d
Hidrocarburos volátiles	NS	↑ 60 d	↑ 60 d	NS
Bencénicos volátiles	↓ 120 d	↓ 120 d	↓ 120 d	↓ 120 d
Azufrados volátiles	↓↓ 120 d	↓↓ 120 d	↓↓ 120 d	↓↓ 120 d
Nitrogenados volátiles	↓↓ 120 d	↓↓ 120 d	↓↓ 120 d	↓↓ 120 d

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos. OPA; *o*-ftalaldehído (técnica espectrofotométrica).

La reducción de los niveles de microorganismos, especialmente de *P. camemberti*, por las altas presiones junto a la inactivación de enzimas debidas a los tratamientos de 600 MPa, consiguieron atenuar la fuerte hidrólisis de α_S-, β- y κ-caseínas característica del queso control, aunque no lograron mantener niveles similares a los del queso control de 30 días durante todo el periodo de almacenamiento en refrigeración. Las altas presiones provocaron un aumento de los péptidos hidrófilos, que alcanzaron niveles superiores a los del queso control de 30 días durante el almacenamiento en refrigeración, mientras que mantuvieron los péptidos hidrófobos a niveles similares a los del queso control de 30 días, de forma que el ratio de péptidos hidrófobos/hidrófilos de los quesos tratados fue menor que el del queso control de 30 días, evitando el aumento de sabor amargo que se dio en el control. La reducción de la actividad aminopeptidasa provocada por las altas presiones no se vio reflejada en una disminución de los niveles de aminoácidos libres, de manera similar a lo ocurrido en queso Hispánico (Ávila *et al.*, 2006). La inactivación de las enzimas responsables del catabolismo de aminoácidos posiblemente hizo que éstos se acumulasen en mayor cantidad en los quesos tratados, al igual que sucedió con la proteolisis global.

La aplicación de altas presiones provocó un aumento de la actividad esterasa, especialmente en los quesos tratados con 400 MPa. Este aumento no se vio reflejado en la liberación de ácidos grasos de cadena corta, ya que los quesos tratados mantuvieron niveles similares o ligeramente superiores a los del queso control de 30 días durante todo el periodo de almacenamiento en refrigeración. Los quesos tratados a las 2 semanas mostraron niveles de ácidos grasos libres de cadena media y larga inferiores a los del queso control de 30 días, mientras que los niveles de los ácidos grasos de cadena larga de los quesos tratados a las 3 semanas fueron superiores a día 60 a los del queso control de 30 días, aunque posteriormente disminuyeron hasta niveles inferiores a los del control de 30 días. Un patrón similar de ácidos grasos libres se dio en queso de leche de oveja tratado por alta presión (Juan *et al.*, 2007). Los quesos tratados con 600 MPa mantuvieron niveles de ácido etanoico similares a los del queso control de 30 días durante todo el almacenamiento en refrigeración, mientras que los tratados con 400 MPa mostraron niveles superiores. Con las altas presiones se logró evitar el drástico

incremento de ácidos grasos de cadena ramificada que se dio en el control a partir del día 60.

Tabla 13. Tendencias de las características de textura, color y sensoriales del queso Brie tratado por alta presión con respecto al queso control.

Parámetro	400-2S	600-2S	400-3S	600-3S
Fracturabilidad	↑↑ 30 a 120 d	↑↑ 30 a 120 d	↓ 21 d	↓ 21 d
Firmeza	↓ 14 d, ↑↑ 30 a 120 d	↓ 14 d, ↑↑ 30 a 120 d	↓ 21 d, ↑ 60 a 120 d	↓ 21 d, ↑ 60 a 120 d
Elasticidad	↓ 14 d, ↑↑ 30 a 120 d	↓ 14 d, ↑↑ 30 a 120 d	↓ 21 d, ↑ 60 a 120 d	↓ 21 d, ↑ 60 a 120 d
L* interior	↑ 90 y 120 d	↑ 90 y 120 d	↑ 90 y 120 d	↑ 90 y 120 d
a* interior	↓ 90 y 120 d	↓↓ 60 a 120 d	↓ 120 d	↓↓ 60 a 120 d
b* interior	↓ 120 d	↓ 90 y 120 d	↓ 120 d	↓ 90 y 120 d
Calidad sabor	↑↑ 60 a 120 d	↑↑ 60 a 120 d	↑↑ 60 a 120 d	↑↑ 60 a 120 d
Intensidad sabor	↑ 30 d, ↓ 90 y 120 d	↑ 30 y 60 d, ↓ 90 y 120 d	↓ 90 y 120 d	↓ 90 y 120 d
Sabor umami	NS	NS	NS	NS
Sabor ácido	NS	NS	NS	NS
Sabor amargo	↓ 60 a 120 d	↓ 60 a 120 d	↓ 60 a 120 d	↓ 60 a 120 d
Sabor dulce	NS	NS	NS	NS
Sabor salado	NS	NS	NS	NS
Calidad de olor	↑↑ 60 a 120 d	↑↑ 60 a 120 d	↑↑ 60 a 120 d	↑↑ 60 a 120 d
Intensidad de olor	↓ 120 d	↓ 120 d	↓ 120 d	↓ 120 d

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos.

Los tratamientos por altas presiones afectaron de diferente forma a los distintos grupos de compuestos volátiles. Las altas presiones evitaron el fuerte incremento de compuestos volátiles nitrogenados, azufrados y bencénicos que se dio en el queso control, manteniendo estos compuestos en niveles similares a los del queso control de 30 días hasta el final del almacenamiento en refrigeración. Se controló así la aparición de sabores no deseados, tales como los aportados por el dimetildisulfuro cuando se encuentra en concentraciones elevadas. Igualmente sucedió con los niveles de cetonas volátiles en los quesos tratados, que se mantuvieron por debajo de los del queso control de 30 días, excepto en los quesos tratados con 600 MPa a las 3 semanas, que a día 60 tuvieron niveles superiores, aunque a día 120 descendieron por debajo de los niveles del queso control de 30 días. Por otro lado los niveles de ácidos volátiles, alcoholes, aldehídos, ésteres y éteres de los quesos tratados fueron superiores durante todo el almacenamiento en refrigeración a los del queso control de 30 días.

Tabla 14. Valores de las características microbiológicas y químicas en queso Brie control de 30 días, quesos tratados por alta presión de 30-120 días y queso control de 60-120 días (sobremadurado).

Parámetro	Control 30 días	400-2S 30 a 120 d (rango)	600-2S 30 a 120 d (rango)	400-3S 30 a 120 d (rango)	600-3S 30 a 120 d (rango)	Control 60 a 120 d (rango)
ES (%)	**45,3**	46,8 - 50,5	47,8 - 49,5	46,0 - 49,4	45,7 - 48,4	42,1 - 42,4
pH	**6,3**	5,3 - 5,5	5,4 - 5,5	5,5 - 5,7	5,7 - 5,8	7,6 - 7,9
Aerobios totales[a]	**8,3**	6,3 - 6,6	3,1 - 4,8	6,8 - 7,7	4,6 - 6,5	7,9 - 8,3
Bacterias lácticas[a]	**8,3**	6,4 - 6,7	2,6 - 4,5	6,9 - 7,7	5,0 - 5,4	7,9 - 8,3
Lactobacilos[a]	**8,0**	6,0 - 6,3	2,3 - 4,0	6,3 - 7,2	4,4 - 5,0	7,3 - 8,1
P. camemberti[a]	**6,2**	nd - 4,5	nd	nd - 3,0	nd	6,0 - 7,0
Levaduras[a]	**nd**	nd - 2,6	nd	nd - 2,3	nd	nd - 6,9
α-caseína[b]	**87,2**	12,1 - 87,0	25,4 - 98,5	5,2 - 85,2	17,2 - 84,7	1,7 - 68,3
β-caseína[b]	**101,6**	36,4 - 104,1	54,5 - 108,2	19,5 - 95,4	33,7 - 99,7	5,1 - 62,4
κ-caseína[b]	**28,3**	9,6 - 27,9	13,3 - 28,4	2,9 - 25,8	3,4 - 25,7	1,9 - 21,4
Péptidos hidrófilos[c]	**15,7**	27,8 - 47,4	22,6 - 48,7	17,6 - 44,5	17,8 - 42,5	24,0 - 47,4
Péptidos hidrófobos[c]	**6,3**	5,8 - 7,8	3,7 - 7,6	5,0 - 6,1	4,6 - 5,9	30,3 - 72,4
Ratio hidrófobos/filos	**0,4**	0,16 - 0,22	0,11 - 0,21	0,12 - 0,26	0,14 - 0,26	0,8 - 1,7
Act. aminopeptidasa (Lys)[d]	**5,1**	1,8 - 4,1	1,3 - 3,8	1,7 - 6,4	1,5 - 4,2	5,7 - 7,9
Act. aminopeptidasa (Leu)[d]	**4,2**	1,6 - 3,6	1,1 - 3,5	1,5 - 5,4	1,7 - 3,9	5,1 - 7,9
Aminoácidos libres totales[b]	**11,0**	27,6 - 101,8	17,0 - 78,0	18,3 - 123,4	18,7 - 94,6	28,6 - 78,2
Proteolisis global (OPA)[e]	**0,6**	1,8 - 7,5	1,3 - 5,8	1,4 - 9,6	1,0 - 7,2	1,7 - 5,7
Actividad esterasa[f]	**8,8**	5,0 - 33,3	3,6 - 27,6	12,6 - 44,1	4,7 - 22,1	9,0 - 18,1
Etanoico + propanoico[b]	**0,1**	0,5 - 0,7	0,2 - 0,3	0,3 - 0,8	0,1 - 0,2	0,2 - 0,5
AGL cadena ramificada[b]	**0,03**	0,01 - 0,07	0,01 - 0,18	0,01 - 0,06	0,01 - 0,22	0,16 - 1,00
AGL cadena corta[b]	**1,1**	0,4 - 1,5	0,4 - 1,1	1,2 - 1,6	0,5 - 1,5	2,1 - 3,5
AGL cadena media[b]	**1,5**	0,3 - 1,7	0,2 - 1,1	0,6 - 2,4	0,4 - 2,5	1,8 - 5,0
AGL cadena larga[b]	**9,8**	1,1 - 6,5	1,4 - 5,0	8,9 - 22,1	5,3 - 13,7	11,2 - 21,6
Ácidos volátiles[g]	**796**	1688 - 3294	1272 - 2773	957- 3723	833 - 3206	70 - 85
Alcoholes volátiles[g]	**182**	656 - 1120	715 - 809	745 - 1252	483 - 553	310 - 358
Aldehídos volátiles[g]	**5,8**	17,0 - 43,7	11,1 - 35,0	7,5 - 34,6	6,7 - 73,7	8,7 - 39,1
Cetonas volátiles[g]	**725**	249 - 1458	561 - 688	168 - 344	533 - 1252	322 - 2674
Ésteres volátiles[g]	**14,4**	112,4 - 172,7	234,3 - 302,7	67,1 - 128,4	80,2 - 176,1	12,5 - 14,3
Éteres volátiles[g]	**31,7**	138,9 - 188,5	132,4 - 183,3	91,6 - 161,6	89,6 - 172,3	24,8 - 33,6
Bencénicos volátiles[g]	**18,7**	16,0 - 16,7	13,8 - 16,9	15,8 - 18,0	16,0 - 21,2	16,6 - 31,6
Hidrocarburos volátiles[g]	**24,6**	24,8 - 27,6	25,0 - 28,8	27,1 - 28,2	24,7 - 26,3	19,8 - 21,6
Azufrados volátiles[g]	**8,2**	7,3 - 9,1	8,2 - 9,1	7,8 - 8,7	7,3 - 8,1	9,0 - 232,9
Nitrogenados volátiles[g]	**3,2**	3,5 - 5,7	2,8 - 3,0	3,0 - 5,8	2,4 - 7,4	3,9 - 422,1

nd: no detectado, OPA: *o*-ftalaldehído, [a]: (log ufc/g), [b]: (mg/g ES), [c]: (UA/mg ES), [d]:(nmol de *p*-nitroanilina/min g), [e]: (unidades de absorbancia), [f]: (pmol α-naftol/min g), [g]: (abundancia relativa).

Por lo que respecta a la textura, la mayor concentración de caseínas, junto a los niveles más altos de extracto seco en los quesos tratados y su menor pH (Sousa & McSweeney, 2001) hicieron que en todos los quesos tratados se evitase la pérdida de elasticidad y firmeza que se dio en el queso control, y además en los tratados a las 2

semanas se logró eviar la pérdida de fracturabilidad. Respecto al color, todos los tratamientos evitaron la pérdida de luminosidad del interior que se dio en el queso control, así como el incremento de los parámetros a* y b* del interior del queso. La pérdida de la capa de moho superficial provocada por las altas presiones hizo que los parámetros de color de la superficie fuesen significativamente diferentes a los del queso control. Las altas presiones atenuaron el incremento de intensidad de sabor y olor, así como del sabor amargo, aunque no se mantuvieron en niveles similares a los del control de 30 días durante todo el periodo de almacenamiento en refrigeración. Las altas presiones evitaron la drástica disminución de la calidad de sabor y olor, consiguiendo mantener durante el almacenamiento en refrigeración valores similares o ligeramente inferiores a los del control de 30 días.

Tabla 15. Valores de las características de textura, color y sensoriales en queso Brie control de 30 días, quesos tratados por alta presión de 30-120 días y queso control de 60-120 días (sobremadurado).

Parámetro	Control 30 días	400-2S 30 a 120 d (rango)	600-2S 30 a 120 d (rango)	400-3S 30 a 120 d (rango)	600-3S 30 a 120 d (rango)	Control 60 a 120 d (rango)
Fracturabilidad (N)	**3,4**	2,4 - 6,3	1,0 - 8,4	0,0 - 0,6	0,0 - 0,3	0,0 - 0,0
Firmeza (J)	**0,027**	0,034 - 0,059	0,031 - 0,057	0,010 - 0,034	0,011 - 0,033	0,001 - 0,002
Elasticidad (N/mm^2)	**0,052**	0,074 - 0,103	0,043 - 0,095	0,015 - 0,044	0,020 - 0,054	0,001 - 0,002
L* interior	**86,2**	84,4 - 85,4	83,1 - 84,6	82,1 - 85,5	82,0 - 85,1	75,9 - 83,9
a* interior	**1,5**	1,2 - 1,3	0,4 - 1,4	1,5 - 1,7	0,6 - 1,4	1,2 - 2,7
b* interior	**19,3**	19,2 - 21,5	19,6 - 20,2	18,8 - 21,9	19,0 - 21,1	20,2 - 22,6
Calidad de sabor	**6,0**	4,9 - 6,0	4,6 - 5,9	5,1 - 6,0	3,8 - 6,0	0,2 - 3,4
Intensidad de sabor	**5,0**	6,0 - 6,8	5,9 - 6,9	5,5 - 7,0	5,0 - 7,2	6,3 - 8,6
Sabor amargo	**1,4**	1,3 - 4,3	1,3 - 4,2	1,4 - 3,8	1,1 - 4,4	4,3 - 6,4
Calidad de olor	**6,6**	4,3 - 6,7	4,5 - 6,5	4,9 - 6,9	4,6 - 6,5	1,4 - 5,9
Intensidad de olor	**5,1**	5,4 - 6,8	5,4 - 6,4	5,2 - 6,6	5,3 - 6,7	6,4 - 7,9

El análisis de componentes principales de compuestos volátiles individuales (recogido en el capítulo 8) indicó que los quesos tratados a las 2 semanas mantuvieron un perfil de compuestos volátiles similar a los del control de 30 días durante todo el almacenamiento en refrigeración. En base a todos estos resultados se puede concluir que la presurización aplicada a las 2 semanas permite obtener quesos que mantienen en gran medida las características del queso Brie en su momento óptimo de consumo (30 días) hasta los 4 meses de almacenamiento en refrigeración. Sin embargo, la

pérdida de la capa superficial de moho causada por las altas presiones representaría un serio impedimento a la hora de comercializar esta variedad de queso.

- **Queso Arzúa-Ulloa**

El queso Arzúa-Ulloa es habitualmente un queso tierno de leche cruda o pasteurizada de vaca, que también se puede comercializar como queso curado. En queso de esta variedad, elaborado con leche cruda, se estudió la evolución de las variables microbiológicas, químicas, de textura, color y sensoriales durante la maduración y el almacenamiento en refrigeración hasta el día 240. Mediante los análisis microbiológicos, se observó una reducción de los niveles de bacterias mesófilas totales desde 9,2 log ufc/g a día 1 hasta 8,0 log ufc/g a día 21, manteniéndose posteriormente en niveles estables hasta el final del periodo de refrigeración. Los niveles de bacterias lácticas, igualmente descendieron desde 8,8 log ufc/g a día 1 hasta 8,0 log ufc/g a día 14 y posteriormente se mantuvieron en los mismos niveles hasta el día 240. Los niveles de lactobacilos aumentaron desde 4,1 log ufc/g a día 1 hasta 8,0 log ufc/g a día 60, manteniéndose en los mismos niveles hasta el día 240. Los niveles de enterococos descendieron durante la maduración y el almacenamiento en refrigeración desde 6,31 log ufc/ a día 1 hasta 4,71 log ufc/g a día 240. Los niveles de *Micrococcaceae*, coliformes y bacterias gram negativas descendieron desde 6,92, 7,50 y 7,52 log ufc/g respectivamente a día 1, hasta 4,69, 3,81 y 3,90 log ufc/g a día 60, y a 4,08, 1,46 y 2,04 log ufc/g a día 240. Los niveles de estafilococos coagulasa positivos descendieron entre los días 1 y 14 desde 4,83 hasta 2,66 log ufc/g, no volviéndose a detectar durante el posterior almacenamiento en refrigeración. Los valores de pH aumentaron gradualmente desde 5,26 a día 1, hasta 5,36 a día 60 y 5,68 a día 240, mientras que el extracto seco aumentó desde 47,78 % a día 1, hasta 53,13 % a día 60 y 65,43 % a día 240.

Los niveles de caseínas disminuyeron progresivamente a lo largo de la maduración y del almacenamiento en refrigeración por efecto de la plasmina, el cuajo residual y las enzimas del cultivo iniciador, junto a la actividad proteolítica de microorganismos presentes en la leche como *Enterococcus* y *Micrococcus* (Centeno *et al.*, 1995). Entre los días 1 y 240 las α_S-, β-, κ- y *p*-κ-caseínas sufrieron una reducción del 46, 64, 65 y 35 %, respectivamente. Los péptidos hidrófilos aumentaron durante la maduración y el

almacenamiento, llegando a alcanzar valores de 8,72 UA/mg ES a día 240, mientras que los péptidos hidrófobos aumentaron hasta el día 60 con valores de 7,69 UA/mg ES, reduciéndose posteriormente hasta 4,37 UA/mg ES al final del periodo de almacenamiento en refrigeración. El ratio de péptidos hidrófobos/hidrófilos descendió de forma progresiva, desde 1,13 a día 14 hasta 0,50 a día 240. La actividad aminopeptidasa alcanzó valores de 5,87 nmoles de *p*-nitroanilina/min·g a día 14 con el sustrato Leu-*p*-NA, manteniéndose hasta el día 240 a niveles similares, mientras que con el sustrato Lys-*p*-NA el máximo se alcanzó a día 21 con un valor de 11,39 nmoles de *p*-nitroanilina/min·g, descendiendo posteriormente hasta 6,85 nmoles de *p*-nitroanilina/min·g a día 240. La concentración de aminoácidos libres aumentó desde 2,39 mg/g ES a día 14, valores similares a los obtenidos para esta variedad de queso elaborado con leche pasteurizada (Rodríguez-Alonso *et al.*, 2011), hasta 31,68 mg/g ES a día 240. De igual manera, la proteolisis global aumentó durante la maduración y el almacenamiento en refrigeración.

La actividad tirosina descarboxilasa aumentó desde 0,04 mUA/g ES a día 1 hasta 2,62 mUA/g ES a día 240, provocando un aumento de tiramina desde 0,004 mg/g ES a día 21 hasta 0,372 mg/g ES a día 240. Igualmente, la putrescina aumentó a lo largo del tiempo desde 0,008 mg/g ES a día 21 hasta 0,207 mg/g ES a día 240, mientras que la cadaverina que a día 1 se encontró en una concentración de 0,014 mg/g ES aumentó hasta 0,113 mg/g ES a día 21, y a partir del día 60 se mantuvo en niveles similares. La histamina únicamente se encontró a los 180 y 240 días, en concentraciones de 0,09 y 0,026 mg/g ES respectivamente. La concentración total de aminas biógenas alcanzó a día 240 una concentración de 0,686 mg/g ES.

La actividad esterasa aumentó de 4,63 pmoles de α-naftol/min·g a día 1 hasta 10,75 pmoles de α-naftol/min·g a día 120, estabilizándose en estos niveles hasta el día 240. Los niveles de ácidos grasos libres aumentaron durante la maduración y el almacenamiento en refrigeración. Los ácidos grasos libres de cadena corta aumentaron hasta 0,19 mg/g ES a día 240 y los de cadena media y larga a hasta 0,40 y 1,31 mg/g ES, respectivamente, a día 240. Por otro lado, el ácido etanoico, aumentó hasta 2,10 mg/g ES a día 240. El ácido benzoico, que puede formarse a partir del ácido hipúrico, de la

fenilalanina o de la auto-oxidación del benzaldehído (Sieber *et al.*, 1995), aumentó hasta 0,398 mg/g ES a día 180.

El grupo de compuestos volátiles más abundante en todos los tiempos analizados fue el de los alcoholes, al igual que en otros quesos gallegos (Rodríguez-Alonso *et al.*, 2009), seguido de los ácidos volátiles y de las cetonas. Dentro del grupo de los alcoholes el compuesto más abundante fue el 2-butanol, procedente del metabolismo del diacetilo, que aporta aromas florales (Mallia *et al.*, 2005). Los niveles de ácidos volátiles, cetonas, ésteres, éteres, hidrocarburos, compuestos bencénicos y azufrados descendieron durante el almacenamiento en refrigeración, los aldehídos se mantuvieron en niveles estables y los terpenos aumentaron. El nivel de alcoholes aumentó entre los días 60 y 120, descendiendo posteriormente.

Durante el almacenamiento en refrigeración los parámetros de textura, elasticidad y firmeza aumentaron en función del tiempo hasta el día 180 y descendiendo a día 240, mientras que la fracturabilidad únicamente se pudo determinar a día 240. La luminosidad en el interior del queso descendió a lo largo del tiempo. La tendencia al rojo del interior aumentó gradualmente hasta el día 180, reduciéndose a día 240, mientras que la tendencia al amarillo del interior del queso fue aumentando a lo largo del tiempo hasta el día 240. La intensidad de sabor fue aumentando con el tiempo, mientras que la calidad se mantuvo hasta el día 180, descendiendo a día 240. Los parámetros de sabor amargo y umami aumentaron con el tiempo de almacenamiento.

El almacenamiento en condiciones de refrigeración consiguió mantener la calidad de sabor del queso Arzúa-Ulloa hasta los 6 meses, aunque se siguieron produciendo cambios en los parámetros químicos, de textura y color durante todo este periodo. A los 8 meses se observó una pérdida de la calidad de sabor, acompañada de un aumento de la intensidad de sabor, así como del sabor amargo. Junto a la pérdida de calidad del sabor también se encontraron niveles de aminas biógenas al final del periodo de almacenamiento en refrigeración, que podrían resultar nocivos para la salud del consumidor.

Tabla 16. Tendencias de las características microbiológicas y químicas del queso Arzúa-Ulloa tratado por alta presión con respecto al queso control.

Parámetro	400-2S	600-2S	400-3S	600-3S
ES	NS	NS	NS	NS
pH	↓ 180 a 240 d	↑ 14 d, ↓↓ 180 a 240 d	↓ 240 d	↓↓ 180 a 240 d
Aerobios totales	↓ hasta 21 d	↓↓ hasta 240 d	↓ 21 d	↓↓ hasta 240 d
Bacterias lácticas	↓ hasta 21 d	↓↓ hasta 240 d	↓ 21 d	↓↓ hasta 240 d
Lactobacilos	↓ hasta 21 d	↓↓ hasta 240 d	↓ 21 d	↓↓ hasta 240 d
Micrococos	↓ hasta 240 d	↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Gram negativos	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
Coliformes	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
Estafilococos coagulasa +	↓↓ 14 d	↓↓ 14 d	↓↓ 14 d	↓↓ 14 d
Enterococos	↓ 14, 120 a 240 d	↓↓ hasta 240 d	↓ 120 a 240 d	↓↓ hasta 240 d
Levaduras	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d	↓↓ hasta 240 d
α-caseína	↓ 120 a 240 d	NS	↓ 120 a 240 d	NS
β-caseína	↓ hasta 240 d	↓ 60 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d
κ-caseína	↓ 14, 60 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d
p-κ-caseína	↓ 21 a 240 d	↓ 21 a 240 d	↓ 60 a 240 d	↓ 60 a 240 d
Péptidos hidrófilos	↑ 21 a 240 d	↑ 14, 60 a 240 d	↑ hasta 240 d	↑ hasta 240 d
Péptidos hidrófobos	↑ 60 a 240 d	↑ 60 a 240 d	↑ 60 a 240 d	↑ 60 a 240 d
Ratio hidrófobos/filos	↑ 240 d	↓ 21 d, ↑ 180 y 240 d	↓ 21 d, ↑ 240 d	↓ 21 d, ↑ 120 a 240 d
Act. aminopeptidasa (Lys)	↓ hasta 240 d	↓ hasta 240 d	↓ hasta 240 d	↓ hasta 240 d
Act. aminopeptidasa (Leu)	↓ hasta 240 d	↓ hasta 240 d	↓ hasta 240 d	↓ hasta 240 d
Aminoácidos libres totales	↓ 180 y 240 d	↓↓ 60 a 240 d	↓ 180 y 240 d	↓↓ 60 a 240 d
Proteolisis global (OPA)	↓ 180 y 240 d	↓↓ 21 a 240 d	↓ 180 y 240 d	↓↓ 60 a 240 d
Actividad TDC	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 120 d	↓↓ hasta 240 d
Tiramina	↓ 21, 60 180 y 240 d	↓↓ 21 a 240 d	↓ 60 y 240 d	↓↓ 21 a 240 d
Putrescina	↓ 21, 60 y 240 d	↓↓ 21 a 240 d	↓ 21, 60 y 240 d	↓↓ 21 a 240 d
Cadaverina	↓ 21 a 240 d	↓ 21 a 180 d	↓ 21, 60 180 y 240 d	↓ 21, 120, 180 y 240 d
Histamina	↑ 120 d, ↓ 180 y 240 d	↓ 180 y 240 d	↓ 180 y 240 d	↓ 180 y 240 d
Aminas totales	↓ 21, 60 180 y 240 d	↓↓ 21 a 240 d	↓ 21, 60 y 240 d	↓↓ 21 a 240 d
Actividad esterasa	↓ hasta 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
Etanoico + propanoico	↓ 14, 60 a 240 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
AGL cadena corta	↓ 60 y 240 d	↓↓ 60 a 240 d	↓ 60 y 240 d	↓↓ 60 a 240 d
AGL cadena media	NS	↓ 240 d	↓ 240 d	↓ 240 d
AGL cadena larga	NS	↑ 60 d, ↓ 240 d	↓ 240 d	↓ 240 d
Ácidos volátiles	NS	NS	NS	NS
Alcoholes volátiles	↓ 60 y 120 d	↓↓ hasta 240 d	↓ 60 y 120 d	↓↓ hasta 240 d
Aldehídos volátiles	↑ hasta 240 d	↑↑ hasta 240 d	↑ 60 y 120 d	↑↑ hasta 240 d
Cetonas volátiles	↑↑ 60 d	↑ hasta 240 d	↑↑ 60 d	↑ hasta 240 d
Ésteres volátiles	NS	NS	NS	NS
Éteres volátiles	↑ 120 d	NS	NS	↑↑ 240 d
Hidrocarburos volátiles	↑ 120 d	↑ 240 d	↑ 60 d	↑ 120 y 240 d
Bencénicos volátiles	NS	NS	NS	NS
Azufrados volátiles	↑ 240 d	↑ 240 d	NS	↑ 240 d
Terpenos volátiles	NS	↓ 120 d	NS	↓ 120 d
Otros volátiles	↓ 120 d	↓ hasta 240 d	↓ 60 d	↓ 120 y 240 d

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos. OPA; *o*-ftalaldehído (técnica espectrofotométrica), TDC; tirosina descarboxilasa.

Con el objetivo de evitar la pérdida de calidad de sabor que se dio a los 240 días y la acumulación de aminas biógenas, se aplicaron tratamientos de 400 y 600 MPa a las 2 semanas (400-2S y 600-2S) y a las 3 semanas (400-3S y 600-3S) de maduración. Estos tratamientos indujeron una serie de cambios respecto al control en los diferentes tiempos considerados (Tablas 16 y 17). Por otro lado, comparando los quesos tratados a lo largo del almacenamiento en refrigeración con el queso control en su momento óptimo (21 a 60 días), se puede apreciar que en muchos casos se logró atenuar los efectos negativos asociados a la sobremaduración (Tablas 18 y 19).

Tabla 17. Tendencias de las características de textura, color y sensoriales del queso Arzúa-Ulloa tratado por alta presión con respecto al queso control.

Parámetro	400-2S	600-2S	400-3S	600-3S
Fracturabilidad	↑↑ 180 y 240 d	↑↑ 180 y 240 d	↑↑ 180 y 240 d	↑↑ 180 y 240 d
Firmeza	↓ 14 y 21 d, ↑ 240 d	↑ 120 y 240 d	↓ 21 d	↑ 120 d
Elasticidad	↓ 14 y 21 d, ↑ 180 y 240 d	↑ 240 d	↓ 21 d, ↑ 240 d	↑ 120 d
L* interior	↑ 60 d	↑ 21 a 240 d	↑ 60 d	↑ 21 a 240 d
a* interior	↓ hasta 120 d	↓↓ hasta 240 d	↓ hasta 240 d	↓↓ hasta 240 d
b* interior	NS	↑ 14 d	NS	NS
Calidad sabor	↓ 120 d	NS	NS	NS
Intensidad sabor	NS	NS	NS	NS
Sabor umami	NS	NS	NS	NS
Sabor ácido	NS	NS	NS	NS
Sabor amargo	↑ 60 a 240 d	↑ 240 d	↑ 120 d	NS
Sabor dulce	NS	NS	NS	NS
Sabor salado	NS	NS	NS	NS

↑ , ↓ ; aumento o disminución, respectivamente, significativos respecto al queso control en el tiempo indicado a continuación. NS; ausencia de efecto significativo ($P > 0,05$, test de Tukey) para todos los tiempos.

Los tratamientos de altas presiones disminuyeron los niveles de microorganismos, en especial los tratamientos de 600 MPa. Sin embargo, no se consiguió atenuar la hidrólisis de caseínas, sino que los tratamientos la incrementaron de manera similar a lo observado en queso Cheddar (O'Reilly *et al.*, 2003, Rynne *et al.*, 2008). Las altas presiones pueden modificar la estructura de las caseínas haciéndolas más susceptibles a la hidrólisis por la plasmina, que mantiene su actividad tras la aplicación de tratamientos de hasta 800 MPa (Malone *et al.*, 2003, Huppertz *et al.*, 2004), lo que pudo dar lugar a una mayor hidrólisis de las caseínas en los quesos tratados. Los niveles de péptidos hidrófilos en quesos tratados fueron superiores a los del queso control de 60

días. Los niveles de péptidos hidrófobos también fueron por lo general superiores a los del control de 60 días, sin embargo los quesos tratados con 400 MPa presentaron a los 180 y 240 días niveles similares o ligeramente inferiores a los del queso control de 60 días. El ratio de péptidos hidrófobos/hidrófilos se mantuvo en niveles inferiores a los del queso control de 60 días hasta el final del almacenamiento en refrigeración. La fuerte reducción de la actividad aminopeptidasa que provocaron las altas presiones, consiguió atenuar la formación de aminoácidos libres, que en el caso de los quesos tratados con 600 MPa se encontraron en niveles similares a los del queso control de 60 días hasta el final del periodo de refrigeración, al igual que sucedió con la proteolisis global.

La reducción de la actividad tirosina descarboxilasa por efecto de las altas presiones, en especial los tratamientos de 600 MPa, atenuó la formación de tiramina, que se mantuvo en niveles no detectables en los quesos tratados con 600 MPa hasta el final del almacenamiento en refrigeración. Igualmente, la formación de putrescina se atenuó con los tratamientos de 400 MPa y se eliminó completamente en los quesos tratados con 600 MPa. Por el contrario, la formación de cadaverina no se vio tan afectada por las altas presiones, aunque sí se observó un ligero descenso en los quesos tratados.

La reducción de la actividad esterasa por las altas presiones, en especial con los tratamientos de 600 MPa, consiguió disminuir la formación de ácidos grasos libres de cadena corta, que se mantuvieron en los quesos tratados con 600 MPa en niveles similares o ligeramente superiores a los del queso control de 60 días durante todo el almacenamiento en refrigeración. Por el contrario, los niveles de ácidos grasos libres de cadena media y larga fueron superiores a los del queso control de 60 días, ya que no se observó ningún efecto de las altas presiones sobres estos ácidos grasos, de igual manera que sucedió en queso Cheddar (Rynne *et al.*, 2008). Las altas presiones consiguieron mantener los niveles de ácido etanoico y propanoico por debajo de los del queso control de 60 días, especialmente los tratamientos de 600 MPa. Los niveles de ácido benzoico no se vieron afectados por los tratamientos de altas presiones.

Tabla 18. Valores de las características microbiológicas y químicas en queso Arzúa-Ulloa control de 21 y 60 días, quesos tratados por alta presión de 60-240 días y queso control de 120-240 días.

Parámetro	Control 21 y 60 días	400-2S 60 a 240 d (rango)	600-2S 60 a 240 d (rango)	400-3S 60 a 240 d (rango)	600-3S 60 a 240 d (rango)	Control 120 a 240 d (rango)
pH	**5,4 y 5,4**	5,3 - 5,3	5,3 - 5,7	5,4 - 5,6	5,3 - 5,4	5,4 - 5,7
Aerobios totales[a]	**8,0 y 7,9**	7,7 - 7,9	3,2 - 4,4	7,6 - 7,9	3,4 - 4,7	7,9 - 8,1
Bacterias lácticas[a]	**8,1 y 8,1**	7,8 - 7,9	2,9 - 4,5	7,6 - 7,8	3,1 - 4,7	7,6 - 8,0
Lactobacilos[a]	**7,0 y 8,0**	7,3 - 8,0	2,0 - 4,3	7,6 - 8,1	3,1 - 4,7	7,9 - 8,0
Enterococos[a]	**5,5 y 4,9**	2,0 - 3,9	nd	2,4 - 4,9	nd	4,7 - 5,0
Micrococos[a]	**5,5 y 5,0**	nd - 2,6	nd - 2,2	nd - 3,4	nd	4,1 - 4,7
Gram negativos[a]	**6,2 y 3,9**	nd	nd	nd	nd	2,0 - 3,3
Coliformes[a]	**5,8 y 3,8**	nd	nd	nd	nd	nd - 2,9
Estafilococos coagulasa +[a]	**nd**	nd	nd	nd	nd	nd
Levaduras[a]	**3,5 y 3,2**	nd	nd	nd	nd	2,5 - 3,0
α-caseína[b]	**73,3 y 67,6**	32,9 - 55,2	52,4 - 57,2	30,4 - 48,4	48,4 - 62,5	47,7 - 55,2
β-caseína[b]	**73,6 y 62,8**	7,3 - 25,9	14,0 - 25,3	8,9 - 25,5	14,5 - 29,9	32,4 - 52,3
κ-caseína[b]	**16,7 y 13,7**	5,4 - 8,8	4,4 - 9,3	5,4 - 7,6	5,2 - 9,9	8,1 - 9,8
p-κ-caseína[b]	**12,8 y 12,0**	1,8 - 3,6	3,9 - 4,6	1,9 - 3,5	4,8 - 5,1	10,4 - 10,8
Péptidos hidrófilos[c]	**7,0 y 7,6**	11,7 - 12,5	10,8 - 12,0	10,6 - 12,5	10,8 - 11,7	7,2 - 8,7
Péptidos hidrófobos[c]	**7,5 y 7,7**	7,5 - 10,3	9,5 - 10,0	7,5 - 9,8	9,5 - 10,6	4,4 - 5,9
Ratio hidrófobos/filos	**1,1 y 1,0**	0,6 - 0,9	0,8 - 0,9	0,6 - 0,9	0,8 - 0,9	0,5 - 0,8
Act. aminopeptidasa (Lys)[d]	**11,4 y 9,9**	0,8 - 1,7	0,2 - 1,1	0,7 - 1,7	0,2 - 1,3	6,9 - 9,9
Act. aminopeptidasa (Leu)[d]	**5,5 y 5,4**	0,5 - 0,8	0,3 - 0,6	0,5 - 0,8	0,3 - 0,7	4,6 - 5,0
Aminoácidos libres totales[b]	**3,8 y 8,8**	8,0 - 22,0	6,2 - 11,7	9,8 - 22,8	6,4 - 11,4	16,7 - 31,7
Proteolisis global (OPA)[e]	**0,4 y 1,2**	1,1 - 3,0	0,7 - 1,6	1,1 - 3,2	0,9 - 1,8	2,2 - 4,2
TDC[f]	**0,2 y 0,9**	0,04 - 1,1	0,0 - 0,0	0,08 - 1,9	0,0 - 0,0	1,4 - 2,6
Tiramina[b]	**0,004 y 0,109**	0,012 - 0,176	0,000 - 0,000	0,068 - 0,244	0,000 - 0,005	0,178 - 0,372
Putrescina[b]	**0,008 y 0,097**	0,007 - 0,161	0,000 - 0,001	0,029 - 0,183	0,000 - 0,002	0,147 - 0,207
Cadaverina[b]	**0,113 y 0,088**	0,032 - 0,049	0,044 - 0,081	0,032 - 0,069	0,042 - 0,090	0,057 - 0,082
Histamina[b]	**0,000 y 0,000**	0,000 - 0,005	0,000 - 0,000	0,000 - 0,004	0,000 - 0,000	0,000 - 0,026
Aminas totales[b]	**0,13 y 0,29**	0,07 - 0,35	0,05 - 0,08	0,16 - 0,32	0,04 - 0,10	0,34 - 0,69
Actividad esterasa[g]	**7,7 y 7,9**	3,2 - 4,3	0,7 - 1,8	2,8 - 3,9	0,7 - 1,8	10,7 - 11,2
Etanoico + propanoico[b]	**1,5 y 1,8**	1,4 - 1,7	1,2 - 1,5	1,7 - 1,8	1,3 - 1,5	2,2 - 2,4
AGL cadena corta[b]	**0,04 y 0,07**	0,05 - 0,15	0,04 - 0,09	0,06 - 0,15	0,05 - 0,09	0,11 - 0,19
AGL cadena media[b]	**0,19 y 0,22**	0,23 - 0,38	0,26 - 0,35	0,24 - 0,37	0,24 - 0,36	0,30 - 0,40
AGL cadena larga[b]	**0,65 y 0,75**	0,75 - 1,24	0,87 - 1,14	0,81 - 1,21	0,80 - 1,17	0,98 - 1,31
Alcoholes volátiles[h]	**4209 y 11663**	3432 - 8955	1508 - 2522	4052 - 12055	1998 - 3402	9582 - 16181
Aldehídos volátiles[h]	**26,3 y 20,5**	29,3 - 31-8	38,9 - 42,2	17,4 - 32,7	39,4 - 52,0	19,4 - 19,6
Cetonas volátiles[h]	**8792 y 5211**	759 - 21057	7432 - 14344	980 - 18262	6990 - 10874	1381 - 4049
Éteres volátiles[h]	**106 y 148**	215 - 395	133 - 224	155 - 279	131 - 546	91 - 103
Azufrados volátiles[h]	**23 y 12**	8 - 23	9 - 18	6 - 18	9 - 19	4 - 8
Hidrocarburos volátiles[h]	**51 y 75**	30 - 138	54 - 106	39 - 146	54 - 132	23 - 73

nd: no detectado, OPA: *o*-ftalaldehído, TDC: actividad tirosina descarboxilasa, [a]: (log ufc/g), [b]: (mg/g ES), [c]: (UA/mg ES), [d]:(nmol de *p*-nitroanilina/min g), [e]: (unidades de absorbancia), [f]: (mUA/g ES) [g]: (pmol α-naftol/min g), [h]: (abundancia relativa).

Los tratamientos de altas presiones afectaron de forma diferente a los distintos grupos de compuestos volátiles. Los tratamientos de 600 MPa atenuaron la disminución de cetonas, manteniendo niveles superiores a los del queso control de 60 días y provocaron que los niveles de alcoholes se mantuviesen estables durante el almacenamiento en refrigeración, con niveles inferiores a los del queso control de 60 días. Las altas presiones disminuyeron los niveles de terpenos, manteniendo los quesos tratados con 600 MPa niveles similares a los del queso control de 60 días. Las altas presiones atenuaron la disminución de ácidos volátiles, éteres, hidrocarburos y compuestos azufrados y bencénicos. Únicamente se consiguieron mantener niveles de éteres similares a los del queso control de 60 días, a día 120, en los quesos tratados a las 3 semanas y a día 240, en los tratados con 600 MPa a las 2 semanas y con 400 MPa a las 3 semanas. Los niveles de ácidos volátiles y compuestos azufrados en los quesos tratados se mantuvieron en niveles similares a los del queso control de 60 días únicamente hasta el día 120. Los tratamientos de altas presiones hicieron que los aldehídos se mantuvieran en niveles superiores a los del queso control de 60 días, excepto en los quesos tratados con 400 MPa a las 3 semanas, que a día 240 presentaron niveles similares. Los ésteres de los quesos tratados se mantuvieron en niveles inferiores a los del queso control de 60 días hasta el día 240.

Tabla 19. Valores de las características de textura, color y sensoriales en queso Arzúa-Ulloa control de 21 y 60 días, quesos tratados por alta presión de 60-240 días y queso control de 120-240 días.

Parámetro	Control 21 y 60 días	400-2S 60 a 240 d (rango)	600-2S 60 a 240 d (rango)	400-3S 60 a 240 d (rango)	600-3S 60 a 240 d (rango)	Control 120 a 240 d (rango)
Fracturabilidad (N)	**0,0 y 0,0**	0,0 - 21,2	0,0 - 24,0	0,0 - 19,4	0,0 - 19,0	0,0 - 2,4
Firmeza (J)	**0,03 y 0,04**	0,05 - 0,18	0,05 - 0,20	0,04 - 0,17	0,05 - 0,18	0,07 - 0,18
Elasticidad (N/mm^2)	**0,03 y 0,05**	0,06 - 0,38	0,06 - 0,41	0,05 - 0,37	0,05 - 0,33	0,10 - 0,26
L* en interior	**85,1 y 83,3**	78,0 - 84,4	78,9 - 84,9	77,2 - 84,1	78,0 - 83,8	76,6 - 82,8
a* en interior	**2,0 y 2,1**	1,4 - 2,1	0,7 - 1,5	1,2 - 1,9	0,6 - 1,4	1,6 - 2,1
b* en interior	**19,0 y 19,3**	18,7 - 24,1	19,1 - 23,7	19,3 - 24,0	19,7 - 23,7	22,1 - 24,1
Calidad de sabor	**7,0 y 7,2**	5,5 - 6,3	5,4 - 6,5	5,6 - 6,5	5,5 - 6,7	5,5 - 7,3
Sabor amargo	**1,1 y 1,4**	3,3 - 4,8	2,7 - 4,7	2,9 - 4,3	2,7 - 4,3	1,8 - 2,6

Las altas presiones hicieron que los parámetros de textura (fracturabilidad, firmeza y elasticidad) aumentasen en los quesos tratados en mayor medida que en el queso control, manteniendo en todo momento valores superiores a los del queso control de

60 días. El parámetro b* en el interior del queso no se vio afectado por las altas presiones. La pérdida de luminosidad en el interior del queso control se vio atenuada por los tratamientos de altas presiones y únicamente los quesos tratados con 600 MPa mantuvieron niveles similares a los del queso control de 60 días hasta el día 180. El parámetro a* fue inferior en los quesos tratados, manteniéndose en valores inferiores a los del queso control de 60 días durante todo el periodo de almacenamiento en refrigeración. Las altas presiones no lograron controlar el aumento de la intensidad de sabor ni la disminución de la calidad de sabor que se dio a los 240 días, y además favorecieron el aumento de sabor amargo.

El análisis de componentes principales (recogido en el capítulo 10) indicó que los quesos tratados con 600 MPa mantuvieron hasta los 120 días un perfil de compuestos volátiles muy similar al del queso control de 21 días. De los resultados obtenidos se puede concluir que los tratamientos de 600 MPa ralentizan y atenúan ciertos cambios que se producen durante el almacenamiento en refrigeración, consiguiendo que el queso Arzúa-Ulloa tratado con 600 MPa mantenga en gran medida las características de esta variedad de queso en su momento óptimo (21 a 60 días) hasta los 6 meses de almacenamiento en refrigeración, y evitando la acumulación de aminas biógenas.

- **Bibliografía**

Ávila, M., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy Science* **89**, 2882-2893.

Ávila, M., Calzada, J., Garde, S. & Nuñez, M. (2007). Effect of a bacteriocin-producing *Lactococcus lactis* strain and high-pressure treatment on the esterase activity and free fatty acids in Hispánico cheese. *International Dairy Journal* **17**, 1415-1423.

Cantor, M. D., van den Tempel, T., Hansen, T. K. & Ardö, Y. (2004). Blue cheese. In Cheese: Chemistry, Physics and Microbiology, pp. 175-198: Academic Press.

Carbonell, M., Nuñez, M. & Fernández-García, E. (2002). Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavour characteristics. *Lait* **82**, 683-698.

Centeno, J. A., Cepeda, A. & Rodríguez-Otero, J. L. (1995). Identification and preliminary characterization of strains of enterococci and micrococci isolated from Arzúa raw cows'-milk cheese. *Nahrung-Food* **39**, 55-62.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal* **13**, 841-866.

Creamer, L. K. & Olson, N. F. (1982). Rheological evaluation of maturing cheddar cheese. *Journal of Food Science* **47**, 631-636.

Curioni, P. M. G. & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal* **12**, 959-984.

Daryaei, H., Coventry, M. J., Versteeg, C. & Sherkat, F. (2006). Effects of high-pressure treatment on shelf life and quality of fresh lactic curd cheese. *Australian Journal of Dairy Technology* **61**, 186-188.

Deeth, H. C. (2006). Lipoprotein lipase and lipolysis in milk. *International Dairy Journal* **16**, 555-562.

Delgado, F. J., González-Crespo, J., Ladero, L., Cava, R. & Ramírez, R. (2009). Free fatty acids and oxidative changes of a Spanish soft cheese (PDO 'Torta del Casar') during ripening. *International Journal of Food Science and Technology* **44**, 1721-1728.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J. & Ramírez, R. (2010a). Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. *Food Chemistry* **118**, 182-189.

Delgado, F. J., Rodríguez-Pinilla, J., González-Crespo, J., Ramírez, R. & Roa, I. (2010b). Proteolysis and texture changes of a Spanish soft cheese ('Torta del Casar') manufactured with raw ewe milk and vegetable rennet during ripening. *International Journal of Food Science and Technology* **45**, 512-519.

Delgado, F. J., González-Crespo, J., Cava, R. & Ramírez, R. (2011). Free fatty acids and oxidative changes of a raw goat milk cheese through maturation. *Journal of Food Science* **76**, C669-C673.

Delgado, F. J., González-Crespo, J., Cava, R. & Ramírez, R. (2012). High-pressure treatment applied throughout ripening of a goat cheese caused minimal changes on free fatty acids content and oxidation in mature cheese. *Dairy Science & Technology* **92**, 237-248.

Evert-Arriagada, K., Hernández-Herrero, M. M., Guamis, B. & Trujillo, A. J. (2014). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *LWT-Food Science and Technology* **55**, 498-505.

Fernández, M., Linares, D. M., del Río, B., Ladero, V. & Alvarez, M. A. (2007). HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. *Journal of Dairy Research* **74**, 276-282.

Fernández-García, E., Tomillo, J. & Nuñez, M. (1999). Effect of added proteinases and level of starter culture on the formation of biogenic amines in raw milk Manchego cheese. *International Journal of Food Microbiology* **52**, 189-196.

Fernández-García, E., Tomillo, J. & Nuñez, M. (2000). Formation of biogenic amines in raw milk Hispánico cheese manufactured with proteinases and different levels of starter culture. *Journal of Food Protection* **63**, 1551-1555.

Flórez, A., Ruas-Madiedo, P., Alonso, L. & Mayo, B. (2006). Microbial, chemical and sensorial variables of the Spanish traditional blue-veined Cabrales cheese, as affected by inoculation with commercial *Penicillium roqueforti* spores. *European Food Research and Technology* **222**, 250-257.

Garde, S., Arqués, J. L., Gaya, P., Medina, M. & Nuñez, M. (2007). Effect of high-pressure treatments on proteolysis and texture of ewes' raw milk La Serena cheese. *International Dairy Journal* **17**, 1424-1433.

Gobbetti, M., Burzigotti, R., Smacchi, E., Corsetti, A. & De Angelis, M. (1997). Microbiology and biochemistry of Gorgonzola cheese during ripening. *International Dairy Journal* **7**, 519-529.

Gomez, M. J., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (1997). Relationship between level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk. *Journal of Dairy Research* **64**, 289-297.

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research* **71**, 496-499.

Ismail, B. & Nielsen, S. S. (2010). Invited review: Plasmin protease in milk: Current knowledge and relevance to dairy industry. *Journal of Dairy Science* **93**, 4999-5009.

Juan, B., Ferragut, V., Buffa, M., Guamis, B. & Trujillo, A.-J. (2007). Effects of high-pressure treatment on free fatty acids release during ripening of ewes' milk cheese. *Journal of Dairy Research* **74**, 438-445.

Kinsella, J. E. & Hwang, D. H. (1976). Enzymes of *Penicillium roqueforti* involved in the biosynthesis of cheese flavor. *CRC Critical Reviews in Food Science and Nutrition* **8**, 191-228.

Lenoir, J. & Auberger, B. (1977). Les caractères du système protéolytique de *Penicillium caseicolum*. II. Caractérisation dune protéase neutre. *Le Lait* **57**, 471-491.

Linares, D. M., Martín, M. C., Ladero, V., Álvarez, M. A. & Fernández, M. (2011). Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition* **51**, 691-703.

Macedo, A. C. & Malcata, F. X. (1997). Hydrolysis of alpha(s) and beta-caseins during ripening of Serra cheese. *Food Chemistry* **58**, 43-48.

Madkor, S., Fox, P. F., Shalabi, S. I. & Metwalli, N. H. (1987a). Studies on the ripening of Stilton cheese: Lipolysis. *Food Chemistry* **25**, 93-109.

Madkor, S., Fox, P. F., Shalabi, S. I. & Metwalli, N. H. (1987b). Studies on the ripening of Stilton cheese: Proteolysis. *Food Chemistry* **25**, 13-29.

Mallia, S., Fernández-García, E. & Bosset, J. O. (2005). Comparison of purge and trap and solid phase microextraction techniques for studying the volatile aroma compounds of three European PDO hard cheeses. *International Dairy Journal* **15**, 741-758.

Malone, A. S., Wick, C., Shellhammer, T. H. & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science* **86**, 1139-1146.

McSweeney, P. L. H. & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait* **80**, 293-324.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening: Introduction and overview. *Cheese: Chemistry, Physics and Microbiology*, 347-360.

Molimard, P. & Spinnler, H. E. (1996). Compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. *Journal of Dairy Science* **79**, 169-184.

Mulder, H. (1952). Taste and flavour forming substancesin cheese. *Netherlands Milk and Dairy Journal* **6**, 157 - 167.

O'Reilly, C. E., Kelly, A. L., Oliveira, J. C., Murphy, P. M., Auty, M. A. E. & Beresford, T. P. (2003). Effect of varying high-pressure treatment conditions on acceleration of ripening of cheddar cheese. *Innovative Food Science & Emerging Technologies* **4**, 277-284.

Ordóñez, A. I., Ibáñez, F. C., Torre, P. & Barcina, Y. (1997). Formation of biogenic amines in Idiazabal ewe's-milk cheese: effect of ripening, pasteurization, and starter. *Journal of Food Protection* **60**, 1371-1375.

Prieto, B., Franco, I., Fresno, J. M., Bernardo, A. & Carballo, J. (2000). Picon Bejes-Tresviso blue cheese: an overall biochemical survey throughout the ripening process. *International Dairy Journal* **10**, 159-167.

Rodríguez-Alonso, P., Centeno, J. A. & Garabal, J. I. (2009). Comparison of the volatile profiles of Arzúa-Ulloa and Tetilla cheeses manufactured from raw and pasteurized milk. *LWT - Food Science and Technology* **42**, 1722-1728.

Rodríguez-Alonso, P., Centeno, J. A. & Garabal, J. I. (2011). Biochemical study of industrially produced Arzúa-Ulloa semi-soft cows' milk cheese: Effects of storage under vacuum and modified atmospheres with high-nitrogen contents. *International Dairy Journal* **21**, 261-271.

Rynne, N. M., Beresford, T. P., Guinee, T. P., Sheehan, E., Delahunty, C. M. & Kelly, A. L. (2008). Effect of high-pressure treatment of 1 day-old full-fat Cheddar cheese on subsequent quality and ripening. *Innovative Food Science & Emerging Technologies* **9**, 429-440.

Saldo, J., Fernández, A., Sendra, E., Butz, P., Tauscher, B. & Guamis, B. (2003). High pressure treatment decelerates the lipolysis in a caprine cheese. *Food Research International* **36**, 1061-1068.

Sieber, R., Bütikofer, U. & Bosset, J. O. (1995). Benzoic acid as a natural compound in cultured dairy products and cheese. *International Dairy Journal* **5**, 227-246.

Sousa, M. J. & McSweeney, P. L. H. (2001). Studies on the ripening of Cooleeney, an Irish farmhouse Camembert-type cheese. *Irish Journal of Agricultural and Food Research* **40**, 83-95.

Spinnler, H. E. & Gripon, J. C. (2004). Surface mould-ripened cheeses. In Cheese: Chemistry, Physics and Microbiology, pp. 157-174: Academic Press.

Voigt, D. D., Chevalier, F., Qian, M. C. & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavour compounds in mature blue-veined cheese. *Innovative Food Science & Emerging Technologies* **11**, 68-77.

Voigt, D. D., Chevalier, F., Donaghy, J. A., Patterson, M. F., Qian, M. C. & Kelly, A. L. (2012). Effect of high-pressure treatment of milk for cheese manufacture on proteolysis, lipolysis, texture and functionality of Cheddar cheese during ripening. *Innovative Food Science & Emerging Technologies* **13**, 23-30.

Yvon, M. & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal* **11**, 185-201.

Capítulo 12. Conclusiones.

Fotografía: variedades de queso estudiadas, queso azul (superior izquierda), Torta del Casar (superior derecha), queso Brie (inferior izquierda) y queso Arzúa-Ulloa (Inferior derecha).

1. En el queso azul, únicamente los tratamientos de 600 MPa y el de 400 MPa aplicado a las 3 semanas consiguieron reducir la proteolisis secundaria, manteniendo los niveles de aminoácidos libres y proteolisis global en valores similares a los del queso control de 180 días. Todos los tratamientos redujeron los niveles de tiramina, pero únicamente el tratamiento de 400 MPa aplicado a las 6 semanas consiguió disminuir los niveles de aminas biógenas totales.

2. En el queso azul, la reducción de los niveles de ácidos grasos y compuestos volátiles en los quesos tratados con 600 MPa a las 3 semanas provocó una disminución de la calidad de sabor. Los restantes tratamientos disminuyeron los niveles de numerosos compuestos, a pesar de lo cual se mantuvieron niveles de calidad de sabor similares a los del queso control, que apenas sufrió variaciones durante el almacenamiento.

3. En el queso azul, las altas presiones causaron un aumento del índice de hundimiento en los quesos. En general, los tratamientos de altas presiones empleados no representaron ninguna mejora adicional de cara a la conservación de este tipo de queso.

4. En la Torta del Casar, los tratamientos de 600 MPa consiguieron frenar tanto la proteolisis primaria como la secundaria, manteniendo los niveles de caseínas, aunque no los niveles de aminoácidos y proteolisis global, en valores similares a los del queso control de 60 días.

5. En la Torta del Casar, los tratamientos por altas presiones disminuyeron los niveles de tirosina descarboxilasa y los de 600 MPa los niveles de todas las aminas biógenas encontradas. Sin embargo la mayoría de los tratamientos por altas presiones dieron lugar a quesos de textura más firme que la del queso control.

6. En la Torta del Casar, las altas presiones evitaron el incremento de los niveles de ácidos grasos libres y compuestos volátiles, en especial de compuestos azufrados, que se dio en el queso control, consiguiendo que los quesos tratados a las 5 semanas mantuviesen hasta el día 240 un perfil de ácidos grasos libres y compuestos volátiles similar al del queso control de 60 días, previniendo la sobremaduración.

7. En el queso Brie, las altas presiones retrasaron la proteolisis primaria, disminuyendo con 600 MPa la hidrólisis de caseínas y con todos los tratamientos los niveles de péptidos hidrófobos, que aumentaron fuertemente en el queso control. En ningún caso se consiguió reducir la proteolisis secundaria.

8. En el queso Brie, las altas presiones redujeron el incremento de ácidos grasos y, en los quesos tratados a las 2 semanas, mantuvieron un perfil de compuestos volátiles similar al del queso control de 30 días, estabilizando las características organolépticas y evitando así la sobremaduración del queso.

9. En el queso Brie, las altas presiones evitaron la fuerte disminución de fracturabilidad, firmeza y elasticidad que se dio en el queso control, especialmente con los tratamientos aplicados a las 3 semanas. Sin embargo, todos los tratamientos provocaron la pérdida de la capa blanca superficial de moho característica de esta variedad de queso.

10. En el queso Arzúa-Ulloa, las altas presiones aceleraron la proteolisis primaria, pero frenaron la proteolisis secundaria así como la formación de las aminas biógenas. Los quesos tratados con 600 MPa mantuvieron hasta los 240 días niveles de aminas biógenas totales inferiores a los del queso control de 21 días.

11. En el queso Arzúa-Ulloa, las altas presiones redujeron la formación de ácidos grasos. Los tratamientos de 600 MPa mantuvieron además un perfil de compuestos volátiles similar al del control de 21 días hasta el día 120.

12. En el queso Arzúa-Ulloa, las altas presiones indujeron un aumento de fracturabilidad, firmeza y elasticidad. Los cambios químicos ocasionados por las altas presiones no afectaron a la calidad del queso, aunque causaron un cierto aumento de las puntuaciones de amargor.

Capítulo 13. Resumen ampliado.

Fotografía: quesos estudiados, placas de agar para recuento de microorganismos, cromatograma del perfil de volátiles, reacción colorimétrica para determinación de la actividad esterasa y secuencia de ensayo texturométrico por compresión.

- **Introducción**

El queso es un derivado lácteo conocido desde la antigüedad, que ha ido evolucionando hasta dar lugar a los cientos de variedades que se elaboran hoy en día. La producción mundial de queso en 2012 según la FAO, fue de 20,6 millones de toneladas, convirtiéndose en uno de los principales productos agroalimentarios (FAOSTAT, 2014). En la Unión Europea la producción de queso alcanzó 8,7 millones de toneladas en 2013, de los cuales únicamente se produjo en España el 1,66 % (EUROSTAT, 2014). El consumo medio per cápita en España durante el año 2013 se estimó en 8,2 kg/persona y año, lejos aún de la media europea de 17,2 Kg/persona y año (MAGRAMA, 2014).

La transformación de la leche en queso comprende una serie de etapas, de las cuales la maduración es una de las más importantes, ya que es cuando se desarrollan las características organolépticas, de textura y apariencia típicas de cada variedad. Estas características se alcanzan gracias a una serie de reacciones que se pueden agrupar como glicolisis y catabolismo de lactato y citrato, proteolisis y catabolismo de aminoácidos, y lipolisis y catabolismo de ácidos grasos libres. La fermentación de la lactosa por los microorganismos presentes en el queso, principalmente bacterias lácticas, da lugar a la formación de ácido láctico junto a cantidades menores de ácido acético y propiónico, diacetilo, acetoína, etanol y otros compuestos que contribuyen al sabor y aroma. El citrato residual, retenido en la cuajada, puede ser metabolizado por cultivos iniciadores mesófilos para dar diacetilo, acetato, acetoína, 2,3-butanodiol y CO_2 (McSweeney & Fox, 2004). La hidrólisis de triglicéridos por las esterasas y lipasas nativas de la leche o de microorganismos presentes provoca la liberación de ácidos grasos, que contribuyen directamente al aroma y sabor, en especial los de cadena corta y media, y son además precursores de otros compuestos responsables del aroma y sabor (Collins *et al.*, 2003). La hidrólisis de caseínas por las proteasas nativas de la leche, el cuajo o los sistemas proteolíticos de los microorganismos, da lugar a péptidos de gran tamaño, que a su vez son hidrolizados a péptidos de menor tamaño y en última instancia a aminoácidos libres. Tanto péptidos como aminoácidos libres contribuyen al sabor del queso, siendo los aminoácidos además precursores de otros compuestos responsables del sabor y aroma (Sousa *et al.*, 2001, Marilley & Casey, 2004).

Una vez que el queso llega a su punto de maduración óptimo, en el que los compuestos responsables del aroma y sabor alcanzan un equilibrio que da al producto su sabor característico (Mulder, 1952), la maduración continúa durante el almacenamiento, distribución y venta haciendo que el queso llegue al consumidor con un sabor mas fuerte o diferente al deseado por el fabricante (Wick *et al.*, 2004). Este fenómeno conocido como sobremaduración se da principalmente en algunas variedades de queso que experimentan una intensa proteolisis, debido al empleo de enzimas coagulantes de elevada actividad como las cardosinas o de microorganismos con elevada actividad proteolítica como los mohos del género *Penicillium*, y limita la vida útil del producto. Apenas existen trabajos de investigación enfocados a evitar este fenómeno, más allá del empleo de temperaturas de refrigeración, que aunque consiguen ralentizarlo, no lo detienen completamente.

La presencia de microorganismos con actividad descarboxilasa plantea otro problema, como es la presencia de aminas biógenas. Las aminas biógenas son compuestos básicos nitrogenados, de bajo peso molecular, con actividad biológica. Su síntesis y degradación forma parte del metabolismo normal de animales, plantas y microorganismos (ten Brink *et al.*, 1990). Las aminas pueden ser alifáticas (cadaverina, putrescina, espermina y espermidina), aromáticas (tiramina y β-feniletilamina) o heterocíclicas (histamina y triptamina), y pueden clasificarse según el número de grupos amino que contienen como mono, di, o poliaminas (Silla Santos, 1996). A pesar de formar parte del metabolismo normal y de regular diversas funciones indispensables en el organismo, la ingesta de niveles elevados de aminas biógenas puede tener efectos tóxicos tales como aumento de la presión sanguínea, dolores de cabeza, calambres abdominales y urticaria. En mamíferos existe un eficiente sistema de detoxificación en el tracto intestinal y en condiciones normales las aminas exógenas son rápidamente detoxificadas por acción de las amino oxidasas. No obstante, en individuos con un sistema de detoxificación deficiente o bajo tratamiento con fármacos inhibidores de las amino oxidasas, pequeñas cantidades de aminas biógenas pueden provocar episodios de intoxicación que pueden llegar a ser graves (Ladero *et al.*, 2010).

Las aminas biógenas se pueden formar por descarboxilación de aminoácidos o por transaminación de aldehídos y cetonas. El queso es un sustrato ideal para la formación

de aminas, ya que tiene un alto contenido de aminoácidos libres y puede contener microorganismos con actividad descarboxilasa, así como unas condiciones favorables para el crecimiento de estos microorganismos y para la formación de aminas (Loizzo *et al.*, 2013).

Las altas presiones se emplean actualmente como método de pasteurización no térmico, que mejora las características nutricionales de los alimentos frente a los tratamientos térmicos de pasteurización. Además de transmitirse homogéneamente a todo el producto de forma instantánea, otra de las ventajas que ofrecen las altas presiones sobre los tratamientos térmicos es la posibilidad de ser aplicadas directamente al producto elaborado, impidiendo la contaminación posterior del interior del alimento. La demanda existente por parte del consumidor de alimentos más naturales y mínimamente procesados convierte a las altas presiones en una tecnología adecuada para satisfacer esta demanda. Existen en la actualidad gran número de productos tratados por altas presiones en el mercado, como zumos y bebidas, productos lácteos y cárnicos, platos preparados, rellenos para sándwiches, pescados y mariscos, salsas, aguacate y guacamole. Las altas presiones se han aplicado con éxito en leche y queso para la eliminación de microorganismos patógenos contaminantes (Rodriguez *et al.*, 2005, López-Pedemonte *et al.*, 2007, Yang *et al.*, 2012). También se han empleado con el objetivo de acelerar la maduración en diferentes variedades de queso, provocando la lisis de las bacterias lácticas y la liberación de enzimas al medio (O'Reilly *et al.*, 2000, Saldo *et al.*, 2002, Ávila *et al.*, 2006). Únicamente se han empleado altas presiones para alargar la vida útil en queso fresco (Evert-Arriagada *et al.*, 2012, Evert-Arriagada *et al.*, 2014). Las altas presiones además, de la capacidad de eliminar microorganismos, también pueden emplearse para inactivar enzimas, pudiendo evitar el fenómeno de la sobremaduración, así como la formación de aminas biógenas en queso (Novella-Rodríguez *et al.*, 2002, Malone *et al.*, 2003, Huppertz *et al.*, 2004).

- **Objetivo**

El objetivo del presente trabajo fue evaluar el efecto del tratamiento por altas presiones hidrostáticas en diferentes variedades de queso a lo largo de un prolongado periodo de almacenamiento en refrigeración, con el fin de evitar la sobremaduración alargando la vida útil y prevenir la formación y acumulación de aminas biógenas.

- **Material y métodos**

Los tratamientos de altas presiones se aplicaron en cuatro variedades de queso a distintos tiempos de maduración, escogidos en función de la variedad. En queso azul de leche pasteurizada de oveja, con *Penicillium roqueforti* en su interior, se aplicaron 400 y 600 MPa durante 5 minutos a las 3 (400-3S y 600-3S), 6 (400-6S y 600-6S) y 9 semanas (400-9S y 600-9S) de maduración y se realizaron los análisis inmediatamente después de los tratamientos y a los 3, 6, 9 y 12 meses. En Torta del Casar, elaborada con leche cruda de oveja y cuajo vegetal de cardo (*Cynara cardunculus*), se aplicaron 400 y 600 MPa durante 5 minutos a las 3 (400-3S y 600-3S) y 5 semanas (400-5S y 600-5S) de maduración y se realizaron los análisis inmediatamente después de los tratamientos y a los 2, 4, 6 y 8 meses. En queso Brie de leche de vaca pasteurizada, con *Penicillium camemberti* en su superficie, se aplicaron 400 y 600 MPa durante 5 minutos a las 2 (400-2S y 600-2S) y 3 semanas (400-3S y 600-3S) de maduración y se realizaron los análisis inmediatamente después de los tratamientos y a los 1, 2, 3 y 4 meses. En el queso Arzúa-Ulloa elaborado con leche cruda de vaca, se aplicaron 400 y 600 MPa durante 5 minutos a las 2 (400-2S y 600-2S) y 3 semanas (400-3S y 600-3S) de maduración y se realizaron los análisis inmediatamente después de los tratamientos y a los 2, 4, 6 y 8 meses.

Para evaluar el efecto de las altas presiones sobre la microbiota del queso se realizaron análisis microbiológicos mediante recuento en medio sólido de bacterias mesófilas totales (PCA), bacterias lácticas (M17 y MRS agar), lactobacilos (Rogosa agar), enterococos (KF agar), bacterias gram negativas (MacConkey agar), coliformes (VRBA), *Micrococcaceae* (MSA), estafilococos coagulasa positivos (Baird-Parker + RPF II), *Listeria monocytogenes* (Palcam agar), mohos y levaduras (CGA). Se analizó el pH mediante un pH-metro acoplado a un electrodo de penetración y el extracto seco (ES) mediante desecación a 102 ºC hasta peso constante. La proteolisis se evaluó mediante el análisis de caseínas por electroforesis capilar en gel, péptidos y aminoácidos libres por cromatografía líquida (HPLC), y proteolisis global mediante ensayo espectrofotométrico. La actividad aminopeptidasa se determinó mediante ensayo colorimétrico medido por espectrofotometría. La lipolisis se evaluó determinando los ácidos grasos libres mediante extracción en fase sólida y análisis por cromatografía de gases (GC). La

actividad esterasa se determinó mediante ensayo colorimétrico medido por espectrofotometría. Para evaluar el efecto de las altas presiones sobre la formación de aminas biógenas, se midió la actividad tirosina descarboxilasa y la concentración de aminas biógenas mediante HPLC. Se analizaron los compuestos volátiles mediante microextracción en fase sólida (SPME), seguida de cromatografía de gases-espectrometría de masas (GC-MS). Se determinaron los parámetros de textura (fracturabilidad, firmeza y elasticidad) por compresión mediante un texturómetro y los parámetros colorimétricos (L*, a* y b*) mediante un colorímetro. Finalmente, se llevó a cabo el análisis sensorial de sabor y olor, mediante evaluación de diversas características y descriptores por un panel entrenado de catadores.

- **Resultados**

- Queso azul

El queso control, sin tratar, sufrió una reducción de los niveles de bacterias lácticas y bacterias mesófilas totales desde 9,54 y 8,76 log ufc/g respectivamente a día 1 hasta 6,57 y 5,57 log ufc /g a día 360, mientras que los niveles de *P. roqueforti* descendieron desde 7,73 log ufc/g a día 21 hasta 5,66 log ufc/g a día 360. Los tratamientos de altas presiones redujeron los niveles de bacterias lácticas, bacterias mesófilas totales y *P. roqueforti*, en especial los tratamientos de 600 MPa que llegaron a reducir la población de *P. roqueforti* hasta niveles no detectables

La fuerte proteolisis primaria que se dio en el queso control, con reducciones de las α_S-, β-, κ- y *p*-κ-caseínas de 93, 87, 78 y 46 %, respectivamente, entre los días 1 y 21, no pudo evitarse con los tratamientos de altas presiones. En cuanto a los niveles de péptidos hidrófilos e hidrófobos, que aumentaron en el queso control, únicamente se consiguieron reducir los niveles péptidos hidrófilos con los tratamientos de 600 MPa aplicados a las 3 y 6 semanas. La actividad aminopeptidasa se redujo durante el almacenamiento en refrigeración, y las altas presiones provocaron la reducción de esta actividad. Los niveles de aminoácidos del queso control aumentaron durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar una concentración de 116,76 mg/g ES a día 360, que únicamente los tratamientos de 600 MPa y el de 400 MPa aplicado a las 3 semanas consiguieron frenar, manteniendo hasta

el día 360 niveles de aminoácidos libres y proteolisis global similares a los del queso control de 180 días.

Las aminas biógenas en el queso control alcanzaron concentraciones de 0,06, 0,07, 0,05, 0,03 y 0,01 mg/g ES de β-feniletilamina, triptamina, tiramina, putrescina y espermidina, respectivamente, a día 360. Los tratamientos de altas presiones únicamente consiguieron reducir los niveles de tiramina.

Los tratamientos de 600 MPa y el de 400 MPa aplicado a las 3 semanas redujeron la actividad esterasa. Los ácidos grasos libres en el queso control aumentaron durante la maduración y el almacenamiento en refrigeración, alcanzando concentraciones de 8,88, 18,31 y 56,63 mg/g ES de ácidos grasos libres de cadena corta ($C_{4:0}$ – $C_{8:0}$), media ($C_{10:0}$ – $C_{14:0}$) y larga ($C_{16:0}$ – $C_{18:3}$), respectivamente, a día 360. Excepto en los quesos tratados con 400 MPa a las 6 y 9 semanas, el resto de tratamientos redujeron la formación de todos los grupos de ácidos grasos libres. Únicamente los quesos tratados con 400 MPa a las 3 semanas mantuvieron niveles de ácidos grasos libres de cadena corta similares o inferiores a los del queso control de 180 días. Estos quesos, junto a los quesos tratados con 600 MPa a las 6 y 9 semanas, mantuvieron niveles de ácidos grasos libres de cadena media y larga similares a los del control de 180 días. Por otro lado, los niveles de los tres grupos de ácidos grasos libres en los quesos tratados con 600 MPa a las 3 semanas se mantuvieron muy por debajo de los niveles del control de 180 días. El ácido etanoico aumentó hasta el día 63, descendiendo posteriormente. Excepto los tratamientos de 400 MPa aplicados a las 6 y 9 semanas, el resto redujeron los niveles de este ácido.

El grupo mayoritario de compuestos volátiles fue el de los ácidos, que aumentó durante el almacenamiento en refrigeración, seguido de alcoholes y cetonas, que también aumentaron, aunque en menor medida. Igualmente, aumentaron los niveles de ésteres, terpenos, compuestos azufrados y nitrogenados, mientras que los aldehídos y compuestos bencénicos descendieron, y los hidrocarburos no variaron. Las altas presiones redujeron los niveles de ésteres, alcoholes, hidrocarburos, compuestos bencénicos y nitrogenados, especialmente en los quesos tratados con 600 MPa, en los cuales además se redujo la formación de cetonas. En los quesos tratados a las 3 semanas se redujeron los niveles de ácidos volátiles, compuestos azufrados y terpenos.

El análisis de componentes principales de los grupos de ácidos grasos libres y los grupos de compuestos volátiles discriminó única y escasamente entre tiempos, indicando que el efecto de las altas presiones sobre el queso azul fue leve.

Únicamente el tratamiento de 600 MPa aplicado a las 3 semanas redujo el ligero aumento de intensidad de sabor que se dio en el queso control durante el almacenamiento en refrigeración. El queso control no sufrió variaciones en la calidad de sabor durante el almacenamiento en refrigeración y todos los quesos tratados mantuvieron niveles similares a los del queso control, excepto los tratados con 600 MPa a las 3 semanas que obtuvieron puntuaciones más bajas.

En el queso control se observó un hundimiento de la parte central del queso, que llegó a ser de un 15 % respecto al borde. Las altas presiones provocaron un aumento de este hundimiento, que en el caso de los quesos tratados con 400 MPa a las 3 semanas llegó a ser de hasta un 36 %.

- Torta del Casar

En el queso control se observó un descenso de los niveles de bacterias mesófilas totales, bacterias lácticas y enterococos desde 9,51, 9,46 y 7,32 log ufc/g respectivamente a día 21, hasta 8,61, 8,42 y 6,80 log ufc/g a día 240. Los niveles de lactobacilos aumentaron hasta 8,13 log ufc/g a día 35 y se mantuvieron en esos niveles durante todo el almacenamiento en refrigeración. Las altas presiones redujeron los niveles de bacterias mesófilas, bacterias lácticas, lactobacilos y enterococos, especialmente con los tratamientos de 600 MPa. Los niveles de *Micrococcaceae*, coliformes y bacterias gram negativas descendieron desde 6,39, 5,52 y 6,01 log ufc/g respectivamente a día 1, hasta 5,57, 2,87 y 5,37 log ufc/g a día 240. Las altas presiones redujeron los niveles de *Micrococcaceae*, especialmente con los tratamientos aplicados a las 5 semanas, mientras que todos los tratamientos, excepto el de 400 MPa aplicado a las 3 semanas, redujeron por debajo del límite de detección los niveles de coliformes y bacterias gram negativas. Los niveles de estafilococos coagulasa positivos descendieron en el queso control desde 5,23 log ufc/g a día 1 hasta 2,30 a día 180, no detectándose a día 240, mientras que en los quesos presurizados estuvieron desde el día 21 por debajo del límite de detección. En el queso control el pH aumentó durante la maduración y el

almacenamiento en refrigeración hasta alcanzar valores de 6,13, siendo muy inferior el aumento en los quesos tratados por altas presiones.

Las α_S- y β-caseínas del queso control sufrieron reducciones del 79 y 62 %, respectivamente, entre los días 1 y 35, mientras que la κ-caseína se redujo en un 96 % entre los días 1 y 21, no volviéndose a detectar a partir del día 35. Los tratamientos de 600 MPa consiguieron frenar la proteolisis primaria, reduciendo la hidrólisis de α_S- y β-caseínas así como la formación de péptidos hidrófilos, que sí aumentaron en el queso control. La actividad aminopeptidasa disminuyó más rápidamente en los quesos tratados por altas presiones que en el queso control. La concentración de aminoácidos libres aumentó durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar 23,30 mg/g ES a día 360. Los tratamientos de 600 MPa redujeron la proteolisis global, además el tratamiento aplicado a las 3 semanas redujo la formación de aminoácidos libres, aunque para ambos parámetros los niveles obtenidos fueron superiores a los del queso control de 60 días.

La actividad tirosina descarboxilasa se triplicó en el queso control entre los días 60 y 180. Todos los tratamientos de altas presiones consiguieron reducirla. El incremento de tiramina llegó hasta 1,09 mg/g ES a día 240 en el queso control, y se vio reducido con los tratamientos de 600 MPa. La putrescina, triptamina, β-feniletilamina, cadaverina e histamina aumentaron hasta alcanzar 0,95, 0,83, 0,29, 0,69 y 0,30 mg/g ES, respectivamente, a día 240. Todos los tratamientos de altas presiones consiguieron reducir los niveles de cadaverina. Los niveles de putrescina, triptamina y β-feniletilamina se vieron igualmente reducidos, excepto en los quesos tratados con 400 MPa a las 3 semanas. Únicamente los tratamientos de 600 MPa consiguieron reducir los niveles de histamina.

La actividad esterasa en el queso control disminuyó a partir del día 60. Los tratamientos de 600 MPa lograron reducir esta actividad durante todo el almacenamiento en refrigeración. Los niveles de ácidos grasos libres del queso control aumentaron a lo largo de la maduración y del almacenamiento en refrigeración, llegando a alcanzar concentraciones de ácidos grasos libres de cadena corta, media y larga de 8,69, 0,88 y 3,02 mg/g ES, respectivamente, a día 240. Todos los tratamientos redujeron la formación de ácidos grasos libres y únicamente los ácidos grasos libres de

cadena corta mantuvieron hasta el día 240 niveles similares a los del queso control de 60 días. Los ácidos etanoico, propanoico y de cadena ramificada también aumentaron, alcanzando 5,82, 2,99 y 1,15 mg/g ES a día 240. Todos los tratamientos redujeron los niveles de ácido propanoico y ácidos de cadena ramificada, y únicamente los tratamientos aplicados a las 3 semanas los de ácido etanoico, logrando mantener niveles similares o inferiores a los del queso control de 60 días durante todo el almacenamiento.

El grupo mayoritario de compuestos volátiles en el queso control fue el de los ácidos hasta el día 180, mientras que a día 240 fue el grupo de alcoholes. Durante el almacenamiento en refrigeración, únicamente disminuyó el grupo de ácidos volátiles, mientras que el resto aumentaron, con un fuerte incremento de compuestos azufrados que se dio en el queso control. Todos los tratamientos de altas presiones consiguieron reducir los niveles de ésteres, aldehídos y especialmente de compuestos azufrados. Los tratamientos de 600 MPa redujeron los niveles de alcoholes, mientras que los tratamientos aplicados a las 3 semanas redujeron los niveles de ácidos volátiles. Mediante el análisis de componentes principales de los grupos de ácidos grasos y los grupos de compuestos volátiles se vio que los quesos tratados a las 5 semanas de maduración mantuvieron hasta el día 240 un perfil similar al del queso control de 60 días.

Durante el almacenamiento en refrigeración descendieron los parámetros de firmeza y elasticidad en el queso control. Ninguno de los tratamientos consiguió estabilizar ni mantener valores similares a los del queso control de 60 días. En el queso control se produjo un incremento de la intensidad de sabor y olor así como de sabor amargo y de olor a pútrido y rancio durante el almacenamiento, provocando un fuerte descenso de la calidad de sabor y olor. Todos los tratamientos redujeron el incremento de la intensidad de olor pero únicamente los de 600 MPa consiguieron reducir la intensidad de sabor. Las altas presiones frenaron también el incremento de olor pútrido y rancio, evitando la fuerte pérdida de calidad de sabor y olor que se dio en el queso control.

- Queso Brie

El queso control sufrió una reducción de los niveles de bacterias lácticas y bacterias mesófilas totales desde 8,99 y 8,75 log ufc/g respectivamente a día 1, hasta 7,88 y 7,90 log ufc/g a día 120, mientras que los niveles de *P. camemberti* se mantuvieron estables a lo largo de todo el periodo de estudio. Los tratamientos de altas presiones redujeron los niveles de bacterias lácticas, bacterias mesófilas totales y *P. camemberti*, en especial los tratamientos de 600 MPa, que redujeron los niveles de *P. camemberti* por debajo del límite de detección. El pH del queso control sufrió un drástico aumento a partir del día 30, alcanzando valores de 7,87 a día 120. Con los tratamientos de altas presiones se consiguió que el pH se mantuviera en valores estables durante todo el almacenamiento en refrigeración.

La proteolisis primaria que se dio en el queso control, con reducciones de las α_S-, β- y κ-caseínas del 41, 37 y 36 %, respectivamente, entre los días 1 y 14, únicamente se vio reducida por los tratamientos de 600 MPa, que frenaron la hidrólisis de α_S- y β-caseína. Todos los tratamientos evitaron el fuerte incremento de péptidos hidrófobos que se dio en el queso control. Los tratamientos de altas presiones lograron reducir la actividad aminopeptidásica durante todo el almacenamiento en refrigeración, excepto el de 400 MPa aplicado a las 3 semanas que únicamente consiguió valores más bajos que el queso control a día 120. Los niveles de aminoácidos libres del queso control aumentaron durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar una concentración de 78,24 mg/g ES a día 120. Las altas presiones no frenaron la proteolisis secundaria, sino que causaron una mayor acumulación de aminoácidos libres, así como una mayor proteolisis global.

La actividad estearasa en el queso control aumentó durante el almacenamiento. Las altas presiones no consiguieron evitar este aumento y en algunos casos la aumentaron. Los ácidos grasos libres aumentaron en el queso control durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar concentraciones de 3,48, 5,03 y 21,58 mg/g ES de ácidos grasos libres de cadena corta, media y larga respectivamente, a día 120. Las altas presiones redujeron la formación de ácidos grasos libres de cadena corta, y los tratamientos aplicados a las 2 semanas redujeron además los niveles de ácidos grasos libres de cadena media y larga, mientras que los tratamientos aplicados a

las 3 semanas no afectaron a los ácidos grasos de cadena media hasta el día 60 y provocaron un aumento de los de cadena larga, reduciendo posteriormente los niveles de estos dos grupos hasta niveles similares o inferiores a los del queso control de 30 días. Los niveles de ácidos etanoico y de cadena ramificada aumentaron hasta 0,40 y 1,03 mg/g ES, respectivamente, a día 120. Las altas presiones redujeron la formación de los ácidos libres de cadena ramificada pero únicamente los tratamientos de 600 MPa redujeron la formación de ácido etanoico.

El grupo de compuestos volátiles más abundante en el control a día 30 fue el de los ácidos, mientras que a día 60 y 120 fueron las cetonas, que sufrieron un descenso entre los días 30 y 60, aumentando posteriormente. Los niveles de alcoholes, aldehídos, compuestos bencénicos, nitrogenados y azufrados aumentaron durante el almacenamiento en refrigeración. Las altas presiones evitaron el incremento de cetonas, compuestos bencénicos, nitrogenados y azufrados, y provocaron una mayor acumulación de ácidos volátiles, alcoholes, éteres y ésteres. Mediante el análisis de componentes principales de los compuestos volátiles individuales se vio que los quesos tratados a las 2 semanas mantuvieron hasta el día 120 un perfil de compuestos volátiles similar al del queso control de 30 días.

Los valores de fracturabilidad, firmeza y elasticidad descendieron durante la maduración, manteniéndose en valores muy bajos durante el almacenamiento en refrigeración. Las altas presiones evitaron el descenso de elasticidad y firmeza, y únicamente los tratamientos aplicados a las 2 semanas frenaron el descenso de la fracturabilidad. En el queso control se dio un ligero aumento del parámetro de color b* (tendencia al amarillo) tanto en la superficie como en el interior, un fuerte aumento entre los días 90 y 120 del parámetro a* (tendencia al rojo) y una disminución de la L* (luminosidad). Las altas presiones frenaron el aumento de b* y a* del interior y evitaron la pérdida de luminosidad del interior del queso. Las altas presiones provocaron la pérdida de la capa superficial de moho característica de esta variedad de queso, haciendo que los parámetros de color de la superficie fuesen muy diferentes de los del queso control. Durante el almacenamiento en refrigeración se produjo una pérdida de calidad de olor y sabor acompañada de un fuerte incremento de intensidad de olor y sabor, así como de los sabores amargo y umami. Las altas presiones evitaron el

aumento de intensidad de sabor y olor y de sabor amargo, evitando la pérdida de calidad de sabor y olor, que se mantuvieron en niveles similares a los del queso control de 30 días.

- Arzúa-Ulloa

El queso control sufrió una reducción de los niveles de bacterias mesófilas totales desde 9,2 log ufc/g a día 1 hasta 8,0 log ufc/g a día 21, permaneciendo en los mismos niveles hasta el día 240. Los niveles de bacterias lácticas descendieron desde 8,8 log ufc/g a día 1 hasta 8,0 log ufc/g a día 14, manteniéndose en los mismos valores hasta el día 240. Por otro lado los niveles de lactobacilos aumentaron, desde 4,1 log ufc/g a día 1 hasta 8,0 log ufc/g a día 60, no variando posteriormente. Los tratamientos de 600 MPa consiguieron reducir y mantener durante el almacenamiento en refrigeración los niveles de bacterias mesófilas totales, lácticas y lactobacilos. Los niveles de enterococos, *Micrococcaceae*, coliformes y bacterias gram negativas descendieron desde 6,31, 6,92, 7,50 y 7,52 log ufc/g respectivamente a día 1, hasta 4,71, 4,08, 1,46 y 2,04 log ufc/g a día 240, mientras que los niveles de estafilococos coagulasa positivos descendieron desde 4,83 log ufc/g a día 1 hasta 2,66 log ufc/g a día 14, no volviéndose a detectar a partir del día 21. Todos los tratamientos de altas presiones consiguieron reducir los niveles de enterococos, *Micrococcaceae*, coliformes, gram negativas y estafilococos, manteniendo por debajo del límite de detección los niveles de coliformes, gram negativas y estafilococos. Para los enterococos este efecto sólo se consiguió con los tratamientos de 600 MPa. El pH del queso control aumentó levemente durante el almacenamiento en refrigeración, alcanzando un valor de 5,68 a día 240. Los tratamientos de 600 MPa frenaron este aumento manteniendo valores similares a los del queso control de 60 días.

El queso control sufrió una progresiva proteolisis primaria, con reducciones de los niveles de las α_S-, β-, κ- y *p*-κ-caseínas del 46, 64, 65 y 35 %, respectivamente, entre los días 1 y 240. Las altas presiones no sólo no frenaron la proteolisis primaria sino que la aceleraron, provocando una mayor hidrólisis de β-, κ- y *p*-κ-caseínas. Los tratamientos de 400 MPa aumentaron además la hidrólisis de α_S-caseína. Esta mayor hidrólisis de caseínas en los quesos tratados se vio reflejada en una mayor acumulación de péptidos hidrófilos e hidrófobos que en el queso control. Todos los tratamientos de altas

presiones consiguieron reducir la actividad aminopeptidasa. La concentración de aminoácidos libres del queso control aumentó a lo largo de la maduración y del almacenamiento en refrigeración, llegando a alcanzar niveles de 31,68 mg/g ES. Las altas presiones consiguieron frenar la proteolisis secundaria, reduciendo los niveles de aminoácidos libres y la proteolisis global, y manteniendo los quesos tratados con 600 MPa niveles similares a los del queso control de 60 días hasta el final del almacenamiento en refrigeración.

La actividad tirosina descarboxilasa aumentó en el queso control a lo largo de la maduración y el almacenamiento en refrigeración, reduciéndose con los tratamientos de altas presiones, excepto con el tratamiento de 400 MPa aplicado a las 3 semanas en los días 180 y 240. En el queso control la tiramina y la putrescina aumentaron durante la maduración y el almacenamiento en refrigeración, llegando a alcanzar concentraciones de 0,372 y 0,207 mg/g ES, respectivamente, a día 240, mientras que la cadaverina aumentó hasta 0,113 mg/g ES a día 21, descendiendo posteriormente y manteniéndose en valores de 0,800 mg/g ES hasta el día 240. La histamina no se detectó hasta el día 180, con una concentración de 0,009 mg/g ES que a día 240 aumentó hasta 0,026 mg/g ES. Las altas presiones redujeron los niveles de tiramina y putrescina, manteniendo los tratamientos de 600 MPa niveles no detectables. Las altas presiones consiguieron además reducir los niveles de histamina, mientras que los de cadaverina no mantuvieron una tendencia clara a lo largo del almacenamiento en refrigeración.

La actividad esterasa, que aumentó en el queso control a lo largo del tiempo, se vio reducida por los tratamientos de altas presiones. Durante la maduración y el almacenamiento en refrigeración, en el queso control se produjo un aumento de ácidos grasos libres de cadena corta, media y larga y de ácido etanoico, hasta concentraciones de 0,190, 0,400, 1,307 y 2,102 mg/g ES, respectivamente, a día 240. Las altas presiones redujeron la formación de ácido etanoico y ácidos grasos libres de cadena corta, pero no de los de cadena media y larga, que únicamente mostraron niveles menores a día 240 en los quesos tratados con 600 MPa y los tratados con 400 MPa a las 3 semanas. En el queso control se observó un aumento de ácido benzoico que llegó a alcanzar una concentración de 0,398 mg/g ES a día 180. Únicamente se vio una reducción de los niveles de este ácido a día 180 en los quesos tratados a las 2 semanas.

El grupo de compuestos volátiles mayoritario en el queso control fue el de los alcoholes, que aumentó entre los días 60 y 120, descendiendo posteriormente. Los niveles de ácidos, cetonas, ésteres, éteres, hidrocarburos, compuestos bencénicos y azufrados descendieron durante el almacenamiento en refrigeración, mientras que los aldehídos se mantuvieron en los niveles estables y los terpenos aumentaron. Las altas presiones evitaron parcialmente el descenso de ácidos volátiles, éteres y compuestos azufrados. Además, en los quesos tratados con 600 MPa se evitó el descenso de cetonas e hidrocarburos. Mediante el análisis de componentes principales de los distintos grupos de compuestos volátiles se vio que los quesos tratados con 600 MPa mantuvieron hasta los 120 días un perfil similar al del queso control de 21 días.

En el queso control la elasticidad y la firmeza aumentaron hasta el día 180, mientras que la fracturabilidad únicamente pudo determinarse a día 240. Las altas presiones aumentaron la fracturabilidad y no lograron evitar el aumento de elasticidad y firmeza. La intensidad de sabor aumentó durante el almacenamiento en refrigeración, al igual que aumentaron el sabor amargo y umami, mientras que la calidad de sabor se mantuvo hasta día 180, descendiendo levemente a día 240. Las altas presiones no lograron frenar el aumento de la intensidad de sabor, ni la disminución de la calidad de sabor que se dio a los 240 días, sino que por el contrario favorecieron el aumento de sabor amargo.

- **Conclusiones**

En el queso azul, el queso control experimentó cambios químicos menores durante el almacenamiento en refrigeración que no afectaron a la calidad ni a la intensidad de sabor. Los tratamientos de altas presiones de 600 MPa y el de 400 MPa aplicado a las 3 semanas consiguieron reducir la proteolisis secundaria y, además, los tratamientos aplicados a las 3 semanas consiguieron reducir la lipolisis. El efecto de las altas presiones sobre la proteolisis y la lipolisis junto al efecto que ejercieron en la formación de compuestos volátiles, únicamente provocó una pérdida de la calidad e intensidad de sabor en los quesos tratados con 600 MPa a las 3 semanas, mientras que en el resto de quesos no supuso ninguna diferencia. De modo que las altas presiones no representan un beneficio adicional en la conservación del queso azul bajo las condiciones empleadas en este trabajo.

En la Torta del Casar, el queso control experimentó fuertes cambios durante el almacenamiento en refrigeración que provocaron una pérdida de la calidad de sabor y olor, además de producirse una gran acumulación de aminas biógenas. Los tratamientos de altas presiones evitaron el aumento de pH que se dio en el queso control y además, los tratamientos de 600 MPa redujeron la proteolisis. Las altas presiones consiguieron reducir la lipolisis, así como la formación de aminas biógenas y la formación de compuestos volátiles responsables de la pérdida de calidad que se dio en el queso control. De modo que las altas presiones, en especial los tratamientos aplicados a las 5 semanas, supusieron una mejora sustancial para la conservación de la Torta del Casar, alargando su vida útil hasta 240 días, además de reducir la acumulación de aminas biógenas.

En el queso Brie, el queso control experimentó marcados cambios químicos durante el almacenamiento en refrigeración que provocaron una fuerte pérdida de calidad, así como un notable incremento de la intensidad de sabor y olor. Los tratamientos de altas presiones evitaron el fuerte incremento de pH que se dio en el control, además de reducir la proteolisis primaria, evitando la acumulación de péptidos hidrófobos. Las altas presiones también consiguieron frenar la lipolisis, así como la formación de compuestos volátiles responsables de la pérdida de calidad del queso control, principalmente compuestos azufrados. Por otro lado, los tratamientos de altas presiones provocaron la pérdida de la capa superficial de moho. De modo que las altas presiones, en especial los tratamientos aplicados a las 2 semanas, supusieron una mejora sustancial para la conservación del queso Brie, aumentando su vida útil hasta 120 días, aunque la pérdida de la capa superficial de moho supone una limitación a la hora de su comercialización.

En el queso Arzúa-Ulloa, el queso control experimentó cambios químicos durante el almacenamiento en refrigeración que únicamente provocaron la pérdida de calidad de sabor en el día 240, aunque se dio un aumento de intensidad de sabor y olor, así como de sabor amargo y umami, a lo largo de este periodo. Los tratamientos de altas presiones redujeron el aumento de pH que tuvo lugar en el queso control. A pesar de acelerar la proteolisis primaria, los tratamientos consiguieron frenar la proteolisis secundaria. Las altas presiones disminuyeron levemente la lipolisis, especialmente la

formación de ácidos grasos libres de cadena corta, y redujeron la formación de aminas biógenas, en especial los tratamientos de 600 MPa. El efecto de las altas presiones sobre los compuestos volátiles hizo que los quesos tratados con 600 MPa mantuviesen hasta el día 120 un perfil de volátiles similar al del queso control de 21 días. Las altas presiones no influyeron sobre la calidad ni la intensidad de sabor del queso, a pesar de que provocaron un aumento del sabor amargo. De modo que las altas presiones no representaron una mejora de cara a la conservación del queso desde el punto de vista de sus características sensoriales, aunque sí que lograron evitar la acumulación de aminas biógenas.

- **Bibliografía**

Ávila, M., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy Science* **89**, 2882-2893.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal* **13**, 841-866.

EUROSTAT (2014). http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home/

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

Evert-Arriagada, K., Hernández-Herrero, M. M., Guamis, B. & Trujillo, A. J. (2014). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *LWT-Food Science and Technology* **55**, 498-505.

FAOSTAT (2014). http://faostat3.fao.org/faostat-gateway/go/to/home/E

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research* **71**, 496-499.

Ladero, V., Calles-Enríquez, M., Fernández, M. & Alvarez, M. A. (2010). Toxicological effects of dietary biogenic amines. *Current Nutrition & Food Science* **6**, 145-156.

Loizzo, M. R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U. G. & Restuccia, D. (2013). Technological aspects and analytical determination of biogenic amines in cheese. *Trends in Food Science & Technology* **30**, 38-55.

López-Pedemonte, T., Roig-Sagués, A. X., De Lamo, S., Gervilla, R. & Guamis, B. (2007). High hydrostatic pressure treatment applied to model cheeses made from cow's milk inoculated with *Staphylococcus aureus*. *Food Control* **18**, 441-447.

MAGRAMA (2014). http://www.magrama.gob.es/

Malone, A. S., Wick, C., Shellhammer, T. H. & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science* **86**, 1139-1146.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology* **90**, 139-159.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In Cheese: Chemistry, Physics and Microbiology, pp. 361-371: Academic Press.

Mulder, H. (1952). Taste and flavour forming substances in cheese. *Netherlands Milk and Dairy Journal* **6**, 157 - 167.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Saldo, J. & Vidal-Carou, M. C. (2002). Effects of high hydrostatic pressure treatments on biogenic amine contents in goat cheeses during ripening. *Journal of Agricultural and Food Chemistry* **50**, 7288-7292.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L. & Beresford, T. P. (2000). The effect of exposure to pressure of 50 MPa on Cheddar cheese ripening. *Innovative Food Science & Emerging Technologies* **1**, 109-117.

Rodriguez, E., Arques, J. L., Nuñez, M., Gaya, P. & Medina, M. (2005). Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli* O157:H7 in raw-milk cheese. *Applied and Environmental Microbiology* **71**, 3399-3404.

Saldo, J., McSweeney, P. L. H., Sendra, E., Kelly, A. L. & Guamis, B. (2002). Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening. *International Dairy Journal* **12**, 35-44.

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology* **29**, 213-231.

Sousa, M. J., Ardö, Y. & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal* **11**, 327-345.

ten Brink, B., Damink, C., Joosten, H. & JHJ., H. i. t. V. (1990). Occurrence and formation of biologically-active amines in foods. *International Journal of Food Microbiology* **11**, 73-84.

Wick, C., Nienabert, U., Anggraeni, O., Shellhammer, T. H. & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial cheddar cheeses treated with high pressure. *Journal of Dairy Research* **71**, 107-115.

Yang, B. W., Shi, Y., Xia, X. D., Xi, M. L., Wang, X., Ji, B. Y. & Meng, J. H. (2012). Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. *Food Control* **28**, 273-278.

Capítulo 14. Extended abstract.

Fotografía: cuñas de los quesos estudiados, determinación de pH, liofilizado de péptidos, colonias de *P. roqueforti,* equipo de altas presiones y extracción de ácidos grasos libres mediante separación en fase solida.

- **Introduction**

Cheese is a dairy product known since ancient times that has evolved to give rise to the hundreds of varieties that are currently manufactured. According to FAO, world cheese production in 2012 was 20.6 million tons, making cheese one of the main food products (FAOSTAT, 2014). In the EU cheese production reached 8.7 million tonnes in 2013, out of which only 1.66 % was made in Spain (EUROSTAT, 2014). The averaged per capita consumption in Spain in 2013 was estimated in 8.2 kg/person per year, still far from the European average of 17.2 kg/person per year (MAGRAMA, 2014).

The transformation of milk into cheese comprises different stages, out of which ripening is one of the most important, since organoleptic characteristics, texture and typical appearance of each variety develop during this phase. These characteristics are achieved by a series of reactions which can be grouped as glycolysis and catabolism of lactate and citrate, proteolysis and amino acid catabolism, and lipolysis and free fatty acids catabolism. Lactose fermentation by the microorganisms present in cheese, especially lactic bacteria, results in the formation of lactic acid and minor amounts of acetic and propionic acids, diacetyl, acetoin, ethanol and other compounds that contribute to cheese flavour and aroma. The residual citrate, retained in the curd, can be metabolized by mesophilic starter cultures, yielding diacetyl, acetate, acetoin, 2,3-butanediol and CO_2 (McSweeney & Fox, 2004). Triglyceride hydrolysis by esterases and lipases from milk or microorganisms causes the release of fatty acids, which contribute directly to aroma and flavour, especially the short and medium chain free fatty acids, and are also precursors of other compounds responsible for aroma and flavour (Collins *et al.*, 2003). Hydrolysis of casein by native milk proteases, rennet or proteolytic systems of microorganisms, leads to the generation of large peptides, which are further hydrolyzed into smaller peptides and finally, into free amino acids. Both, peptides and free amino acids, contribute to the flavour of cheese, being amino acids also precursors of flavour and aroma compounds (Sousa *et al.*, 2001, Marilley & Casey, 2004).

Once cheese reaches its optimum ripening time, at which the compounds responsible for flavour and aroma reach an equilibrium which confers the product its characteristic flavour (Mulder, 1952), ripening continues during storage, distribution and sale, causing the cheese to reach the consumer with a stronger or different flavour than

the manufacturer intended (Wick *et al.*, 2004). This phenomenon, known as over-ripening, mainly occurs in some cheese varieties which undergo an intense proteolysis, due to the use of coagulating enzymes of high proteolytic activity as cardosins or microorganisms with high proteolytic activity as moulds from the genus *Penicillium*, and it limits the shelf-life of product. There is hardly any research leading to avoid this phenomenon, just the use of refrigeration temperatures, which allows to slow it down but not to stop it.

The presence in cheese of microorganisms with decarboxylase activity poses another problem, the presence of biogenic amines. Biogenic amines are nitrogenous basic compounds of low molecular weight with biological activity. Their synthesis and degradation is part of the normal metabolism of animals, plants and microorganisms (ten Brink *et al.*, 1990). Biogenic amines can be aliphatic (cadaverine, putrescine, spermine and spermidine), aromatic (tyramine and β-phenylethylamine) or heterocyclic (histamine and tryptamine) and can be classified by the number of amino groups as mono, di, or polyamines (Silla Santos, 1996). Although biogenic amines are involved in normal metabolism and regulate various essential functions in the body, the intake of high levels of biogenic amines may have toxic effects such as increased blood pressure, headache, abdominal cramps and urticaria. In mammals, there is an efficient detoxification system in the intestinal tract and under normal conditions exogenous biogenic amines are rapidly detoxified by the action of amine oxidases. However, in individuals with a deficient detoxification system or under treatment with amine oxidases inhibitory drugs, small amounts of biogenic amines can induce episodes of intoxication that can become serious (Ladero *et al.*, 2010).

Biogenic amines are formed by decarboxylation of amino acids or by transamination of aldehydes and ketones. Cheese is an ideal substrate for amines formation, due to its high content of free amino acids and because it may contain microorganisms with decarboxylase activity, and it presents favourable conditions for the growth of these microorganisms and for the formation of amines (Loizzo *et al.*, 2013).

High hydrostatic pressure is currently used as a non-thermal pasteurization procedure, which improves the nutritional characteristics of foods over thermal

pasteurization treatments. Besides of the fact that the pressure is rapidly and homogeneously transferred to the entire product, another advantage offered by high pressure over thermal treatments is the possibility of being directly applied to the elaborated product, preventing further contamination of the food interior. Consumers demand more natural and minimally processed foods, and high pressure processing represents an appropriate technology to meet this demand. Currently, there are many products treated by high pressure in the market, such as juices and beverages, meat and dairy products, prepared meals, sandwich fillings, fish and shellfish, sauces, avocado and guacamole. High pressure treatments have been successfully applied to milk and cheese for elimination of foodborne pathogens (Rodriguez *et al.*, 2005, López-Pedemonte *et al.*, 2007, Yang *et al.*, 2012). High pressure treatments have also been used in order to accelerate ripening in different cheese varieties, causing lysis of lactic acid bacteria and releasing enzymes into the cheese matrix (O'Reilly *et al.*, 2000, Saldo *et al.*, 2002, Ávila *et al.*, 2006). But high pressure treatments have been used to extend shelf life only in fresh cheese (Evert-Arriagada *et al.*, 2012, Evert-Arriagada *et al.*, 2014). In addition to the ability of high pressure treatments to eliminate microorganisms, they can also be used to inactivate enzymes and might avoid the phenomenon of over-ripening as well as the formation of biogenic amines in cheese (Novella-Rodríguez *et al.*, 2002, Malone *et al.*, 2003, Huppertz *et al.*, 2004).

- **Objetive**

The aim of this study was to evaluate the effect of high hydrostatic pressure treatments on different cheese varieties throughout a prolonged period of refrigerated storage, in order to avoid over-ripening, extending shelf-life and preventing the formation and accumulation of biogenic amines.

- **Material and methods**

High pressure treatments were applied on four cheese varieties at different times of ripening, that were selected according to each variety. In blue cheese, made from pasteurized ewe's milk and ripened with *Penicillium roqueforti* in the interior, 400 and 600 MPa were applied for 5 minutes at 3 (400-3W and 600-3W), 6 (400-6W and 600-6W) and 9 weeks (400-9W and 600-9W) of ripening, and analyses were performed immediately after treatment and at 3, 6, 9 and 12 months. In Torta del Casar, made from

raw ewe's milk and vegetable rennet from cardoon (*Cynara cardunculus*), 400 and 600 MPa were applied for 5 minutes at 3 (400-3W and 600-3W), and 5 weeks (400-5W and 600-5W) of ripening, and analyses were performed immediately after treatment and at 2, 4, 6 and 8 months. In Brie cheese, made from pasteurized cow's milk and ripened with *P. camemberti* on its surface, 400 and 600 MPa were applied for 5 minutes at 2 (400-2W and 600-2W) and 3 weeks (400-3W and 600-3W) of ripening, and analyses were performed immediately after treatment and at 1, 2, 3 and 4 months. In Arzúa-Ulloa cheese, made from raw cow's milk, 400 and 600 MPa were applied for 5 minutes at 2 (400-2W and 600-2W) and 3 weeks (400-3W and 600-3W) of ripening, and analyses were performed immediately after treatment and at 2, 4, 6 and 8 months.

To evaluate the effect of high pressure on cheese microbiota, microbiological analyses were performed by counting on agar plates the following microbial groups: total aerobic mesophilic bacteria (PCA), lactic acid bacteria (M17 and MRS agar), lactobacilli (Rogosa agar), enterococci (KF agar), gram negative bacteria (MacConkey agar), coliforms (VRBA), *Micrococcaceae* (MSA), coagulase-positive staphylococci (Baird-Parker + RPF II), *Listeria monocytogenes* (Palcam agar), moulds and yeasts (CGA). The pH was measured by using a pH-meter coupled to a penetration electrode and dry matter (DM) was determined by drying at 102 °C to constant weight. Proteolysis was evaluated by analyses of caseins by capillary gel electrophoresis, peptides and free amino acids by liquid chromatography (HPLC) and overall proteolysis by a spectrophotometric assay. Aminopeptidase activity was determined by a colorimetric assay, measured spectrophotometrically. Lipolysis was evaluated by determining the free fatty acids by solid phase extraction followed by gas chromatography. Esterase activity was determined by colorimetric assay, measured spectrophotometrically. To evaluate the effect of high pressure on the formation of biogenic amines, tyrosine decarboxylase activity and biogenic amines concentration were determined by HPLC. Volatile compounds were analysed by solid phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS). Texture parameters (fracturability, firmness and elasticity) were determined by using a compression texturometer and the colorimetric parameters (lightness: L*, redness: a* and yellowness: b*) were estimated by

using a colorimeter. Finally, sensory analyses of flavour and odour were carried out through the evaluation of different characteristics and descriptors by a trained panel.

- **Results**

- Blue cheese

Control cheese, non-treated, underwent a reduction in the counts of lactic acid bacteria and mesophilic bacteria from 9.54 and 8.76 log cfu/g on day 1 to 6.57 and 5.57 log cfu/g on day 360, respectively, while *P. roqueforti* counts declined from 7.73 log cfu/g on day 21 to 5.66 log cfu/g on day 360. High pressure treatments reduced the counts of lactic acid bacteria, total mesophilic bacteria and *P. roqueforti*, especially the 600 MPa treatments which reduced *P. roqueforti* population below the detection level.

The strong primary proteolysis occurring in control cheese, with reductions between days 1 and 21 of 93, 87, 78 and 46 %, for α_S-, β-, κ- and *p*-κ-caseins, respectively, could not be avoided by high pressure treatments. The levels of hydrophilic and hydrophobic peptides increased in control cheese, and high pressure treatments were only able to reduce the hydrophilic peptides with 600 MPa treatments applied at 3 and 6 weeks. The aminopeptidase activity decreased in control cheese during refrigerated storage, and high pressure caused the reduction of this activity. Free amino acids levels in control cheese increased during ripening and refrigerated storage, reaching a concentration of 116.76 mg/g DM on day 360, and only 600 MPa treatments and 400 MPa treatment applied at 3 weeks were able to reduce the formation of free amino acids, maintaining until day 360 levels of free amino acids and overall proteolysis similar to those of control cheese at 180 days.

Biogenic amines in the control cheese reached on day 360 concentrations of 0.06, 0.07, 0.05, 0.03 and 0.01 mg/g DM for β-phenylethylamine, tyramine, tryptamine, putrescine and spermidine, respectively. High pressure treatments only reduced the levels of tyramine.

Esterase activity was reduced by 600 MPa treatments and 400 MPa treatment applied at 3 weeks. Free fatty acids in the control cheese increased during ripening and refrigerated storage, reaching on day 360 concentrations of 8.88, 18.31 and 56.63 mg/g DM of short ($C_{4:0}$ - $C_{8:0}$), medium ($C_{10:0}$ - $C_{14:0}$) and long chain ($C_{16:0}$ - $C_{18:3}$) free fatty acids,

respectively. Excepting the treatments at 400 MPa at 6 and 9 weeks, high pressure treatments reduced the formation of all the groups of free fatty acids. Only cheeses treated at 400 MPa at 3 weeks showed levels of short chain free fatty acids similar or lower than those of control cheese at 180 days. These cheeses, together with those treated with 600 MPa at 6 and 9 weeks, showed levels of medium and long chain fatty acids similar to those of control cheese at 180 days. Moreover, the levels of the three groups of free fatty acids in cheeses treated with 600 MPa at 3 weeks remained well below those of control cheese at 180 days. Ethanoic acid increased until day 63, decreasing thereafter in control cheese. High pressure treatments reduced the levels of this acid, with the exception of 400 MPa applied at 6 and 9 weeks.

The major group of volatile compounds in control cheese was the volatile acids group, which increased during refrigerated storage, followed by alcohols and ketones, which also increased during storage but to a lesser extent. Levels of esters, terpenes, sulphur and nitrogen compounds also increased during storage, while aldehydes and benzenic compounds declined and hydrocarbons remained stable. High pressure treatments reduced the levels of esters, alcohols, hydrocarbons, nitrogen and benzenic compounds, especially the 600 MPa treatments which also reduced the formation of ketones. In cheeses treated at 3 weeks the levels of volatile acids, sulphur compounds and terpenes were reduced by high pressure treatments. Principal component analysis of free fatty acids groups and volatile compounds groups only scarcely discriminated between times of storage, indicating a very mild effect of high pressures on blue cheese.

Only the treatment of 600 MPa applied at 3 weeks reduced the slight increase in flavour intensity that occurred in control cheese during refrigerated storage. Control cheese showed stable values for flavour quality during refrigerated storage, and all treated cheeses showed similar scores to those of the control cheese, with the exception of cheeses treated with 600 MPa at 3 weeks, which obtained lower scores.

Control cheese showed subsidence at the centre that reached 15 % with respect to the edge. High pressure greatly increased it, reaching 36 % in the case of cheeses treated with 400 MPa at 3 weeks.

- Torta del Casar

There was a decrease of mesophilic bacteria, lactic acid bacteria and enterococci counts in control cheese from 9.51, 9.46 and 7.32 log cfu/g on day 21 to 8.61, 8.42 and 6.80 log cfu/g on day 240, respectively. Lactobacilli counts increased to 8.13 log cfu/g on day 35 and remained at these levels throughout the refrigerated storage. High pressure treatments reduced the counts of mesophilic bacteria, lactic acid bacteria, lactobacilli and enterococci, especially 600 MPa treatments. *Micrococcaceae*, coliform and gram negative bacteria counts decreased from 6.39, 5.52 and 6.01 log cfu/g on day 1 to 5.57, 2.87 and 5.37 log cfu/g on day 240, respectively. High pressure reduced *Micrococcaceae* counts, especially with treatments applied at 5 weeks, whereas all treatments, with the exception of 400 MPa applied at 3 weeks, reduced coliforms and gram negative bacteria counts below the detection level. Coagulase positive staphylococci counts decreased in control cheese from 5.23 log cfu/g on day 1 to 2.30 on day 180 and below the detection level on day 240, whereas in pressurized cheeses, they were below the detection level since day 21. In control cheese pH increased during ripening and refrigerated storage reaching values of 6.13, and in treated cheeses pH also increased but to a lesser extent.

Control cheese α_S- and β-caseins suffered reductions of 79 and 62 %, respectively, between days 1 and 35, while κ-casein suffered a reduction of 96 % between days 1 and 21, remaining below the detection level from day 35 onwards. The 600 MPa treatments successfully arrested primary proteolysis reducing α_S- and β-caseins hydrolysis and the formation of hydrophilic peptides, which increased in control cheese. Aminopeptidase activity decreased more rapidly in high pressure treated cheeses than in control cheese. Free amino acids concentration increased in control cheese during ripening and refrigerated storage, reaching 23.30 mg/g DM on day 360. The 600 MPa treatments reduced the overall proteolysis and in addition, the 600 MPa applied at 3 weeks treatment reduced the formation of free amino acids, but for both parameters their levels were higher than in the 60-day control cheese.

Tyrosine decarboxylase activity tripled in control cheese between days 60 and 180, and all high pressure treatments reduced it. Tyramine increased in control cheese reaching 1.09 mg/g DM on day 240, and 600 MPa treatments lowered its levels.

Putrescine, tryptamine, β-phenylethylamine, cadaverine and histamine increased to 0.95, 0.83, 0.29, 0.69 and 0.30 mg/g DM, respectively, on day 240. All high pressure treatments reduced cadaverine levels. The levels of putrescine, tryptamine and β-phenylethylamine were similarly reduced by high pressure treatments, with the exception of 400 MPa at 3 weeks. Only 600 MPa treatments reduced the levels of histamine.

Esterase activity in control cheese decreased since day 60. The 600 MPa treatments reduced this activity throughout refrigerated storage. The levels of free fatty acids in control cheese increased throughout ripening and refrigerated storage reaching concentrations of short, medium and long chain free fatty acids of 8.69, 0.88 and 3.02 mg/g DM, respectively, on day 240. All treatments reduced the formation of free fatty acids, and only short chain free fatty acids levels remained in treated cheeses until day 240 at similar levels to those of control cheese on day 60. Ethanoic, propanoic and branched chain acids also increased, reaching 5.82, 2.99 and 1.15 mg/g DM, respectively. All treatments reduced the levels of propanoic and branched-chain acids and only the treatments applied at 3 weeks reduced the levels of ethanoic acid, maintaining throughout refrigerated storage similar or lower levels than those of control cheese on day 60.

The major group of volatile compounds in control cheese was the volatile acids group until day 180, while on day 240 it was the group of alcohols. During refrigerated storage, only the volatile acids group decreased, while the rest of groups increased, with a marked increase of sulphur compounds in control cheese. High pressure treatments reduced the levels of esters, aldehydes, and especially sulphur compounds. The 600 MPa treatments reduced the levels of alcohols, while treatments applied at 3 weeks reduced the levels of volatile acids. Principal component analysis of free fatty acids groups and volatile compounds groups indicated that cheeses treated at 5 weeks of ripening showed until day 240 a similar profile to that of the 60-day control cheese.

During refrigerated storage, firmness and elasticity parameters decreased in control cheese. None of the treatments was able to stabilize or maintain similar values to those of control cheese on day 60. In control cheese flavour and odour intensity, bitter taste and putrid and rancid odour increased during storage, causing a marked decline in

flavour and odour quality. All treatments reduced the increase in odour intensity but only 600 MPa treatments were able to reduce flavour intensity. High pressure also slowed the increase in putrid and rancid odour, preventing the drstic loss of flavour and odour quality that occurred in control cheese.

- Brie Cheese

In control cheese, lactic acid bacteria and mesophilic bacteria counts decreased from 8.99 and 8.75 log cfu/g on day 1 to 7.88 and 7.90 log cfu/g on day 120, respectively, while *P. camemberti* counts remained stable throughout storage. High pressure treatments reduced counts of lactic acid bacteria, mesophilic bacteria and *P. camemberti*, especially 600 MPa treatments which reduced *P. camemberti* counts below the detection level. The pH of control cheese increased drastically from day 30 onwards, reaching values of 7.87 at day 120. High pressure treatments maintained the same pH values throughout refrigerated storage.

Primary proteolysis of control cheese, with reductions between days 1 and 14 of 41, 37 and 36 % for α_S-, β- and κ-casein, respectively, was only successfully arrested by 600 MPa treatments, which reduced the hydrolysis of α_S- and β-caseins. All treatments prevented the marked increase of hydrophobic peptides that occurred in control cheese. High pressure treatments reduced aminopeptidase activity throughout refrigerated storage, with the exception of 400 MPa applied at 3 weeks, which only on day 120 yielded lower values than control cheese. Free amino acid levels in control cheese increased during ripening and refrigerated storage, reaching a concentration of 78.24 mg/g DM on day 120. High pressure treatments did not reduce secondary proteolysis, on the contrary, they caused a greater accumulation of free amino acids as well as a higher overall proteolysis.

Esterase activity increased in control cheese during refrigerated storage. High pressure treatments did not reduce this increase and in some cases they increased it. Free fatty acids accumulated in control cheese during ripening and refrigerated storage, reaching concentrations of 3.48, 5.03 and 21.58 mg/g DM of short, medium and long chain free fatty acids, respectively, on day 120. High pressure treatments reduced the formation of short chain free fatty acids, and treatments applied at 2 weeks also reduced the levels of medium and long chain free fatty acids, while treatments applied

at 3 weeks did not affect medium chain free fatty acids and caused an increase of long chain free fatty acids until day 60, reducing subsequently the levels of these two groups to similar or lower values than those of control cheese on day 30. Ethanoic and branched chain acid levels increased to 0.40 and 1.03 mg/g DM, respectively, on day 120. High pressure treatments reduced the formation of branched chain acids, but only 600 MPa treatments reduced the formation of ethanoic acid.

The major group of volatile compounds in control cheese on day 30 was the volatile acids group, while on days 60 and 120 it was the ketones group, which suffered a decline between days 30 and 60, increasing thereafter. The levels of alcohols, aldehydes, benzenic, nitrogen and sulphur compounds increased during refrigerated storage. High pressure treatments prevented the increase of ketones, benzenic, nitrogen and sulphur compounds, and caused a higher accumulation of volatile acids, alcohols, ethers and esters. Principal component analysis of individual volatile compounds indicated that cheeses treated at 2 weeks showed until day 120 a profile of volatile compounds similar to that of 30-day control cheese.

Fracturability, firmness and elasticity values decreased during ripening, remaining at very low values during refrigerated storage. High pressure treatments prevented the decrease of elasticity and firmness, and only treatments applied at 2 weeks slowed the decline of fracturability. In control cheese there was a slight increase in the colour parameter b* on the surface and the interior, a marked increase between days 90 and 120 of the parameter a* and a decrease of lightness. High pressure slowed the increase of a* and b* parameters in the interior and prevented the loss of lightness in the cheese interior. High pressure treatments caused the loss of the mould surface layer that is characteristic of this cheese variety, making the colour parameters of the surface very different from those of control cheese. During refrigerated storage there was a loss of flavour and odour quality, accompanied by a strong increase of flavour and odour intensity as well as of bitter and umami flavours. High pressures prevented the increase of flavour and odour intensity and bitter flavour, avoiding the loss of flavour and odour quality and maintaining treated cheeses with similar scores to those of control cheese on day 30.

- Arzúa-Ulloa

Mesophilic bacteria counts decreased in control cheese between days 1 and 21 from 9.2 log cfu/g to 8.0 log cfu/g, and then remained at stable levels until day 240. Lactic acid bacteria levels decreased between days 1 and 14 from 8.8 log cfu/g to 8.0 log cfu/g, remaining at the same values until day 240. Furthermore lactobacilli counts increased between days 1 and 60 from 4.1 log cfu/g to 8.0 log cfu/g, maintaining the same levels until day 240. The 600 MPa treatments reduced and maintained the levels of mesophilic bacteria, lactobacilli and lactic acid bacteria throughout refrigerated storage. Enterococci, *Micrococcaceae*, coliforms and gram negative bacteria counts decreased between days 1 and 240 from 6.31, 6.92, 7.50 and 7.52 log cfu/g to 4.71, 4.08, 1.46 and 2.04 log cfu/g, respectively, while coagulase positive staphylococci counts decreased between days 1 and 14 from 4.83 log cfu/g to 2.66 log cfu/g, and were below the detection level from day 21 onwards. High pressure treatments reduced enterococci *Micrococcaceae*, coliforms, gram negative bacteria and staphylococci counts, and kept below the detection level the counts of coliforms, gram negative bacteria and staphylococci. Only 600 MPa treatments maintained enterococci counts below the detection level. The pH of control cheese slightly increased during refrigerated storage, reaching a value of 5.68 on day 240. The 600 MPa treatments reduced this increase, maintaining similar values to those of control cheese on day 60.

Control cheese suffered a progressive primary proteolysis, with reductions of 46, 64, 65 and 35 % for α_S-, β-, κ- and *p*-κ-casein, respectively, between days 1 and 240. High pressure treatments not only did not reduce primary proteolysis but increased it, causing a higher hydrolysis of β-, κ- and *p*-κ-caseins. The 400 MPa treatments also increased the hydrolysis of α_S-casein. This higher hydrolysis of caseins in treated cheeses resulted in a greater accumulation of hydrophilic and hydrophobic peptides, than in control cheese. High pressure treatments reduced aminopeptidase activity. The concentrations of free amino acids increased in control cheese throughout ripening and refrigerated storage, reaching levels of 31.68 mg/g DM. High pressure treatments slowed secondary proteolysis, reducing free amino acids and overall proteolysis level, and showing 600 MPa treated cheeses similar levels to those of 60-day control cheese until the end of refrigerated storage.

Tyrosine decarboxylase activity increased in control cheese during ripening and refrigerated storage. High pressure treatments reduced this activity, with the exception of 400 MPa applied at 3 weeks on days 180 and 240. In control cheese, tyramine and putrescine increased during ripening and refrigerated storage, reaching concentrations of 0.372 and 0.207 mg/g DM on day 240 respectively, while cadaverine increased to 0.113 mg/g DM on day 21, decreasing thereafter and maintaining values of 0.800 mg/g up to day 240. Histamine was only detected on days 180 and 240, with concentrations of 0.009 mg/g DM and 0.026 mg/g DM respectively. High pressure treatments reduced the levels of tyramine and putrescine, and 600 MPa treatments kept their concentrations below the detection level. High pressure treatments also reduced histamine levels, whereas cadaverine levels did not show a clear trend throughout refrigerated storage.

Esterase activity, which increased in control cheese throughout storage, was reduced by high pressure treatments. During ripening and refrigerated storage short, medium, long chain free fatty acids and ethanoic acid increased in control cheese, reaching concentrations of 0.190, 0.400, 1.307 and 2.102 mg/g DM on day 240, respectively. High pressure treatments reduced the formation of ethanoic acid and short chain free fatty acids, but not medium and long chain free fatty acids that only showed lower levels on day 240, in cheeses treated with 400 MPa at 3 weeks and in 600 MPa cheeses. In control cheese, benzoic acid increased reaching a concentration of 0.398 mg/g DM on day 180. High pressure treatments applied at 2 weeks reduced the levels of benzoic acid on day 180.

The major group of volatile compounds in control cheese was the alcohols, that increased between days 60 and 120 and decreased thereafter. The levels of acids, ketones, esters, ethers, hydrocarbons, benzenic and sulphur compounds decreased during refrigerated storage, while aldehydes remained at the same levels and terpenes increased. High pressure treatments partially prevented the decrease of volatile acids, ethers, and sulphur compounds. Furthermore, in cheeses treated with 600 MPa the decrease of ketones and hydrocarbons was avoided. Principal component analysis of the different volatile compound groups indicated that cheeses treated at 600 MPa showed until day 120 a similar profile to that of 21-day control cheese.

In control cheese elasticity and firmness increased until day 180, while fracturability could only be determined at day 240. High pressure treatments increased fracturability and could not avoid the increase of elasticity and firmness. Flavour intensity increased during refrigerated storage in control cheese as well as bitter and umami flavour, while flavour quality maintained similar scores until day 180, suffering a slightly decrease on day 240. High pressure treatments did not avoid the increase of flavour intensity or the reduction in flavour quality which control cheese suffered on day 240, and on the contrary, they favoured the increase of bitter flavour.

- **Conclusions**

In blue cheese, control cheese underwent some minor chemical changes during refrigerated storage, which did not affect flavour quality or intensity. High pressure treatments of 400 MPa applied at 3 weeks and 600 MPa reduced secondary proteolysis, and treatments applied at 3 weeks also reduced lipolysis. The effect of high pressure treatments on proteolysis and lipolysis together with the effect exerted on the formation of volatile compounds only caused a loss of flavour quality and intensity in cheeses treated with 600 MPa at 3 weeks, whereas in the rest of treated cheeses it did not cause differences. High pressure treatments did not represent an additional benefit for preserving blue cheese under the conditions employed in this work.

In Torta del Casar, control cheese underwent strong changes during refrigerated storage which caused a loss of flavour and odour quality, along with a considerable accumulation of biogenic amines. High pressure treatments prevented the increase of pH that occurred in control cheese, and treatments of 600 MPa also reduced proteolysis. High pressure treatments were able to reduce lipolysis, biogenic amines levels and the formation of volatile compounds, which were responsible for the quality loss in control cheese. High pressure treatments, especially those applied at 5 weeks, represented a substantial improvement for the conservation of Torta del Casar, extending its shelf life up to 240 days and reducing the accumulation of biogenic amines.

In Brie cheese, control cheese underwent chemical changes during refrigerated storage which caused a strong loss of quality, as well as a strong increase of flavour and odour intensity. High pressure treatments prevented the marked increase of pH that

occurred in control cheese, and reduced the primary proteolysis impeding the accumulation of hydrophobic peptides. High pressure treatments were also able to reduce lipolysis and the formation of volatile compounds, mainly sulphur compounds which were responsible for the quality loss of control cheese. Furthermore, high pressure treatments caused the loss of the mould surface layer. High pressures, especially treatments applied at 2 weeks, represented a substantial improvement for the preservation of Brie cheese, extending its shelf life up to 120 days, although the loss of the mould surface layer may hinder its marketing.

In Arzúa-Ulloa cheese, control cheese underwent chemical changes during refrigerated storage, which only caused the loss of flavour quality on day 240, although flavour and odour intensity, as well as bitter and umami flavour, increased throughout this period. High pressure treatments reduced the increase of pH that occurred in control cheese. Despite the acceleration of primary proteolysis by high pressure treatments, they were able to reduce secondary proteolysis. High pressure treatments slightly reduced lipolysis, especially the formation of short chain free fatty acids, and reduced the formation of biogenic amines, especially treatments of 600 MPa. Because of the effect of high pressure treatments on volatile compounds, 600 MPa cheeses maintained until day 120 a similar volatile profile to that of 21-day control cheese. High pressure treatments did not influence the flavour quality and intensity of the cheeses, although they caused an increase of bitterness. High pressure treatments, from the standpoint of the sensory characteristics, did not represent an improvement for cheese preservation, but avoided the accumulation of biogenic amines.

- **Bibliography**

Ávila, M., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (2006). Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. *Journal of Dairy Science* **89**, 2882-2893.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal* **13**, 841-866.

EUROSTAT (2014). http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home/

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

Evert-Arriagada, K., Hernández-Herrero, M. M., Guamis, B. & Trujillo, A. J. (2014). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *LWT-Food Science and Technology* **55**, 498-505.

FAOSTAT (2014). http://faostat3.fao.org/faostat-gateway/go/to/home/E

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004). Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure. *Journal of Dairy Research* **71**, 496-499.

Ladero, V., Calles-Enríquez, M., Fernández, M. & Alvarez, M. A. (2010). Toxicological effects of dietary biogenic amines. *Current Nutrition & Food Science* **6**, 145-156.

Loizzo, M. R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U. G. & Restuccia, D. (2013). Technological aspects and analytical determination of biogenic amines in cheese. *Trends in Food Science & Technology* **30**, 38-55.

López-Pedemonte, T., Roig-Sagués, A. X., De Lamo, S., Gervilla, R. & Guamis, B. (2007). High hydrostatic pressure treatment applied to model cheeses made from cow's milk inoculated with *Staphylococcus aureus*. *Food Control* **18**, 441-447.

MAGRAMA (2014). http://www.magrama.gob.es/

Malone, A. S., Wick, C., Shellhammer, T. H. & Courtney, P. D. (2003). High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science* **86**, 1139-1146.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology* **90**, 139-159.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In Cheese: Chemistry, Physics and Microbiology, pp. 361-371: Academic Press.

Mulder, H. (1952). Taste and flavour forming substances in cheese. *Netherlands Milk and Dairy Journal* **6**, 157 - 167.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Saldo, J. & Vidal-Carou, M. C. (2002). Effects of high hydrostatic pressure treatments on biogenic amine contents in goat cheeses during ripening. *Journal of Agricultural and Food Chemistry* **50**, 7288-7292.

O'Reilly, C. E., O'Connor, P. M., Murphy, P. M., Kelly, A. L. & Beresford, T. P. (2000). The effect of exposure to pressure of 50 MPa on Cheddar cheese ripening. *Innovative Food Science & Emerging Technologies* **1**, 109-117.

Rodriguez, E., Arques, J. L., Nuñez, M., Gaya, P. & Medina, M. (2005). Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli* O157:H7 in raw-milk cheese. *Applied and Environmental Microbiology* **71**, 3399-3404.

Saldo, J., McSweeney, P. L. H., Sendra, E., Kelly, A. L. & Guamis, B. (2002). Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening. *International Dairy Journal* **12**, 35-44.

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology* **29**, 213-231.

Sousa, M. J., Ardö, Y. & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal* **11**, 327-345.

ten Brink, B., Damink, C., Joosten, H. & JHJ., H. i. t. V. (1990). Occurrence and formation of biologically-active amines in foods. *International Journal of Food Microbiology* **11**, 73-84.

Wick, C., Nienabert, U., Anggraeni, O., Shellhammer, T. H. & Courtney, P. D. (2004). Texture, proteolysis and viable lactic acid bacteria in commercial cheddar cheeses treated with high pressure. *Journal of Dairy Research* **71**, 107-115.

Yang, B. W., Shi, Y., Xia, X. D., Xi, M. L., Wang, X., Ji, B. Y. & Meng, J. H. (2012). Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. *Food Control* **28**, 273-278.