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Departamento de Higiene y Tecnología de los Alimentos

TESIS DOCTORAL

Enrichment on bioactive fatty acids of sheep milk cheese through the study and improvement of the factors involved in the technology of its production

Enriquecimiento en ácidos grasos bioactivos del queso de oveja a través del estudio y mejora de los factores implicados en la tecnología de su elaboración

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"Science has not yet taught us if madness is or is not the sublimity of the intelligence"

Edgar Allan Poe (1809-1849)

"No es verdad que las personas dejen de perseguir sueños porque se hacen viejas, sino que se hacen viejas porque dejan de perseguir sus sueños"

Gabriel García Márquez (1927-2014)

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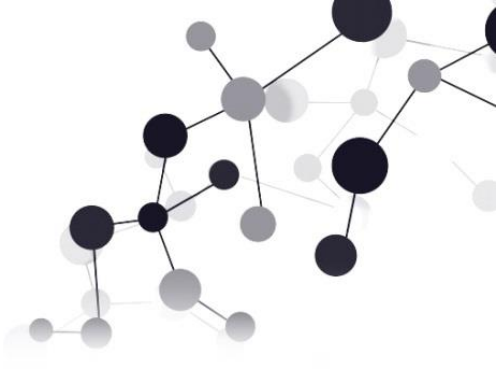

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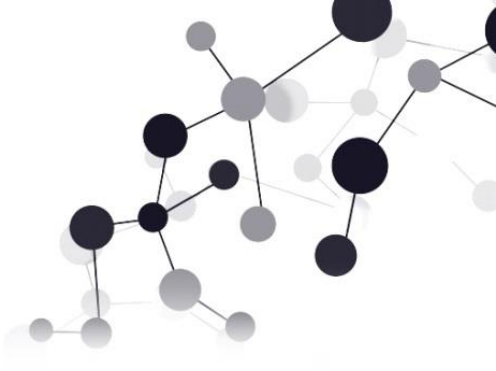


RESUMEN/SUMMARY

En la actualidad la creciente demanda de alimentos con efectos beneficiosos sobre la salud humana, está dando lugar a que en la industria alimentaria se tenga la necesidad de investigar nuevos métodos para satisfacer las exigencias del consumidor.

En este sentido, el queso, que supone un porcentaje importante del comercio agrícola mundial, ha suscitado en los últimos años una connotación negativa por parte del consumidor debido a la relación del contenido en ácidos grasos saturados de este alimento y el desarrollo de algunas enfermedades. Sin embargo, el queso también presenta compuestos con efectos beneficiosos sobre la salud del consumidor, como es el caso del ácido linoleico conjugado (CLA).

Ante la situación planteada, el objetivo general de esta Tesis Doctoral fue abordar la posible mejora del perfil nutricional del queso de oveja. En consecuencia, los métodos estudiados para alcanzar este objetivo abarcaron aspectos que van desde la producción animal hasta la tecnología de elaboración del queso.

En primer lugar, se estudió el efecto de la alimentación, de la etapa de lactación, del mes de muestreo y del rebaño en el perfil de ácidos grasos, con especial énfasis en el CLA, de la leche de tanque procedente de granjas de ovino comerciales. En este estudio se observó que en las granjas de ovino en las que se practicaba un régimen alimentario basado en un 50% de pastoreo, la leche de tanque que se obtenía presentaba mayor proporción de CLA (1,16%) así como los mejores valores para la relación *omega*-6/*omega*-3 (1,90) e índice de aterogenicidad (1,59) en comparación con la leche de tanque procedente de granjas en las que a las ovejas no les era permitido el acceso al pasto (0,79%, 3,41 y 1,88, respectivamente). Resulta oportuno indicar que el efecto de la etapa de lactación sobre la proporción de ácidos grasos presentes en la leche de oveja fue menos importante que el del resto de los factores estudiados ya que explicó menos del 4,18% de la varianza.

En segundo lugar, es conocido el papel que pueden jugar los microorganismos durante la elaboración de los quesos en la mejora de su calidad nutricional y sensorial. Por ello, se analizó la capacidad para sintetizar CLA y ácido gamma-aminobutírico (GABA) en 85 cepas de bacterias ácido lácticas aisladas de quesos artesanales. Como resultado de este estudio se observó que seis cepas de *Lactobacillus brevis* y cuatro cepas de *Lactococcus lactis* subsp. *lactis* fueron capaces de sintetizar GABA en medio de cultivo suplementado con glutamato monosódico (5 mg/mL) y que la mayor concentración (2524,05 µg/mL) producida por estas cepas fue detectada tras 72 h de incubación. Así mismo, cuatro cepas de *Lactobacillus plantarum* y dos cepas de *Lactobacillus casei* subsp. *casei* fueron identificadas como productoras de CLA mediante detección espectrofotométrica de los dobles enlaces conjugados. Posteriormente, se analizó la capacidad de estas 6 cepas autóctonas para producir CLA en medio de cultivo y leche desnatada suplementados con ácido linoleico (0,5 mg/mL) bajo diferentes tiempos de incubación (24, 48 y 72 h). En particular, *Lactobacillus plantarum* TAUL 1588 fue la cepa que produjo la mayor concentración de los isómeros *cis*-9,*trans*-11 $C_{18:2}$ (23,73 µg/mL), *trans*-10,*cis*-12 $C_{18:2}$ (3,37 µg/mL) y *trans*-9,*trans*-11 $C_{18:2}$ (27,97 µg/mL) en medio de cultivo tras 48 h de incubación. Sin embargo, *Lactobacillus casei* subsp. *casei* SS 1644 fue

la cepa que sintetizó mayor concentración de los isómeros *cis*-9,*trans*-11 $C_{18:2}$ (18,33 µg/mL) y *trans*-9,*trans*-11 $C_{18:2}$ (35,05 µg/mL) en leche desnatada.

En tercer lugar, y en base a la buena capacidad para sintetizar CLA por parte de estas dos cepas autóctonas de *Lactobacillus*, se diseñaron cuatro cultivos con posible aplicación en la elaboración de queso de oveja. En este estudio se observó que el cultivo compuesto por la combinación de estas cepas produjo la mayor concentración (56,51 µg/mL) de CLA en leche desnata suplementada con ácido linoleico. A su vez, los cultivos compuestos por cepas de *Lactobacillus* productoras de CLA en combinación con cepas de *Lactococcus lactis* autóctonas con adecuada aptitud tecnológica fueron más efectivos en la producción de CLA que los cultivos que incluyeron cepas comerciales de *Lactococcus lactis* y los *Lactobacillus* productores de CLA.

Finalmente, se elaboraron cuatro lotes de queso de oveja a partir de leche procedente de una de las granjas en la cual las ovejas fueron alimentadas bajo régimen de pastoreo y se emplearon diferentes cultivos constituidos por dos cepas autóctonas de *Lactococcus lactis* y las dos cepas de *Lactobacillus* mencionadas previamente. Cabe destacar que la combinación de estas cuatro cepas en el cultivo empleado en la elaboración de queso fue la que generó mayor concentración de ácido vacénico (1,55%), CLA (0,69%) y ácidos grasos *omega*-3 (0,66%) sin apreciar cambios significativos en las características sensoriales en comparación con el lote control, elaborado únicamente con las cepas autóctonas de *Lactococcus lactis*. Así mismo, tras 240 días de maduración de los cuatro lotes de queso, se detectó una alta concentración de GABA (1296,75 mg/kg queso) y ornitina (2355,76 mg/kg queso), los cuales se ha descrito que tienen efectos beneficiosos sobre la salud humana. De igual manera, se observó que el lote de queso que incluía la cepa *Lactobacillus plantarum* TAUL 1588 y las dos cepas autóctonas de *Lactococcus lactis*, mostró 2,37 veces menos de aminas biógenas que el lote que se elaboró con el cultivo que contenía las cepas de *Lactococcus lactis* pero no la cepa de *Lactobacillus plantarum*.

En definitiva, esta Tesis Doctoral demuestra la gran influencia que ejercen las prácticas de manejo del ganado ovino en el perfil de la grasa láctea así como la repercusión que tiene el empleo de cultivos lácticos seleccionados en función de su capacidad para sintetizar compuestos bioactivos con efectos beneficiosos sobre la salud humana en el desarrollo de productos lácteos con calidad nutricional mejorada.

SUMMARY

Currently, the growing demand for foods with beneficial effects on human health is generating the need to investigate new innovations in the food industry in order to meet the consumer demand.

In this context, cheese, which represents a significant percentage of the world agricultural trade, has generated a negative connotation on consumers in recent years due to the relationship between the saturated fatty acids in cheese and the development of some diseases. However, cheese contains other compounds that can improve the consumer health, such as the conjugated linoleic acid (CLA).

In the face of this situation, the general aim of this Doctoral Thesis was to study the potential for improvement of the nutritional value of sheep milk cheese. Consequently, the methods studied to achieve this aim included aspects related to the animal production and to the cheese-making process.

First, the effect of the feeding regimen, lactation stage, sampling month and flock on the fatty acid profile, with special emphasis on CLA, of bulk tank milk from commercial sheep farms was studied. It was observed that in sheep farms where a feeding regimen based on 50% grazing was practiced, the bulk tank milk had a higher proportion of CLA (1.16%) as well as the improved *omega*-6/*omega*-3 ratio (1.90) and atherogenicity index (1.59) compared to the bulk tank milk from farms where sheep did not graze (0.79%, 3.41 and 1.88, respectively). The effect of lactation stage on the fatty acid proportions of sheep milk was less important than that of the other factors studied, since it explained less than 4.18% of the variance.

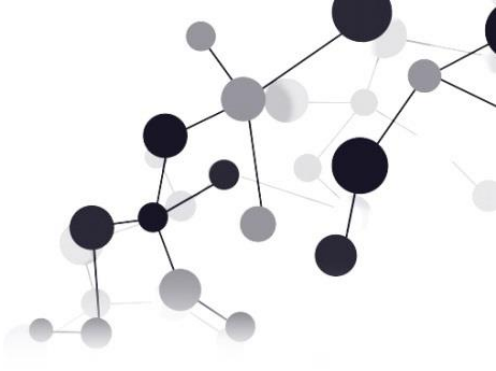
Second, the role that microorganisms can play during the cheese-making process for the improvement of the nutritional and sensory quality of cheese is well known. Thus, 85 strains of lactic acid bacteria isolated from artisanal cheeses were studied to determine their ability to synthesize CLA and gamma-aminobutyric acid (GABA). It was observed that six *Lactobacillus brevis* and four *Lactococcus lactis* subp. *lactis* strains were able to produce GABA in culture medium supplemented with monosodium glutamate (5 mg/mL) and the highest concentration (2524.05 µg/mL) of GABA was found after 72 h of incubation. Likewise, four *Lactobacillus plantarum* and two *Lactobacillus casei* subsp. *casei* strains were identified as CLA-producers by spectrophotometric detection of the conjugated double bonds. Subsequently, the ability of these 6 autochthonous strains to produce CLA in culture medium and in skim milk supplemented with linoleic acid (0.5 mg/mL) under different incubation times (24, 48 and 72 h) was analyzed. In particular, *Lactobacillus plantarum* TAUL 1588 was the strain that produced the highest concentration of the isomers *cis*-9,*trans*-11 $C_{18:2}$ (23.73 µg/mL), *trans*-10,*cis*-12 $C_{18:2}$ (3.37 µg/mL) and *trans*-9,*trans*-11 $C_{18:2}$ (27.97 µg/mL) in culture medium after 48 h incubation. However, *Lactobacillus casei* subsp. *casei* SS 1644 was the strain that synthetized the highest concentration of the isomers *cis*-9,*trans*-11 $C_{18:2}$ (18.33 µg/mL) and *trans*-9,*trans*-11 $C_{18:2}$ (35.05 µg/mL) in skim milk.

Third, based on the demonstration of the ability of these two *Lactobacillus* strains to synthesize CLA, four cultures were designed with possible application for the production of sheep milk cheese. In this study, it was observed that the culture including the

combination of these strains produced the highest concentration (56.51 µg/mL) of CLA in skim milk supplemented with linoleic acid. Additionally, the cultures including these CLA-producing *Lactobacillus* strains in combination with the autochthonous *Lactococcus lactis* strains (with adequate technological aptitude) were more effective in CLA production than the cultures including commercial strains of *Lactococcus lactis* and the CLA-producing *Lactobacillus* strains.

Finally, four batches of sheep milk cheese were manufactured with the bulk tank milk from one of the farms in which sheep were grazed and using four different cultures, consisting on the two autochthonous *Lactococcus lactis* strains and the two CLA-producing *Lactobacillus* strains mentioned previously. Significantly, the combination of the four strains in the culture used for cheese-making produced higher concentrations of vaccenic acid (1.55%), CLA (0.69%) and *omega*-3 fatty acids (0.66%) without appreciating important changes on the sensory characteristics compared to the control batch, which was produced only with the autochthonous *Lactococcus lactis* strains. Likewise, after 240 days of ripening, a high concentration of GABA (1296.75 mg/kg cheese) and ornithine (2355.76 mg/kg cheese) were detected in the four cheese batches. As has been described previously, these compounds have beneficial effects on human health. In addition, it was observed that the batch produced with the culture including the *Lactobacillus plantarum* TAUL 1588 strain and the two *Lactococcus lactis* strains, showed 2.37 fold reduced biogenic amines concentration with respect to the batch made with the culture including the *Lactococcus lactis* strains but not the *Lactobacillus plantarum* strain.

In conclusion, this Doctoral Thesis shows the great influence of sheep management practices on the fatty acid profile of milk as well as the repercussion from the use of lactic acid bacteria cultures selected for their ability to synthetize bioactive compounds, with beneficial effects on human health, on the development of dairy products with improved nutritional quality.



INTRODUCCIÓN GENERAL

1.1. Importancia de la leche y queso de oveja en la industria alimentaria

La leche y los productos lácteos representan cerca del 14% del comercio agrícola mundial. Tradicionalmente, la leche de vaca es la de mayor consumo suponiendo un 85% de la producción mundial de leche. Sin embargo, la producción láctea de pequeños rumiantes, como la leche de oveja, representa una parte importante de la economía agraria de varios países principalmente situados en la región Mediterránea y Oriente Medio. En 2015, la producción mundial de leche de oveja alcanzó 13 millones de toneladas, siendo los mayores productores: China (12%), Turquía (8,5%), Grecia (5,5%), Rumanía (5%) y España (4,7%) (FAO, 2015). En España la comunidad con mayor producción de leche de oveja es Castilla y León con un 64% seguida por Castilla-La Mancha con un 27% (MAPAMA, 2016). La mayor parte de la producción de leche de oveja es destinada a la elaboración de queso. En este sentido, España abarca el 35% de la producción de queso de oveja en la UE.

En la última década, los requisitos del consumidor en relación con los alimentos están cambiando. Los alimentos no son sólo utilizados para satisfacer el hambre sino además para prevenir enfermedades relacionadas con la alimentación y para mejorar la salud física y mental (Bigliardi y Galati, 2013). Como consecuencia, el desarrollo de alimentos que satisfagan estas nuevas necesidades del consumidor es de gran interés en la industria alimentaria. En este sentido, los productos lácteos elaborados a partir de leche de oveja, en especial el queso, han ganado amplitud de mercado debido a la calidad y al valor nutricional de los mismos. Este alto valor nutricional es debido a las mayores concentraciones de proteína, grasa, vitaminas y minerales presentes en estos productos lácteos en comparación con los elaborados a partir de leche procedente de otros mamíferos (Balthazar et al., 2017).

En lo que respecta a los ácidos grasos saturados presentes en el queso, éstos han adquirido una connotación negativa por parte del consumidor por su relación con el desarrollo de algunas enfermedades, principalmente de tipo cardiovascular (Elwood et al., 2010). Sin embargo, el queso, también presenta compuestos que pueden mejorar la salud del consumidor, como es el caso del ácido linoleico conjugado (Diana et al., 2014; Koba y Yanagita, 2014; Tanaka, 2005). Por consiguiente, el diseño de estrategias enfocadas a incrementar el contenido de estos compuestos bioactivos en el queso tiene un especial interés en el desarrollo de productos lácteos funcionales.

1.2. Ácido linoleico conjugado

1.2.1. Aspectos generales y biosíntesis

El ácido linoleico conjugado (conjugated linoleic acid; CLA) hace referencia a un grupo de isómeros posicionales y geométricos del ácido octadecadienoico con un sistema de dobles enlaces conjugados. Ha sido descrito que estos dobles enlaces pueden encontrarse entre las posiciones 6-8 y 13-15 con 4 isomerías geométricas (*cis-cis*, *cis-trans*, *trans-cis*, *trans-trans*) (Sehat et al., 1998). La leche y los derivados lácteos son los que, de

forma natural, aportan a la dieta la mayor cantidad de CLA, y según la especie animal, se ha observado que la grasa de la leche de oveja presenta una mayor concentración de CLA que la de vaca o la de cabra (Park et al., 2007). De todos los isómeros CLA presentes en la grasa de la leche, el *cis*-9,*trans*-11 $C_{18:2}$, también llamado ácido ruménico, es el más abundante ya que representa entre el 70 – 90% del contenido total de CLA en la grasa láctea (Parodi, 1977). En los rumiantes la biosíntesis del CLA, específicamente del isómero *cis*-9,*trans*-11 $C_{18:2}$, tiene lugar en el rumen (síntesis ruminal) y en la glándula mamaria (síntesis endógena) (**Figura 1**). En el rumen, el CLA se produce como intermediario de la biohidrogenación incompleta de los ácidos grasos poliinsaturados, específicamente del ácido linoleico y del ácido α-linolénico, por acción de las enzimas de distintas bacterias anaerobias ruminales, siendo *Butyrivibrio fibrisolvens* una de las bacterias más importantes implicadas en este mecanismo. En esta ruta biosintética, se produce en primer lugar la isomerización del doble enlace *cis* en posición 12 del sustrato (ácido linoleico o ácido α-linolénico), pasando a tener configuración *trans* en el carbono 11. En la siguiente etapa, el doble enlace en posición *cis*-9 experimenta una hidrogenación, formándose el ácido vacénico. Si la biohidrogenación fuese completa, el producto final sería el ácido esteárico pero esta bioconversión se ve parcialmente impedida por la gran estabilidad del doble enlace en la posición *trans*-11 (Jenkins et al., 2008; Kraft et al., 2003). Entre el 64 y 98% del ácido ruménico presente en la grasa láctea es generado en la glándula mamaria por acción de la enzima Δ-9 desaturasa sobre el ácido vacénico (*trans*-11 $C_{18:1}$), el cual se ha observado que es un intermediario producido en la biohidrogenación ruminal (Rodríguez-Castañedas et al., 2011). Apenas existen estudios que detallen la biosíntesis de los otros isómeros del CLA debido a que estos no se encuentran en cantidades significativas en la grasa láctea y a que la funcionalidad de muchos de ellos aún no ha sido establecida (Gómez-Cortés, 2010).

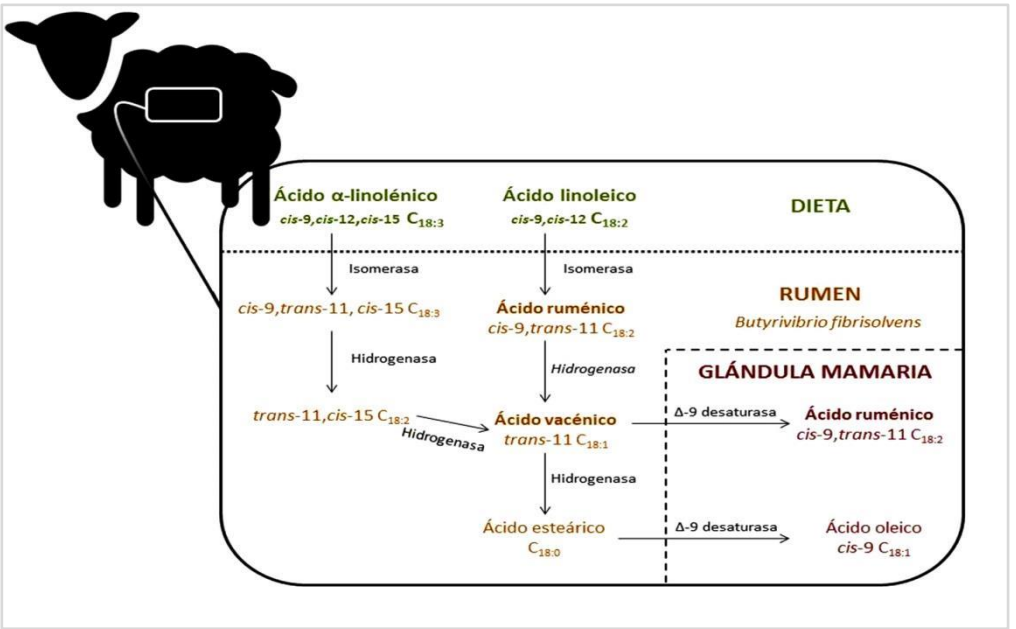


Figura 1. Biosíntesis del ácido ruménico (*cis*-9,*trans*-11 $C_{18:2}$) en el rumen y en la glándula mamaria. Adaptada de Gómez-Cortés (2010).

1.2.2. Efectos fisiológicos del CLA y concentración de este compuesto en leche y queso

El CLA ha suscitado gran interés por sus potenciales efectos beneficiosos sobre la salud, entre los cuales destacan los efectos anticarcinogénicos, antitrombogénicos, reducción de la aterosclerosis, estimulación de la mineralización ósea, modulación del sistema inmune, disminución de la resistencia a la insulina, reducción de la glucosa en sangre y de la grasa corporal (Lehnen et al., 2015). Se ha observado que la actividad biológica del CLA está relacionada en particular con dos isómeros: el *cis*-9,*trans*-11 $C_{18:2}$ y el *trans*-10,*cis*-12 $C_{18:2.}$

El efecto fisiológico más estudiado del CLA es su capacidad para modificar la composición corporal, promoviendo la reducción de los niveles de grasa corporal, como un agente antiobesidad (Churruca et al., 2009). Los resultados iniciales fueron descritos en modelos animales (Botelho et al., 2005, Kloss et al., 2005); ahora bien, investigaciones más recientes en humanos sugieren que el CLA puede actuar reduciendo la grasa corporal modulando el metabolismo lipídico. Los posibles mecanismos de acción implican cambios metabólicos hacia la reducción de la lipogénesis y la mejora de la lipólisis (Martins et al., 2015).

La información disponible sobre los niveles de CLA requeridos para lograr este efecto fisiológico es limitada. Se ha descrito que la reducción de la grasa corporal puede observarse cuando tiene lugar una ingesta de CLA entre 3 y 6 g al día (Iwata et al., 2007; Lehnen et al., 2015). Curiosamente, la ingesta de CLA durante un periodo inferior a 4 semanas en humanos no proporcionó efectos positivos en la reducción de grasa corporal (Pariza, 2009). Sin embargo, en estudios de más de 6 meses de duración, se observó una mayor eficacia del CLA en la modulación de la grasa corporal (Dilzer y Park, 2012).

Otro beneficio potencial del CLA es el efecto antidiabético. Se habla de efecto potencial porque sólo se han realizado estudios en animales, como por ejemplo, el llevado a cabo por Song et al. (2016) en el que se administró a ratones con diabetes tipo II, una leche fermentada que contenía CLA (0,01% o 0,03%) durante 6 semanas. Como resultado de este experimento se observó que los niveles de glucosa en sangre en ayunas, insulina sérica y leptina disminuyeron significativamente en los ratones alimentados con la leche fermentada que contenía la mayor concentración de CLA en comparación con los ratones que recibieron placebo. Por consiguiente, estos resultados sugieren el posible desarrollo de productos lácteos con CLA como alimentos funcionales para contribuir al tratamiento de la diabetes mellitus tipo II (Song et al., 2016). Aun así, es conveniente indicar que sería necesario llevar a cabo estudios en humanos en relación con los posibles efectos antidiabéticos de este ácido graso bioactivo.

La capacidad anticarcinógena del CLA ha sido observada en modelos animales con tumores de mama inducidos químicamente, cuando se les administraba dosis de CLA entre el 0,5% y el 1% (Ip et al., 1994). En consecuencia, se ha tratado de conocer este efecto fisiológico en humanos a través de estudios epidemiológicos pero no ha sido posible extraer conclusiones claras sobre el efecto preventivo del CLA sobre el cáncer. A pesar de estas limitaciones, Pariza (2004) indicó que una persona de 70 kg tendría que consumir 3 g de CLA al día para conseguir los efectos beneficiosos que ejerce el CLA en la inhibición de la carcinogénesis mamaria. En cambio, Mohan et al. (2013) señalaron que una concentración inferior de CLA (0,42 g de CLA al día) a la recomendada por Pariza (2004)

podría ser la dosis efectiva para alcanzar los efectos anticarcinogénicos descritos en humanos.

Cabe destacar que el contenido en CLA en leche y queso varía entre un 0,10% y un 2,86% de los ácidos grasos totales (**Tabla 1**). Lo que quiere decir que la ingesta estimada actual de CLA en personas a partir de las fuentes alimentarias es insuficiente para obtener los efectos potenciales contra el cáncer, la aterosclerosis y la obesidad observados en los estudios *in vivo* (Watkins y Li, 2003). Por este motivo, se están realizando estudios para incrementar la concentración de CLA en la leche y en los productos lácteos, ya que sin duda puede repercutir de forma favorable en su calidad nutricional y por lo tanto en el valor añadido de los alimentos que lo contengan.

Tabla 1. Contenido total de ácido linoleico conjugado (g CLA/100 g ácidos grasos totales) en leche y queso.

Alimento	Intervalo de variación		Referencia
	Mínimo	Máximo	
Leche de vaca entera	0,41	0,93	Ruiz et al., 2016
Leche de oveja entera	0,79	2,00	De La Fuente et al., 2009; Tsiplakou et al., 2006
Leche de cabra entera	0,40	0,62	Cossignani et al., 2014; Tsiplakou et al., 2006
Queso de vaca	0,10	1,30	El-Salam and El-Shibiny, 2014
Queso de oveja	0,38	2,86	El-Salam and El-Shibiny, 2014
Queso de cabra	0,20	0,84	El-Salam and El-Shibiny, 2014

1.2.3. Estrategias para incrementar el contenido en CLA de la leche y del queso

1.2.3.1. Producción animal

Con el fin de modificar la composición en ácidos grasos de la leche de oveja para mejorar el perfil nutricional del queso, fundamentalmente a través del incremento de los niveles de CLA, es necesario conocer los diferentes factores que afectan al perfil lipídico de la misma. De todos ellos, el factor que más influencia tiene sobre el contenido en CLA de la leche es el tipo de alimentación proporcionada a los animales. En los últimos años se han llevado a cabo numerosas investigaciones para estudiar dicho efecto (Chilliard et al., 2007; Hervás et al., 2008; Kalač y Samková, 2010; Stanton et al., 2003). La clave para incrementar la concentración de CLA en la leche se encuentra en el aumento de la producción de ácido vacénico en el rumen, permitiendo de esta manera la síntesis endógena de CLA en la glándula mamaria. El incremento de la producción ruminal de ácido vacénico puede ser logrado de dos maneras: mediante el aumento del suministro de ácidos grasos poliinsaturados precursores, como es el caso del ácido α-linolénico, y por inhibición de la reducción del ácido vacénico en ácido esteárico, siendo la primera vía la más fácil de llevar a cabo (Lock y Bauman, 2004).

Se ha observado que la leche obtenida de animales alimentados a base de pasto fresco presenta mayor concentración de CLA que aquella obtenida de animales estabulados alimentados a base de ensilados o con dietas a base de concentrados (van Wijlen y Colombani, 2010). El uso incrementado en la alimentación de ensilados y concentrados, así como el abandono de la estacionalidad a favor de los sistemas de producción continua, son factores que afectan a la calidad del perfil lipídico de la leche

de oveja (Pellattiero et al., 2015). En la región Mediterránea la mayoría de los sistemas de producción de ovino son semiextensivos, lo que significa que el pasto fresco juega un papel importante en la alimentación de los animales, el cual presenta un bajo contenido lipídico pero aproximadamente un 63% de este está constituido por ácidos grasos precursores del CLA (Cabiddu et al., 2005). De los anteriores planteamientos se deduce que el aumento del tiempo de pastoreo de los rumiantes puede ser una de las formas más importantes para incrementar la concentración de CLA en la leche (Tsiplakou et al., 2008). Así mismo, se ha observado que la concentración de este ácido graso en el pasto se ve principalmente afectada por la etapa fenológica en la que se encuentran las plantas que lo componen. En este sentido, se ha visto que en la etapa vegetativa presentan mayor concentración de ácido α-linolénico que en la etapa reproductiva. Esto puede explicar que, de manera general, los mayores niveles de este ácido graso se observen en pastos primaverales y, por consiguiente, que los rumiantes alimentados con ellos produzcan una leche con mayor concentración de CLA durante esta época del año que en cualquier otra (Rodríguez-Castañedas, 2012).

De La Fuente et al. (2009) han señalado que hay otros factores que también afectan en menor medida al perfil lipídico de la leche de ovino, tales como el rebaño, la edad, la etapa de lactación y el día de muestreo. A su vez, también hay diversos trabajos que han tratado de estudiar el efecto que ejerce la raza sobre el contenido en CLA de la leche. Tsiplakou et al. (2008) estudiaron el efecto de diferentes razas de ovino, bajo el mismo sistema de manejo y alimentación, sobre el contenido en ácidos grasos de la leche, concluyendo que la interacción entre la raza y la dieta tuvo un efecto significativo sobre la mayoría de los ácidos grasos, pero que la raza por sí misma no tuvo un efecto significativo sobre el contenido en CLA. Signorelli et al. (2008) en un estudio llevado a cabo para promover ciertas razas de ovino autóctonas, observaron diferencias significativas en el perfil lipídico de la leche de las diferentes razas estudiadas. Como puede deducirse de los resultados de estos trabajos de investigación, en este ámbito de estudio hay cierta controversia. Este hecho puede deberse a que como Lock y Bauman (2004) señalaron, estos trabajos a menudo incluían pocos animales en su estudio o no separaban bien el efecto que tiene la alimentación animal, haciendo necesario llevar a cabo más estudios para poder establecer si realmente la raza es un factor que puede condicionar el contenido en CLA de la leche.

La gran cantidad de investigaciones llevadas a cabo para estudiar los diferentes factores del sistema de manejo de los animales que puedan mejorar el perfil de ácidos grasos de la leche pone de manifiesto que las estrategias para mejorar el perfil lipídico de los productos lácteos deben comenzar desde el proceso de producción de la materia prima. Sin embargo, también resulta posible modificar el perfil de ácidos grasos de los quesos actuando sobre el propio proceso de elaboración, tal y como se describe a continuación.

1.2.3.2. Tecnología de la elaboración: cultivos iniciadores o adjuntos

Según lo descrito en mayor detalle en el apartado anterior, las bacterias presentes en el rumen son las principales responsables de la síntesis de ácido ruménico y de ácido vacénico a partir del ácido linoleico y α-linolénico procedentes de la alimentación animal. Este hecho ha llevado a varios investigadores a plantearse si

otras bacterias frecuentemente utilizadas en la elaboración de productos lácteos fermentados, tales como las bacterias ácido lácticas o las bifidobacterias, serían capaces de sintetizar CLA a partir de ácidos grasos poliinsaturados como sustrato y de esta forma incrementar la concentración de este compuesto bioactivo en diversos productos lácteos (Gorissen et al., 2010; Nieuwenhove et al., 2007; Rodríguez-Alcalá et al., 2011; Terán et al., 2015).

En lo referente a la biosíntesis del CLA a partir del ácido linoleico por las bacterias ácido lácticas, se ha observado que puede tener lugar aparentemente a partir de dos rutas metabólicas distintas: isomerización directa del ácido linoleico o vía ácido 10-hidroxi-octadecenoico (**Figura 2**).

En esta última ruta metabólica, tiene lugar una hidratación del ácido linoleico dando lugar a 10-hidroxi-*cis*-12-$C_{18:1}$ en equilibrio con 10-hidroxi-*trans*-12-$C_{18:1}$ seguida de una deshidratación e isomerización de estos ácidos grasos hidróxidos a CLA (*cis*-9,*trans*-11 $C_{18:2}$ en equilibrio con *trans*-9,*trans*-11 $C_{18:2}$) (Andrade et al., 2012). Cabe destacar, que estas rutas metabólicas sólo son posibles si en los microorganismos se encuentra presente la linoleato isomerasa, la cual ha sido descrita por Ogawa et al. (2005) como un complejo enzimático constituido por tres enzimas que requieren cofactores redox como el NADH y o el FAD y que, a su vez, es dependiente de la cepa.

En este contexto, varios estudios han demostrado que diversas cepas principalmente pertenecientes a las especies *Bifidobacterium*, *Lactobacillus*, *Lactococcus* y *Propionibacterium* pueden transformar eficientemente el ácido linoleico en CLA en ensayos realizados en medio de cultivo o leche desnatada a los que se les añadió ácido linoleico como sustrato (**Tabla 2**).

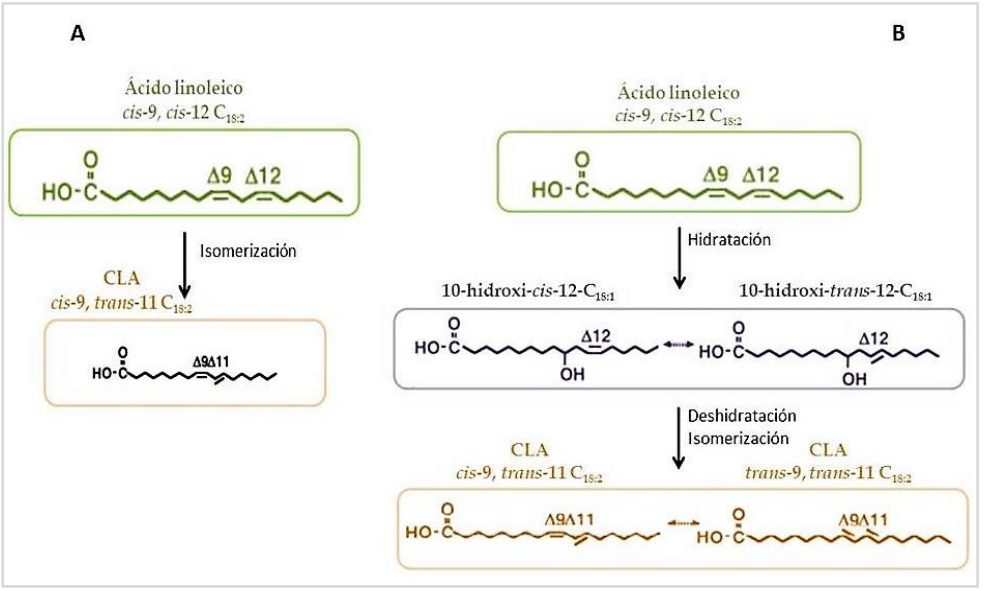


Figura 2. Rutas metabólicas propuestas en los microorganismos con respecto a la síntesis del ácido linoleico conjugado a partir del ácido linoleico. A: isomerización directa; B: vía ácido 10-hidroxi-octadecenoico. Adaptado de Ogawa et al. (2005).

Tabla 2. Producción de ácido linoleico conjugado (CLA; mg/L) por diversas cepas de microorganismos.

Cepa	Medio[1]	Sustrato[2]	CLA total	Referencia
Bifidobacterium				
B. breve WC0420	MRS	LA (0,5g/L)	132	Raimondi et al. (2016)
B. breve WC0421			881	
B. animalis BLC	MRS/LD	LA (1g/L)	7/48	Rodríguez-Alcalá et al. (2011)
B. animalis Bb12			7/42	
B. bifidum LMG10645	MRS	LA (0,5g/L)	207	Gorissen et al. (2010)
B. breve LMG11080			272	
B. breve LMG11613			99	
B. bifidum NCFB795			1	
B. breve NCFB2258	MRS	LA (0,5g/L)	398	Coakley et al. (2003)
B. breve NCIMB8807			128	
Lactobacillus				
L. acidophilus CRL44	MRS	LA (0,3g/L)	112	Terán et al. (Terán et al., 2015)
L. acidophilus CRL1063			44	
L. curvatus CRL1629			364	
L. plantarum CRL100			84	
L. plantarum CRL1935			350	
L. sakei CRL1470			488	
L. acidophilus Ki	MRS	LA (1g/L)	9	Rodríguez-Alcalá et al. (2011)
L. plantarum ATCC8014	MRS	LA (0,5g/L)	23	Gorissen et al. (2011)
L. sakei LMG13558			21	
Lactococcus				
Lc. lactis biovar *diacetylactis* CRL967	MRS	LA (0,3g/L)	50	Terán et al. (Terán et al., 2015)
Lc. lactis biovar diacetylactis CRL1061			98	
Lc. lactis LMG S 19870	MRS/LD	LA (1g/L)	7/46	Rodríguez-Alcalá et al. (2011)
Lc. lactis spp. cremoris CCRC12586	LD	LA (1g/L)	63	Lin et al. (1999)
Lc. lactis spp. *lactis* CCRC10791			78	
Propionibacterium				
Prop. freudenreichii spp. shermanii JS	MRS	LA (0,5g/L)	49	Hennesy et al. (2012)
Prop. freudenreichii spp. *shermanii* 9093			259	
Prop. freudenreichii spp. *freudenreichii* ATCC6207			8	
Prop. freudenreichii spp. *shermanii* B6022	MRS	LA (1g/L)	163	Das et al. (2005)
Prop. freudenreichii spp. *shermanii* B6026			190	
Prop. freudenreichii spp. *shermanii* B6028			190	

[1]MRS: De Man, Rogosa and Sharpe; LD: leche desnatada.
[2]LA: ácido linoleico.

Este hecho ha dado lugar a nuevas líneas de investigación encaminadas a aumentar el contenido en CLA de los quesos mediante el uso de cultivos iniciadores o adjuntos capaces de sintetizar el CLA *in vitro*. Por ejemplo, el empleo de bacterias ácido lácticas autóctonas en la elaboración de queso de cabra o vaca dio lugar a unos resultados prometedores en relación con el incremento del contenido en CLA en estos quesos (Mohan et al., 2013; Taboada et al., 2015). No

obstante, es importante indicar que sería necesario realizar más estudios al respecto ya que la información disponible es limitada.

Atendiendo a lo anteriormente descrito, el diseño de cultivos productores de CLA con aplicación en la fabricación de queso podría fomentar la diversidad de cultivos disponibles para la industria láctea así como el posible desarrollo de productos lácteos funcionales. Además, las cepas que forman parte de estos cultivos también pueden sintetizar otros compuestos, aparte del CLA, de gran interés desde el punto de vista de la salud humana. La revisión realizada acerca de estos aspectos ha sido publicada y se presenta a continuación con el mismo contenido que el artículo.

1.3. Lactic acid bacteria and bifidobacteria with potential to design natural biofunctional health-promoting dairy foods

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Abstract

Consumer interest in healthy lifestyle and health-promoting natural products is a major driving force for the increasing global demand of biofunctional dairy foods. A number of commercial sources sell synthetic formulations of bioactive substances for use as dietary supplements. However, the bioactive-enrichment of health-oriented foods by naturally occurring microorganisms during dairy fermentation is in increased demand. While participating in milk fermentation, lactic acid bacteria can be exploited *in situ* as microbial sources for naturally enriching dairy products with a broad range of bioactive components that may cover different health aspects. Several of these bioactive metabolites are industrially and economically important, as they are claimed to exert diverse health-promoting activities on the consumer, such as anti-hypertensive, anti-inflammatory, and anti-diabetic, anti-oxidative, immune-modulatory, anti-cholesterolemic, or microbiome modulation. This review aims at discussing the potential of these health-supporting bacteria as starter or adjunct cultures for the elaboration of dairy foods with a broad spectrum of new functional properties and added value.

Keywords: lactic acid bacteria, bifidobacteria, health, bioactive, probiotic, biofunctional food.

Biofunctional foods

Today foods are not intended to only satisfy hunger and to provide necessary nutrients for humans, but also to prevent nutrition-related diseases and improve

consumers' health (Gortzi et al., 2015; Siró et al., 2008). Increasing consumer demand and interest in obtaining additional benefits from food has stimulated functional foods to emerge on the market, with USA, Europe, and Japan being the dominant markets.

Although there is no unitary accepted definition, functional foods can be described as an ordinary food that has components or ingredients added to provide a specific health benefit, other than a purely nutritional effect. Ideally, functional food refers to an existing traditional food product that is intended to be consumed as part of a normal diet and has a demonstrated added physiological benefit (Siró et al., 2008). Therefore, it could not be in the form of pill or capsule. The concept of biofunctional foods is generally used when this desirable biological, medical, or physiological effect is exerted by microorganisms (Gobbetti et al., 2010). The health benefits of these microorganisms can be exerted either directly through the interactions of ingested live microorganisms with the host (probiotic effect), or indirectly by ingestion of the microbial metabolites synthesized during fermentation (bioactive effect) (Gobbetti et al., 2010; Joshi, 2015; Stanton et al., 2005).

Probiotic foods

Lactic acid bacteria (LAB) have been used to ferment foods for at least 4000 years (Rotar et al., 2007). Although the probiotic concept has expanded more recently, we have been unconsciously ingesting beneficial microbes with traditional fermented foods since ancient times. Fermented foods are the main carriers to deliver probiotics (**Figure 3**). Among them, dairy products (in particular fermented milks and yogurt) are by far the most efficient and widely used (Giraffa, 2012). Cheese is a dairy product which has a good potential for the incorporation of probiotic cultures due to its specific chemical and physical characteristics compared to fermented milks (higher pH value and lower titrable acidity, higher buffering capacity, greater fat content, higher nutrient availability, lower oxygen content, and denser texture). These conditions facilitate survivability of probiotic strains and tolerance to the low pH conditions encountered during gastric transit (Karimi et al., 2011). Utilization of probiotics has been optimized in several cheese varieties such as Cheddar, Gouda, Camembert, Cottage type, white-brined, and traditional cheeses, among others (Araujo et al., 2012; Giraffa, 2012; Martinovic et al., 2016; Oh et al., 2016). Kefir is another milk-fermented product that has health promoting bacteria (Prado et al., 2015). Other non-fermented dairy foods such as low-fat ice cream, chocolate mousse, coconut flan, or infant milk formula have also been supplemented with probiotic strains (Aragon-Alegro et al., 2007; Baglatzi et al., 2016; Corrêa et al., 2008; Davidson et al., 2000).

Probiotic microorganisms are generally LAB belonging to the species *Lactobacillus acidophilus*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. (para) casei*, *L. reuteri*, *L. plantarum*, *L. rhamnosus*, and *L. fermentum*, while members of the genus *Bifidobacterium* are also used, e.g., *Bifidobacterium bifidum*, *B. longum*, *B. animalis*, and *B. breve* (Castro et al., 2015; Linares et al., 2016b; Tamime et al., 2005). On the basis of the currently available literature, probiotics can balance intestinal microbiota, and thereby regulate proper intestinal function and be effective in the prevention or treatment of several gastrointestinal disorders such as infectious diarrhea, antibiotic-related diarrhea, irritable bowel syndrome or Crohn's disease (Vanderhoof and Young, 1998). Other examples of health benefits promoted by probiotics supplied via dairy products are

inmmunomodulatory effects (*L. casei* CRL431), reduction of serum cholesterol level (*L. reuteri* NCIMB 30242) and antihypertensive effects (*L. plantarum* TENSIATM) (Aragón et al., 2014; EFSA, 2011; Jones et al., 2012).

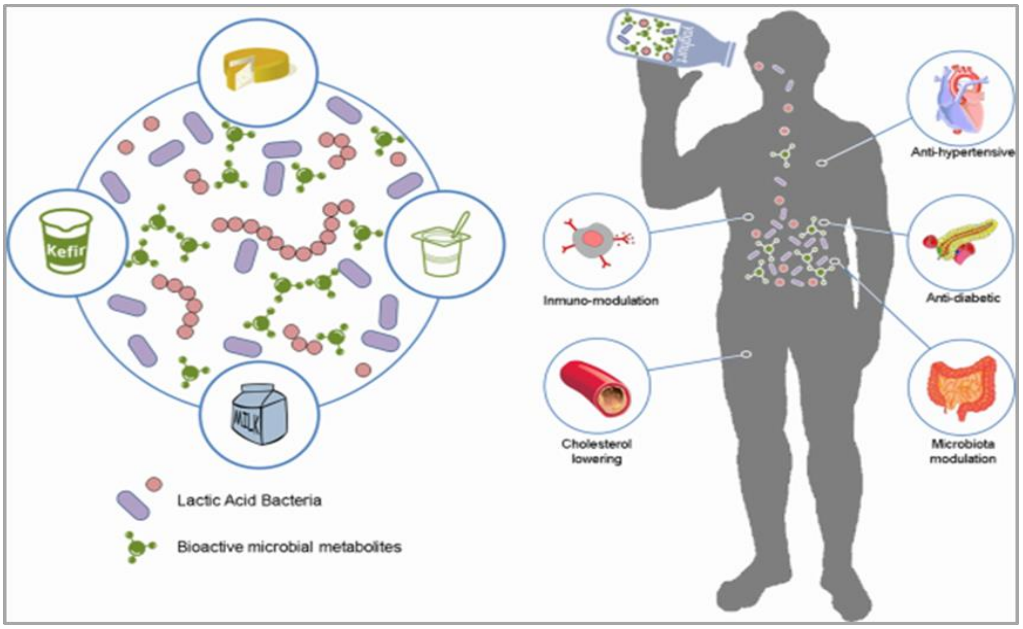

Figure 3. Beneficial effects resulting from the consumption of biofunctional fermented dairy foods. Lactic acid bacteria participating in milk fermentation *in situ* release and naturally enrich the fermented dairy product with a broad range of bioactive metabolites. Subsequent ingestion of this product can exert important health-promoting activities on the consumer, such as anti-hypertensive, and anti-diabetic, immune-modulatory, anti-cholesterolemic or microbiome modulation.

Probiotics are defined as 'live micro-organisms, which when consumed in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). However, regarding probiotic foods, some considerations are of paramount importance. Firstly, effective levels of the live probiotic in the corresponding food matrix at the time of ingestion are required. In this regard, the minimum effective dose which affects the intestinal environment and provides beneficial effects on human health is considered to be 10^6–10^9 live microbial cells per day, although this depends on the particular strain and foodstuff (Karimi et al., 2012; Watson and Preedy, 2015; Williams, 2010). Since probiotics show beneficial health effects on the host once consumed, another precondition for a bacterial strain to be called probiotic is the ability to survive and colonize (at least transiently) the gastrointestinal tract (GIT), which is in part helped by the buffering capacity of the food matrix. In some particular cases, bacterial viability may not be strictly required. As an example, inactivated and dead *L. rhamnosus* GG cells can maintain immunological and health-promoting effects (Ghadimi et al., 2008; Lopez et al., 2008).

Bioactive compounds derived from microbes

Microorganisms involved in dairy fermentations can produce biologically active molecules and enzymes, giving the final food product an additional health value. Unlike

the probiotic concept (the bacteria must be ingested alive and produce the beneficial metabolite in the body), the biofunctional concept is generally used when the healthy metabolite emerge in the food product itself during the fermentation process as a consequence of the bacterial metabolic activity. Consequently, the bacteria can act as a microbial factory to enrich foodstuff, for which bacterial viability through the GIT or during the product storage is not absolutely required (Farnworth and Champagne, 2015). The main bioactive compounds produced by LAB during dairy fermentation are vitamins, gamma-aminobutyric acid, bioactive peptides, bacteriocins, enzymes, conjugated linoleic acid, and exopolysaccharides (**Table 3**).

Vitamins

There are 13 vitamins that must be obtained exogenously due to the inability of humans to synthesize them; thereby they are essential nutrients in the human diet, and although in small amounts, a daily requirement is necessary to prevent deficiencies. Although most vitamins are present in a variety of foods, human vitamin deficiencies still occur in many countries, mainly because of malnutrition, not only as a result of insufficient food intake but also because of unbalanced diets (LeBlanc et al., 2002).

Although milk contains many vitamins fermentation by LAB and bifidobacteria constitute an effective way to increase vitamin levels in milk (Laiño et al., 2013). Some bacterial strains included in the genera *Lactobacillus* and *Bifidobacterium* can provide an additional source of B vitamins (thiamine, riboflavin, cobalamin, folate, and biotin) during dairy fermentation. Deficiencies in riboflavin (vitamin B2) or thiamine (vitamin B1) can lead to both liver and skin disorders and alterations in brain glucose metabolism, respectively (Russo et al., 2014). In this regard, *L. casei* KNE-1 has been shown to produce thiamine and riboflavin in fermented milk drinks (Drywien et al., 2015). *B. infantis* CCRC14633 and *B. longum* B6 strains have been reported to produce riboflavin and thiamine during soymilk fermentation (Tamime, 2006). It was recently indicated that soymilk fermented by the riboflavin-producing strain *L. plantarum* CRL2130 was able to prevent ariboflavinosis and experimental colitis in a murine model (Juarez del Valle et al., 2016; Levit et al., 2016). Some propionibacteria can produce cobalamin, folic acid, and biotin (Hugenholtz et al., 2002).

Folate (vitamin B9) is involved in several vital processes and its deficiency is generally linked to neural tube defects, certain forms of cancer, poor cognitive performance and coronary heart diseases. Even though vitamins are widely present in foods, the prevalence of folate deficiency -especially among women of child bearing age- is a growing concern and thereby folate fortification programs have been implemented in various countries (Divya and Nampoothiri, 2015). Rather than incorporating synthetic folate, foods can be naturally fortified with folate synthesized by LAB and bifidobacteria during manufacture of fermented foods (Lin and Young, 2000; Saubade et al., 2017). The strains *Streptococcus thermophilus* CRL803/CRL415 and *L. bulgaricus* CRL871 were reported to be suitable for the elaboration of yogurt naturally bio-enriched in this vitamin (Laiño et al., 2013). High folate concentration (up to 150 mg/l) can be reached in yogurt as a result of the ability of *S. thermophilus* to produce this vitamin (Hugenholtz et al., 2002).

Table 3. Some strains of lactic acid bacteria, bifidobacteria and propionibacteria with potential to biosynthesize health-promoting compounds in fermented dairy products.

Bioactive	Producer strain	Food product	Health effect	Reference
Thiamine/Riboflavin	*Lactobacillus casei KNE-1*	Fermented milk	Vitamin enrichment	Drywlen et al. (2015)
	Bifidobacterium infantis CCRC14633	Fermented soymilk	Vitamin enrichment	Tamine (2006)
	Bifidobacterium longum B6	Fermented soymilk	Vitamin enrichment	Tamine (2006)
	Lactobacillus plantarum CRL 2130	Fermented soymilk	Vitamin enrichment	Levit et al. (2016)
Biotin	*Lactobacillus helveticus* MTCC5463	Fermented milk	Vitamin enrichment	Patel et al. (2013)
Cobalamin	*Propionibacterium freudenreichii*	Kefir	Vitamin enrichment	Van Wyk et al. (2011)
	Bifidobacterium animalis Bb12	Fermented milk	Vitamin enrichment	Patel et al. (2013)
	Lactobacillus reuteri ZJ03	Soy-yogurt	Vitamin enrichment	Gu et al. (2015)
Folic acid	*Streptococcus thermophilus CRL803*	Yogurt	Vitamin enrichment	Laiño et al. (2014)
	Lactobacillus bulgaricus CRL871	Fermented milk	Vitamin enrichment	Laiño et al. (2014)
	Bifidobacterium lactis CSCC5127	Fermented milk	Vitamin enrichment	Crittenden et al. (2003)
	Bifidobacterium infantis CSCC5187	Fermented milk	Vitamin enrichment	Crittenden et al. (2003)
	Bifidobacterium breve CSCC5181	Fermented milk	Vitamin enrichment	Crittenden et al. (2003)
	Lactobacillus amylovorus CRL887	Fermented milk	Vitamin enrichment	Laiño et al. (2014)
GABA	*Lactobacillus casei* Shirota	Fermented milk	Antidiabetic, blood pressure	Inoue et al. (2003)
	Streptococcus salivarus fmb5	Fermented milk	Antidiabetic, blood pressure	Chen et al. (2016)
	Lactobacillus plantarum NDC75017	Fermented milk	Antidiabetic, blood pressure	Shan et al. (2015)
	Lactobacillus brevis OPY-1	Fermented soymilk	Antidiabetic, blood pressure	Park and Oh (2007)
	Streptococcus thermophilus APC151	Yogurt	Antidiabetic, blood pressure	Linares et al. (2016a)
Bioactive peptides	*Lactobacillus helveticus* Evolus^{R}	Fermented milk	Anti-hypertensive	EFSA (2008)
	Lactobacillus helveticus Calpis^{TM}	Fermented milk	Anti-hypertensive	Dzulba and Dziuba (2014)
	Lactobacillus bulgaricus LB340	Fermented milk/Yogurt	Anti-hypertensive	Qian et al. (2011)

Table 3 (continuation). Some strains of lactic acid bacteria, bifidobacteria and propionibacteria with potential to biosynthesize health-promoting compounds in fermented dairy products.

Bioactive	Producer strain	Food product	Health effect	Reference
Bactericins	*Lactococcus lactis* CNZR150/TAB50	Camembert cheese	Pathogen inhibition	Arques et al. (2015)
	Lactococcus lactis DPC3147	Cheddar cheese	Pathogen inhibition	Ross et al. (1999)
	Lactobacillus acidophilus CH5	Yogurt	Pathogen inhibition	Ahmed et al. (2010)
	Pediococcus acidilactici CHOOZIT™	Cheddar cheese	Pathogen inhibition	Arques et al. (2015)
	Lactobacillus plantarum WHE92	Munster cheese	Pathogen inhibition	Arques et al. (2015)
CLA	*Lactococcus lactis* Cl4b	Cheedar cheese	Cholesterol lowering	Mohan et al. (2013)
	Lactobacillus rhamnosus C14 *Lactobacillus casei* CRL431 *Streptococcus thermophilus* CRL728 *Bifidobacterium bifidum* CRL1399	Buffalo cheese	Cholesterol lowering	Van Niewenhove et al. (2007a)
	Lactococcus lactis LMG *Lactobacillus acidophilus* Lac1 *Lactobacillus plantarum*-2 *Bifidobacterium animalis* Bb12	Fermented buffalo milk	Cholesterol lowering	Van Niewenhove et al. (2007b)
	Lactobacillus bulgaricus LB430	Yogurt	Cholesterol lowering	Sosa-Castañeda et al. (2015)
Exopolysaccarides	*Lactobacillus bulgaricus* OLL1073R-1	Yogurt	Immunostimulatory	Makino et al. (2016)
	Lactobacillus mucosae DPC6426	Yogurt/Cheddar cheese	Hypocholesterolemic	Ryan et al. (2015)
	Propionibacterium freudenreichii KG15	Turkish cheese	Microbiota modulation	Darilmaz and Gumustekin (2012)
	Lactococcus lactis SMQ-461	Cheddar cheese	Microbiota modulation	Dabour et al. (2005)
	Lactobacillus plantarum YW11	Kefir	Microbiota modulation	Wang et al. (2015)
	Bifidobacterium longum CCUG52486	Yogurt	Immune modulation	Prassanas et al. (2013)
	Streptococcus thermophilus zwTM11	Yogurt	Microbiota modulation	Han et al. (2016)
	Streptococcus thermophilus FD-DVSST	Fermented ice-cream	Microbiota modulation	Dertli et al. (2016)

Among bifidobacteria, *B. catenulatum* ATCC 27539 was shown to produce high levels of folate in vitro (D'Aimmo et al., 2012), and *B. lactis* CSCC5127, *B. infantis* CSCC5187, and *B. breve* CSCC5181 strains increased folate concentration during fermentation of reconstituted skim milk (Crittenden et al., 2003). Similarly, *L. amylovorus* CRL887 can be used for natural folate bio-enrichment of fermented milk (Laiño et al., 2014).

The deficiency of cobalamin (vitamin B12) can be common, particularly in vegetarians who avoid ingestion of animal protein and use soymilk as an alternative to dairy milk (Gu et al., 2015). Animals, plants and fungi are incapable of producing this vitamin, and hence, it is exclusively produced by microorganisms (LeBlanc et al., 2011). It has been demonstrated that cobalamin can be synthesized by some bacteria such as *L. reuteri* ZJ03, *Propionibacterium freudenreichii*, *B. animalis* Bb12 in soy-yogurt, kefir and fermented milk, respectively (Gu et al., 2015; Moslemi et al., 2016; Patel et al., 2013; Van Wyk et al., 2011).

Microorganisms can biosynthesize two different isoforms, the vitamin and the pseudovitamin. For example, in a recent work, the production of vitamin and pseudovitamin B12 by *P. freudenreichii* was quantified specifically and shows that at the initial phase of the fermentation both isoforms are biosynthesized at similar levels; however, by the end of the fermentation the pseudovitamin is not detected, most likely because it is converted to the vitamin form (Deptula et al., 2017). It seems crucial to differentiate between the two isoforms of this vitamin, as the transporter protein in the human GIT has very low affinity for the pseudovitamin, making it un-available to humans (Varmanen et al., 2016).

Biotin (vitamin B7) deficiency can be caused by inadequate dietary intake or some inborn genetic disorders that affect its metabolism. Subclinical deficiency can cause mild symptoms, such as hair thinning or skin rash typically on the face. Biotin can be synthesized by some LAB in dairy products, e.g., *L. helveticus* MTCC 5463 increased biotin content in fermented milks (Patel et al., 2013). Some propionibacteria can also produce biotin (Hugenholtz et al., 2002).

Vitamin K is an important promoter of bone and cardiovascular health. It has been associated with the inhibition of arterial calcification and stiffening, and the reduction of vascular risk. This vitamin is nearly non-existent in junk food; with little being consumed even in a healthy Western diet (Maresz, 2015). Its deficiency has been implicated in several clinical ailments such as intracranial hemorrhage in newborn infants and possible bone fracture resulting from osteoporosis (LeBlanc et al., 2011). Vitamin K occurs in two forms: firstly, phylloquinone (vitamin K1), which is present in green plants, and secondly, menaquinone (vitamin K2), which is produced by some intestinal bacteria (LeBlanc et al., 2011). Menaquinone can be biosynthesized by some LAB species (mainly *Lactococcus lactis*) commonly used in industrial fermentation of cheese, buttermilk, sour cream, cottage cheese, and kefir (Walther et al., 2013). Other LAB have been screened for the ability to produce menaquinone; these included strains from the genera *Lactococcus*, *Bifidobacterium*, *Leuconostoc*, and *Streptococcus* (Morishita et al., 1999). Although the MK forms are ubiquitous in bacteria, it should be noted that some genera such as *Lactobacillus* have lost the functional ability to produce them (Lechardeur et al., 2011; Walther et al., 2013).

Gamma-aminobutyric acid

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the central nervous system (CNS). Several important physiological functions of GABA have been characterized, such as neurotransmission, induction of hypotension, diuretic effects, antidiabetic, relaxing and tranquilizer effects (Inoue et al., 2003; Marques et al., 2016). In fact, some $GABA_A$-receptor agonist drugs (e.g., benzodiazepines) are important pharmacological agents used for clinical treatment of anxiety (Foster and Kemp, 2006).

Gamma-aminobutyric acid is biosynthesized through α-decarboxylation of glutamate, an enzymatic conversion which is catalyzed by glutamate decarboxylase (GAD) (Tajabadi et al., 2015). Several food-grade LAB have been reported to exhibit GABA-producing ability. Among them, most of the GABA-producing strains are lactobacilli (*L. brevis*, *L. paracasei*, *L. delbrueckii*, *L. buchneri*, *L. plantarum*, *L. helveticus*), *Streptococcus thermophilus*, and *Lactococcus lactis* (Dhakal et al., 2012; Li and Cao, 2010). Some, *Bifidobacterium* spp. were also reported to produce GABA, although with lower capacity than LAB (Barrett et al., 2012; Park et al., 2005).

Some fermented dairy products enriched in GABA using GABA-producing LAB as starters have been developed. The strains *L. casei* Shirota, *S. salivarius* fmb5 and *L. plantarum* NDC75017 were utilized to ferment and enrich milk in GABA (Chen et al., 2016; Inoue et al., 2003; Shan et al., 2015). More recently, yogurt enriched with 2 mg GABA/ml was produced using the strain *S. thermophilus* APC151 (Linares et al., 2017, 2016a). Also, fermented soya milk (using *L. brevis* OPY-1 as source of GABA) (Park and Oh, 2007), or cheese (*Lactococcus lactis* as source of GABA) (Nomura et al., 1998; Pouliot-Mathieu et al., 2013) have been produced. Thus, GABA has potential as a health-promoting bioactive component in foods (Li and Cao, 2010).

Bioactive peptides

During milk fermentation, LAB, making use of their proteolytic system can transform milk proteins into biologically active peptides. These peptides can exert a wide range of effects, such as antimicrobial, antihypertensive, antithrombotic, immunomodulatory, and antioxidative (LeBlanc et al., 2002; Nongonierma and FitzGerald, 2015). The most studied mechanism of bioactive peptides is the antihypertensive action displayed by the inhibition of the angiotensin-I-convertingenzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) which regulates blood pressure (Fernández et al., 2015). ACE inhibitory peptides have been isolated from a variety of fermented dairy products including cheese, fermented milks and yogurt (Fitzgerald and Murray, 2006; Pritchard et al., 2010). The best known ACE-inhibitory biopeptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), have been identified in milk fermented by *L. helveticus* (Slattery et al., 2010). In addition, other dairy startercultures industrially used in the manufacture of fermented dairy products (e.g., *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus*, *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *Lactococcus lactis,* or *S. thermophilus*) can generate bioactive peptides (Hafeez et al., 2014; Hajirostamloo, 2010). Other ACE-inhibitory peptides such as β-casein f(72-81), Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile (SLVYPFPGPI) have been produced by *L. delbrueckii* ssp.*bulgaricus* LB340 in fermented milk (Qian et al., 2011).

On an industrial scale, two fermented milk products with antihypertensive claims, Calpis™ and Evolus®, have been tested extensively in rats and in clinical trials, and are

commercialized as functional foods (Dziuba and Dziuba, 2014). Evolus[R] is available in the market as a *L. helveticus* fermented milk –produced in Finland- proven to decrease the systolic blood pressure in hypertensive subjects due to the actions of *L. helveticus* bioactive peptides (EFSA, 2008). Calpis[TM] is defined as a milk product marketed in Japan (Calpis Co. Ltd.) with antihypertensive properties. It is prepared by fermenting skimmed milk with *L. helveticus* and *Saccharomyces cerevisiae*, which produce VPP and IPP peptides from β-casein and K-casein (Dziuba and Dziuba, 2014).

Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a particular bacterium that are active against other competitor bacteria; thereby they constitute an important part of the microbial defense system (Nes et al., 2007). Such bacteriocin-producing strains may offer potential as an alternative to antibiotics, and may be useful as a means of controlling pathogen carriage, therefore being highly suitable as microbial food additives (Cotter et al., 2013) (**Table 4**). Bacteriocins from LAB have attracted much interest because they are frequently produced by commercially useful strains that are generally regarded as safe (GRAS) for human consumption (Nes et al., 2007). These antimicrobial molecules are among the beneficial peptides intrinsically synthesized by some LAB during milk fermentation and they have been traditionally used as naturally produced food biopreservatives. In addition, they may function in the GIT as potential natural biotherapeutic agents facilitating the competition of probiotic strains and/or inhibition of pathogens; thereby they are potential contributors to the microbiota balance and human health (Dobson et al., 2012).

Table 4. Characteristic aspects of bacteriocins compared to conventional antibiotics (Adapted from Cleveland et al. 2001).

	Bacteriocins	**Antibiotics**
Application	Foods	Clinical
Bioactivity spectra	Mostly narrow	Mostly broad
Bioactive intensity	nM-μM	μM-mM
Biosynthesis	Ribosomal	Secondary metabolite
Proteolytic degradability	High	None
Thermostability	High	Low
Activity pH range	Wide	Narrow
Target cell resistance development	Adaptation through changes in cell membrane composition	Genetically transferable determinant that inactivates the active compound
Mode of action	Generally, pore formation	Cell membrane or intercellular targets, inhibition of cell wall biosynthesis
Toxicity in eukaryotic cells	None known	Present

Nisin is the most well-known bacteriocin used as food preservative due to its antibacterial effect against *Listeria*, clostridia spores and LAB associated to spoilage. Nisin has been approved as a food additive (E234) in the European Union according to Directive 95/2/EC (EC, 1995) in the following products: semolina and tapioca puddings (3 mg/kg); ripened and processed cheese (12.5 mg/kg), clotted cream (10 mg/kg), and Mascarpone

cheese (10 mg/kg). It is also permitted in over 40 countries world-wide including USA, Australia, South Africa, Russia, and India for use as an antimicrobial agent in a variety of food products (EFSA, 2006). Nisin-containing Camembert and semihard cheeses with prolonged shelf-life were made using *Lactococcus lactis* (strains CNRZ150 or TAB50, respectively) as nisin producers (Arqués et al., 2015). Apart from nisin, plantaricins are very wellknown bacteriocins. For example, plantaricin C is a broad spectrum bacteriocin produced by *L. plantarum*, isolated from ripening cheese (González et al., 1994). Plantaricins have been reported to produce an immunomodulatory effect on dendritic cells (Meijerink et al., 2010). However, bacteriocins other than nisin have so far only few and limited authorized uses in foods (Yang et al., 2015). Consequently, the use of bacteriocin-producing bacteria as starter culture for *in situ* biosynthesis during milk fermentation becomes an effective alternative strategy to incorporate bacteriocins in dairy foods.

Similarly, the lacticin 3147-producing strain *Lactococcus lactis* DPC3147 used as a protective culture in Cheddar cheese reduced numbers of *Listeria monocytogenes* to <10 cfu/g within 5 days at 4ºC (Chen and Hoover, 2003; Ross et al., 1999). Other bacterial species such as *L. acidophilus* can be added as an adjunct in many food fermentation processes to contribute to bacteriocin production and food preservation (Anjum et al., 2014). Other LAB strains such as *L. plantarum* WHE92 used as adjunct to the starter culture reduced *Listeria monocytogenes*, *Listeria innocua*, and *Escherichia coli* O157:H7 counts in cheese as a consequence of the production of plantaricin (Arqués et al., 2015). Using a similar concept, Danisco developed a freeze-dried culture of *Pediococcus acidilactici* (marketed as CHOOZIT Flav 43) for use as a bacteriocin-producer adjunct in Cheddar and semihard cheeses (Mills et al., 2011).

Studies of the direct impact of dairy foods containing bacteriocins on human health and microbiome are still limited. In vivo antimicrobial activity of nisin and lacticin 3147 has been recently demonstrated in a murine infection model. A nisin-producing *Lactococcus lactis* CHCC5826 modified the microbiota composition of human microbiota-associated rats increasing bifidobacteria levels and decreasing *Enterococcus/Streptococcus* populations. Lacticin 3147 has the potential to be employed in the treatment of *Clostridium difficile* diarrhea and to eliminate the pathogen when added to an anaerobic fecal fermentation (Arqués et al., 2015).

Enzymes

Lactic acid bacteria associated to dairy fermentations possess enzymes which can be produced in situ during fermentation of dairy foods and have bioactive potential on the consumer. Examples are hydrolytic enzymes that may exert potential synergistic effects on digestion and alleviate symptoms of intestinal malabsorption (Patel et al., 2013). A well-known example is the β-galactosidase activity, which can achieve lactose degradation and thereby improve health and reduce symptoms of lactose intolerant consumers. Yogurt and other conventional starter cultures and probiotic bacteria in fermented and unfermented milk products improve lactose digestion and alleviate symptoms of intolerance in lactose malabsorbers. These beneficial effects are due to microbial β-galactosidase (de Vrese et al., 2001).

CLA

CLA is a polyunsaturated fatty acid (PUFA) that can be biosynthesized by LAB and bifidobacteria through bioconversion of linoleic acid (LA; *cis*-9,*cis*-12 $C_{18:2}$). The two isomers that have been shown to have bioactive potential are *cis*-9,*trans*-11 and *trans*-10,*cis*-12. The health-promoting properties of CLA include anticarcinogenic, antiatherogenic, anti-inflammatory, and antidiabetic activity, as well as the ability to reduce body fat (Sosa-Castañeda et al., 2015). Although it is a native component of milk, the amount consumed in foods is far from that required in order to obtain desired beneficial effects. Thus, increasing the CLA content in dairy foods through milk fermentation with specific LAB offers a promising alternative. An effective way to increase CLA uptake in humans is to increase CLA levels in dairy products by using strains with high production potential (Lee et al., 2007). A number of food-grade LAB and bifidobacteria were reported to produce CLA in milk products (Sosa-Castañeda et al., 2015; Yang et al., 2015), as is the case of *Lactococcus lactis* LMG, *L. rhamnosus* C14, *L. casei* CRL431, *L. acidophilus* Lac1, *L. plantarum*-2, *B. bifidum* CRL1399 and *B. animalis* Bb12 (Florence et al., 2009; Van Nieuwenhove et al., 2007b). Some of these strains were also used as adjunct cultures for the manufacture of high CLA-content buffalo cheese (Van Nieuwenhove et al., 2007a). The CLA-producing starter culture of *Lactococcus lactis* CI4b enhanced levels of total CLA in Cheddar cheese (Mohan et al., 2013). Similarly, *L. bulgaricus* LB430 and *S. thermophilus* TA040 are suitable for production of CLA-enriched yogurt (Florence et al., 2009).

In addition, it has been shown that specific microorganisms such as *L. plantarum* PL60 or *B. breve* NCIMB702258, are able to produce CLA following dietary administration in animal models (Wall et al., 2012) and following the administration as a freeze-dried product in humans (Lee and Lee, 2009). Thus, intestinal CLA production by bacteria may contribute to enhance CLA supply in addition to the CLA provided by the producing strain in fermented milks during the manufacture (Terán et al., 2015).

Exopolysaccharides

Exopolysaccharides (EPS) are complex extracellular carbohydrate polymers that can be produced by some LAB *in situ* during dairy fermentations. Some of them promote selective growth of bifidobacteria, thus playing a role on the host microbiota and immune system (Fernández et al., 2015; Salazar et al., 2016). In this regard, EPS derived from yogurt fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 exerted immunestimulatory effects in mice (Makino et al., 2016). Yogurt, Swiss-type, and Cheddar cheeses represent suitable food matrices for the delivery of the hypocholesterolemic EPS-producer strain *L. mucosae* DPC 6426 (Ryan et al., 2015). Other microorganisms with potential to produce EPS in cheese are *P. freudenreichii* KG15/KG6, *Lactococcus lactis* SMQ-461 or *S. thermophilus* MR-1C (Dabour et al., 2005; Darilmaz and Gumustekin, 2012). Significant levels of EPS can also be produced in kefir by *L. plantarum* YW11 (Wang et al., 2015). Recently, EPS produced by bifidobacteria have attracted the attention due to their immune modulation capability (Hidalgo-Cantabrana et al., 2012).

Exopolysaccharides can also improve the quality, sensory and rheological properties of the final food product, which in many cases results in a reduction of the amount of chemical stabilizers required, thus favoring a more natural product. For example, strains

of *B. longum* subsp. *infantis* CCUG 52486 and *S. thermophilus* were suitable to produce yogurt and fermented ice-cream with improved viscosity and texture and reduced syneresis as a consequence of their high EPS production (Dertli et al., 2016; Han et al., 2016; Prasanna et al., 2013).

Regulatory aspects

At present, the status of probiotic-based products is full of ambiguities because various regulatory agencies in different countries are defining and categorizing probiotics differently. Despite the emerging interest of consumers toward probiotics and functional foods, in Europe the regulatory framework is still not harmonized and no health claim for probiotics alone (except yogurt starters) has been approved. Paradoxically, probiotics or bioactive bacteria have been introduced into the market as dietary supplements or natural health products (capsules, tablets, and powders) (Arora and Baldi, 2015). Japan was the very first global jurisdiction for implementing a regulatory system for functional foods and nutraceuticals in 1991, and is currently acting as global market leader where probiotics are available as both foods and drugs. The government has designated Foods for Specific Health Uses (FOSHU), which classifies health claims into different subcategories (gastrointestinal health, cholesterol moderation, hypertension moderation, lipid metabolism moderation, sugar absorption moderation, mineral absorption, and bone and tooth health). In China, the State Food and Drug Administration (SFDA) has regulated all health foods including functional foods and nutraceuticals, and a well-developed market for functional foods is established (Arora and Baldi, 2015). Currently USA is regulating probiotics as a variety of products as per their intended usage and regulatory bodies are Dietary Supplement Health and Education Act (DSHEA) and Food and Drug administration (FDA). Dietary supplements are considered as 'foods' and are regulated by DSHEA and do not need FDA approval before being marketed. However, probiotics and dietary supplements containing a new dietary ingredient without a marketing history are regulated by FDA. In conclusion, a harmonized categorization of probiotics and functional foods may help to regulate these products whenever solid clinical documentation is available to support any health effects and health messages in human subjects. The appropriate level of evidence for determining a health benefit for probiotics should always be put ahead of commercial and labeling industrial interests.

Challenges in industry and concluding remarks

A goal of the dairy industry is to develop novel dairy products with increased nutritional and/or health promoting properties. Food-grade bacteria have the potential to fortify fermented dairy food products with bioactive metabolites by a natural process, thereby reducing the need for fortification with costly chemically synthesized supplements. Nowadays, a number of commercial sources have available synthetic formulations of bioactive substances for their use as a dietary supplement. The use of health-supporting bacteria for naturally enriching dairy foods with bioactives could be a suitable alternative to food fortification with chemical formulations.

The starter cultures must be carefully selected, since the ability of microbial cultures to produce bioactive metabolites is generally a strain-dependent trait and varies considerably among strains within the same species. The yield of bioactive synthesis and

the concentration of such compound in dairy products is another critical strain-dependent factor. In this regard, the dose of bioactives ingested with the corresponding food product should remain over the minimum required to meet the human requirements and/or have the claimed therapeutic level on the consumer, according to existing clinical recommendations and studies. An open question when using co-cultures or strain combinations is their interaction in terms of nutrient availability, bacterial growth, as well as the bioactive production yield. In some cases, metabolites (i.e., vitamins etc.) produced by one of the strains could be consumed by the other strains, thus decreasing the final content in food.

Generally, the biosynthetic pathways are genetically encoded. In this regard, the increasing availability of bacterial genome sequences over the last decade has provided a major contribution to the knowledge about microbial production of bioactive molecules. However, the presence of the genes required for the biosynthesis of a particular biomolecule should not be assumed as synonym of metabolite production. Typical exceptions to the correlation genotype-phenotype occur when the genes are not active or when the metabolite is intracellularly biosynthesized and a release system is lacking. This is indeed one of the major bottlenecks during biosynthesis of some vitamins that needs to be overcome through the use of alternative strategies such as autolytic mutants and metabolic engineering (Basavanna and Prapulla, 2013).

Consideration should also be given by manufacturers to the optimum conditions for bioactive compound biosynthesis by LAB during technological processes. The content and activity of a bioactive compound in the dairy fermented foodstuffs is the result of the type of food matrix, the individual bacterial strain properties as well as the processing conditions and storage.

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The references used in this publication are included in the reference section of the introduction (section 1.4).

1.4. Referencias

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JUSTIFICACIÓN Y OBJETIVOS

2. JUSTIFICACIÓN Y OBJETIVOS

Los ácidos grasos saturados del queso han dado lugar a una percepción negativa de este alimento por parte de los consumidores. Sin embargo, se ha observado que el queso puede aportar compuestos bioactivos con efectos beneficiosos sobre la salud humana como el CLA. Actualmente se observan cambios en los hábitos alimentarios del consumidor que se traducen en una demanda creciente de alimentos que mejoren la salud. En relación con esto, el estudio de los factores que puedan generar un incremento de la concentración de CLA en el queso podría tener gran interés en el posible desarrollo de alimentos funcionales.

La leche y productos lácteos, entre ellos el queso, son las principales fuentes de CLA en la alimentación. En este sentido, se ha observado que la grasa de la leche de oveja presenta una mayor concentración de CLA que la de vaca o la de cabra. En esta última década, el CLA ha despertado un gran interés por sus propiedades anticarcinogénicas, antitrombogénicas, antiarterioescleróticas y por su efecto en la reducción de la grasa corporal. Un aspecto a resaltar es que se ha detectado que la concentración de CLA en el queso es la misma que la de la leche empleada como materia prima. Por este motivo, la mejora del perfil lipídico de los productos lácteos ha de comenzar desde la producción de la materia prima, es decir, de la leche. Distintos estudios han puesto de manifiesto la gran importancia de la dieta de los animales rumiantes en el contenido en CLA presente en la grasa láctea. La mayoría de estos trabajos se han llevado a cabo en rebaños de ovino experimentales. Sin embargo, la información sobre el efecto de la alimentación y de otros factores asociados al sistema de manejo empleado en granjas comerciales de ovino sobre el perfil de ácidos grasos de la leche de tanque de oveja es muy escasa. En consecuencia, el control de los factores que conlleven a la obtención de leche de oveja con un mejor perfil nutricional podría permitir a los ganaderos e industria láctea obtener una materia prima con mayor valor añadido sin que suponga un incremento significativo en sus costes de producción.

Por otro lado, la concentración de CLA en queso también puede ser incrementada durante el proceso de elaboración del mismo mediante el empleo de bacterias ácido lácticas con capacidad para producir CLA a partir del ácido linoleico presente en la leche. Desde hace mucho tiempo, las bacterias ácido lácticas juegan un papel esencial en el proceso de fermentación que tiene lugar en la elaboración de algunos alimentos, en especial del queso y han sido generalmente reconocidas como seguras (Generally Recognized As Safe; GRAS) en la elaboración de productos fermentados. En este propósito, las bacterias ácido lácticas contribuyen a la acidificación de la leche y durante la maduración de los quesos ejercen actividades proteolíticas y lipolíticas que contribuyen a la textura, aroma y sabor del queso. Como consecuencia de esto, la selección de cepas de bacterias lácticas para el diseño de nuevos cultivos iniciadores que presenten buenas propiedades tecnológicas y capacidad para sintetizar compuestos beneficiosos para la salud puede dar lugar a la mejora del proceso de fermentación así como a un producto final de mejor calidad.

En la actualidad, la pasterización de la leche para elaborar queso permite asegurar la calidad microbiológica de los mismos. Sin embargo, este hecho hace necesario el empleo de cultivos iniciadores y adjuntos comerciales, que si bien generan un mayor grado de control sobre el proceso de fermentación y estandarización del producto final, también dan lugar a quesos con menor personalidad, los cuales son percibidos por el consumidor como "aburridos". Así mismo, algunas características metabólicas importantes en las bacterias lácticas están codificadas por plásmidos, existiendo el riesgo de que se pierdan debido a la adaptación a la matriz alimentaria. En la industria quesera, este hecho ha conducido a una pérdida de la singularidad de cada variedad de queso. Actualmente, para poder solucionar este problema, se están aislando cepas autóctonas de quesos tradicionales que puedan permitir la producción de queso sin que se pierdan las características sensoriales que los caracterizan. Por consiguiente, el diseño de cultivos compuestos por bacterias lácticas autóctonas con capacidad para sintetizar CLA y con buenas propiedades tecnológicas para elaborar queso permitiría obtener un producto con propiedades sensoriales atractivas y con una composición nutricional mejorada.

Durante el proceso de maduración del queso, como se ha mencionado anteriormente, las bacterias ácido lácticas juegan un papel importante en la proteólisis debido a que estas bacterias contienen proteinasas y peptidasas que dan lugar a la producción de aminoácidos libres, los cuales pueden servir de sustrato en reacciones catabólicas secundarias, también por acción de las bacterias ácido lácticas, generándose compuestos tales como el GABA o la ornitina con efectos fisiológicos positivos sobre la salud humana. Sin embargo, también hay que tener en cuenta que ciertas bacterias ácido lácticas están implicadas en reacciones de descarboxilación que dan lugar a la síntesis de compuestos tóxicos como las aminas biógenas. En consecuencia, el consumo de alimentos con elevada concentración de aminas biógenas puede provocar efectos toxicológicos y estos problemas pueden ser más importantes en consumidores cuyo sistema de detoxificación es menos eficiente por causas genéticas o por tratamientos farmacológicos. Por este motivo, cuando se diseñan nuevos cultivos para elaborar queso es necesario asegurar que las bacterias que forman parte de estos cultivos no generan altas concentraciones de aminas biógenas en el producto final.

Los cultivos son uno de los principales responsables de las características físico-químicas que presenta cada variedad de queso ya que como se ha mencionado anteriormente juegan un papel muy importante tanto en la acidificación de la leche como durante la maduración de los quesos. Como resultado, esta actividad microbiana afecta directa e indirectamente a la microestructura del queso y en consecuencia, también a las características sensoriales del mismo.

En base a lo anteriormente descrito, el objetivo general de esta Tesis Doctoral fue estudiar la repercusión que tuvo la selección de leche de tanque con alto contenido en CLA así como el empleo de cultivos iniciadores y adjuntos capaces de sintetizar CLA y otros compuestos con efectos beneficiosos sobre la salud humana como el GABA sobre el perfil nutricional del queso de oveja.

Para lograr tal fin, los objetivos específicos planteados en el desarrollo de esta Tesis fueron los siguientes:

1. Investigar el efecto de la alimentación, de la etapa de lactación, del mes de muestreo y del rebaño en el perfil de ácidos grasos, con especial énfasis en el CLA, de la leche de tanque de granjas de ovino comerciales.

2. Identificar y estudiar la capacidad de diferentes cepas de bacterias ácido lácticas aisladas de quesos artesanales para producir CLA y GABA.

3. Diseñar varios cultivos iniciadores y/o adjuntos constituidos por combinaciones de dichas cepas de bacterias ácido lácticas con capacidad para sintetizar CLA.

4. Elaborar quesos a partir de leche de oveja con alto contenido en CLA y empleando los diferentes cultivos diseñados con el fin de conocer la evolución de los parámetros físico-químicos y de los principales grupos microbianos de los quesos a lo largo de la maduración.

5. Estudiar el efecto de los cultivos diseñados y del tiempo de maduración sobre el perfil de ácidos grasos del queso de oveja.

6. Estudiar el efecto de los diferentes cultivos diseñados sobre el perfil de aminoácidos libres, con especial énfasis en el GABA y la ornitina, sobre la microestructura y sobre el contenido en aminas biógenas de los quesos de oveja durante la maduración de los mismos.

7. Evaluar las características sensoriales de los quesos mediante análisis instrumental y empleando un panel de catadores.

RESULTADOS

CAPÍTULO 1

Effect of feeding regimen on the fatty acid profile of sheep bulk tank milk

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Abstract

The quality of dairy products is affected by the fatty acid (FA) profile of the milk. The aim of this study was to determine whether the feeding regimen and lactation stage of sheep produced a healthier FA profile of milk. The study was carried out on 30 commercial farms, and the feeding regimens studied were different grazing allowances (50%, G1; 25%, G2; 0%, G3). The variance explained by the lactation stage for the FAs was below 4.18%. The milk from the grazing allowance G1 (50% grazing time per day plus alfalfa silage and barley grains) showed a higher conjugated linoleic acid (1.16%) proportion, followed by the grazing allowances G2 (0.90%) and G3 (0.79%), which showed that grazing improved the nutritional quality of milk fat.

INTRODUCTION

Fatty acids (FAs) in milk and cheese are receiving increasing research attention due to the relationship between consumption of saturated FAs and the risk of developing coronary heart disease (Elwood *et al.* 2010). However, the fat in milk and dairy products also contains compounds that are beneficial for consumer health, such as vaccenic acid, oleic acid, conjugated linoleic acid (CLA) and *omega*-3 polyunsaturated FAs (PUFAs) (Zlatanos *et al.* 2002; Field *et al.* 2009; Sofi *et al.* 2010; Sales-Campos *et al.* 2013; Yang *et al.* 2016). Milk and dairy products are our main natural dietary source of CLA, and it has been observed that the fat in sheep milk has a higher CLA content than that of cow or goat milk (Park *et al.* 2007). Most of the production of sheep milk is used to manufacture cheese. Nudda *et al.* (2005) detected that the CLA content from unprocessed raw milk can be recovered in cheese. Therefore, the production of cheese from sheep milk with increased mono- and polyunsaturated FAs content, especially CLA and *omega*-3, warrants for high nutritional properties of such dairy products (Zlatanos *et al.* 2002; Sofi *et al.* 2010; Koba and Yanagita 2014).

Over the past decade, numerous studies have been conducted on ruminant diet as a means to improve the milk FA profile because ruminant feeding is considered to be the major factor affecting the quality of sheep milk fat and, therefore, of sheep cheese (Stanton *et al.* 2003; Hervás *et al.* 2008; Shingfield *et al.* 2008; Kalac and Samková 2010). Tsiplakaou *et al.* (2008) stated that increasing the supply of grass and thus *omega*-3 PUFAs in the livestock diet represents one of the strategies for enhancing the content of these FAs and CLA in sheep milk. Cabiddu *et al.* (2005) have shown that season is another factor possibly affecting the FA profile of sheep milk because the phenological stage of the botanical species that are part of the pastures differs depending on the time of year. These authors observed that flocks grazing on spring pastures (rich in α-linolenic acid) produced milk with higher CLA and *omega*-3 content compared to the milk obtained from the same flocks when grazed on summer pastures. Some authors have stated that the effect of other factors such as flock, lactation stage or breed on the milk FA profile is not significant compared to the feeding regimen or season effects (Tsiplakou *et al.* 2008; De la Fuente *et al.* 2009). However, little information is available about the effects of the lactation stage and the feeding regimen on sheep commercial farms. This study could thus provide the dairy industry, including farmers and advisors, new knowledge about usual farming practices that could lead to an improvement in sheep milk nutritional quality.

The aim of this study was therefore to investigate the effects of the feeding regimen and lactation stage on the FA profile of sheep bulk tank milk from commercial farms.

MATERIALS AND METHODS

Experimental design

This study was conducted on the sheep bulk tank milk records of 30 commercial farms between April and July 2016. It took place in the prefecture of Zamora in Castilla and León and more specifically in the regions of Villalpando and Villamayor de Campos. This overall region covers a total of 153 km^2, and it is characterized by its cereal fields' predominance at an altitude between 685 and 695 m above sea level and for its warm-

summer Mediterranean climate (Kottek *et al.* 2006). In this geographical area, the parity of sheep takes place during winter and spring, and three feeding regimens coexist.

The farms studied in this work were grouped according to their feeding regimens, and the sheep breeds studied were: Assaf, Awassi and Castellana. The first group (G1; n=10) consisted of sheep flocks with a regimen of 50% grazing time per day plus alfalfa silage (200 g of dry matter/animal and day) and barley grains (250 g of dry matter/animal and day) addition during the experimental period. In the second group (G2; n=10) of flocks, the feeding regimen was 25% grazing time per day with alfalfa silage (600 g of dry matter/animal and day) and hay (600 g of dry matter/animal and day) addition. The third group (G3; n=10) consisted of sheep flocks managed under an intensive system (0% grazing time per day) during the experimental period, fed with alfalfa silage (600 g of dry matter/animal and day), hay (500 g of dry matter/animal and day) and commercial concentrate mix (2 kg of dry matter/animal and day; granulated feed, corn grain, granulated dehydrated alfalfa, cotton seed, beet pulp, treacle).

The lactation stages considered were as follows: 20-60 days postpartum (initial), 60-110 days postpartum (middle) and 110-160 days postpartum (final). Births were grouped in each farm. Therefore, most of the sheep in each farm were in the same lactation stage.

Population and milk sampling

The farms were located in Villalpando (41°51'53"N, 5°24'47"W) and Villamayor de Campos (41°53'56"N, 5°21'33"W). The 30 farms belonged to the Consortium for Ovine Promotion (Zamora, Castilla and León, Spain), which is the largest cooperative in the sheep milk sector in Spain. The size of each flock was between 275 and 975 sheep. All the sheep were milked twice a day during the milking period.

Bulk tank milk samples (100 mL) were taken on the same day in April (n=30), May (n=30), June (n=30) and July (n=30). Sampling was carried out during these months as they represent the most important grazing period. A total of 120 bulk tank sheep milk samples were analysed. They were preserved with bronopol (0.05%) and stored at 4°C until laboratory analysis within 96 h of collection (De La Fuente *et al.* 2009). Analytical determination of 32 FAs per sample was carried out in the Department of Food Hygiene and Technology at University of León (León, Spain).

Physicochemical and microbiological composition of milk

All milk samples were analysed for total solids, fat, protein, lactose and somatic cells by Milkoscan FT2(Foss Electric, Hillerod, Denmark), and for aerobic mesophilic bacteria counts by Bactoscan FC (Foss Electric).

Quantification of FAs in sheep milk

Lipids were extracted from the milk samples using the method described by Bligh and Dyer (1959), and FA methyl esters were prepared by base-catalysed methanolysis of glycerides ($NaOCH_3$), following the method described by Aldai *et al.* (2005).

A Hewlett Packard 6890 Series Gases Chromatography System (Hewlett Packard, Wilmington, DE, USA) equipped with a Hewlett Packard 7683 Series Injector (Hewlett Packard) and a Hewlett Packard 5973 Mass Selective Detector (Hewlett Packard) was used. FAs separation was carried out using a Tekno TR-CN 100 capillary column (0.2 μm,

particle size, 60 m × 0.25 mm I.D.; Teknokroma, Barcelona, Spain). Helium was used as a carrier gas at a flow rate of 1 mL/min. The injection and detector temperatures were 230ºC. The temperature program was as follows: the initial temperature was held at 50ºC for 1 min after injection, then programmed to increase at 15ºC/min to 200ºC, held there for 3 min, and then programmed to increase at 2ºC/min to 200ºC, and held there for 5 min. Samples (1 μL) were injected by split injection (split ratio 10:1).

Each peak was identified and quantified using a 37-component FAME mix standard (Supelco, Sigma-Adrich Co., Saint Louis, USA). CLA, in particular, was identified using a 50:50 mixture from individual standard solutions of the two CLA isomers of interest in this study (*cis*-9,*trans*-11 $C_{18:2}$; *trans*-10,*cis*-12 $C_{18:2}$) (Larodan Fine Chemicals AB, Malmö, Sweden). A nonanoic ($C_{9:0}$) FAME (Sigma-Adrich Co.) was added to the methylated milk fat samples prior to GC analysis and was used as an internal standard for chromatographic analysis. The individual FA proportion in milk samples was expressed as g/100 g of total FAs (g/100 g total FAs).

Studied FAs

Although 32 FAs were studied initially, statistical analysis was primarily restricted to the 12 most important ones ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ *cis*-9,*cis*-12, $C_{18:3}$ *cis*-9,*cis*-12,*cis*-15, $C_{18:2}$ *cis*-9,*trans*-11 (CLA), $C_{18:2}$ *trans*-10,*cis*-12 (CLA), $C_{20:4}$ *cis*-5,*cis*-8,*cis*-11,*cis*-14) from a quantitative and/or biofunctional point of view (De La Fuente *et al.* 2009), and to seven FA groups and five FA indexes, which were all treated as dependent variables. The FA groups based on saturation level and chain length were as follows: the sum of short-chain saturated FAs (C_4 to C_{10}, SCFA); the sum of medium-chain saturated FAs (C_{11} to C_{15}, MCFA); and the sum of long-chain saturated FAs (C_{16} to C_{24}, LCFA). The four remaining groups were as follows: the sum of monounsaturated FAs (MUFA); the sum of PUFA; the sum of *omega*-6 FAs and the sum of *omega*-3 FAs. The five indexes considered were as follows: the unsaturated FAs (UFA)/saturated FAs (SFA) ratio; the *omega*-6/*omega*-3 ratio; the $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratio; the atherogenicity index (AI) defined as $[(C_{12:0} + 4\times C_{14:0} + C_{16:0})/(\Sigma \text{ unsaturated FA})]$ (Ulbricht and Southgate 1991); and the desaturase index (DI) calculated as $C_{14:1}$ *cis*-9/$C_{14:0}$ ratio (Renna *et al.* 2012).

Statistical analysis

Statistical analysis was carried out using the MIXED procedure in SAS (SAS Institute, Inc., Cary, NC), following the mathematical model below:

$$Y_{ijklm} = \mu + R_i + F_{j(i)} + M_k + L_l + (R \times M)_{ik} + e_{ijklm},$$

where Y_{ijklm} refers to the 29 dependent variables; namely six physicochemical and microbiological parameters, 12 FAs, seven groups of FAs and five FA indexes, and μ is the overall mean. The R_i factor refers to the fixed effect of feeding regimen; there were three levels: G1, G2, G3. The $F_{j(i)}$ factor is the fixed effect of flock within feeding regimen; there were 30 levels. M_k refers to the fixed effect of sampling month; there were four levels: April, May, June and July. L_l is the fixed effect of lactation stage; there were three levels: initial, middle and final. $(R \times M)_{ik}$ refers to the effect of interaction between feeding regimen and sampling month, and e_{ijklm} is the residual effect. Other interactions were not statically significant ($P > 0.05$) and consequently removed from the statistical model.

The MIXED procedure was used to study the statistical significance of the variation factors, and the least squares means and contrasts of differences between means were estimated. Following the indicated model, but considering all factors as random, the VARCOMP procedure was used to estimate the percentage of variance explained by each fixed effect (sampling month, flock, lactation stage and feeding regimen) for the 12 FAs, seven groups of FAs and five FA indexes.

Additionally, in order to analyse the flock effect, hierarchical clustering was performed using the Ward method (Euclidean distance squared) using SPSS v.21 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Physicochemical and microbiological composition of the milk

The physicochemical and microbiological composition of milk determines its quality and suitability for use in the manufacture of dairy products. The physicochemical and microbiological parameters studied in the bulk tank milk samples are shown in **Table 1**.

The average concentrations of fat, protein, lactose and total solids in all the milk samples analysed were within the range described by Mayer and Fieschter (2012). Significant differences were observed ($P \leq 0.001$) in the fat and protein concentrations in milk from the three groups studied. The G1 grazing group presented the highest average concentration of fat (73.96 g/kg milk), followed by the G2 (68.23 g/kg milk) and the G3 (66.02 g/kg milk). Similarly, the G1 grazing group presented the highest average concentration of protein (57.05 g/kg milk), followed by the G2 (51.52 g/kg milk) and G3 (51.83 g/kg milk), with no significant differences between the latter ($P > 0.05$). It has been observed that in sheep, management systems focusing primarily on milk yield lead to lower concentrations of fat and protein in milk than in less intensive systems (Morand-Fehr *et al.* 2007).

Table 1. Least squares means and standard errors for the physicochemical and microbiological parameters by feeding regimen in sheep bulk tank milk.

Parameter[x]	Feeding regimen[+]			SE	*P*-value
	G1	G2	G3		
Fat	73.96[a]	68.23[b]	66.02[b]	0.07	***
Protein	57.05[a]	51.52[b]	51.83[b]	0.03	***
Lactose	47.06	47.90	48.32	0.01	***
Total solids	186.57[a]	176.66[b]	174.35[b]	0.08	***
Somatic cell counts	3.64	3.62	3.63	0.08	NS
Mesophilic aerobic microbiota	4.87	4.98	4.90	0.03	NS

[a-b]Same row with different superscripts differ ($P \leq 0.05$).
[x]Fat, protein, lactose and total solids expressed as g/kg milk.
Somatic cell counts expressed as cells/g ($\times 10^5$).
Mesophilic aerobic microbiota expressed as $\log_{10}$ cfu/g.
[+]Diet G1 consisting of 50% grazing + alfalfa silage (200 g of dry matter/animal and day) + barley grains (250 g of dry matter/animal and day); Diet G2 consisting of 25% grazing + alfalfa silage (600 g of dry matter/animal and day) + hay (600 g of dry matter/animal and day); Diet G3 consisting of alfalfa silage (600 g of dry matter/animal and day) + hay (500 g of dry matter/animal and day) + commercial concentrate mix (2 kg of dry matter/animal and day; granulated feed, corn grain, granulated dehydrated alfalfa, cotton seed, beet pulp, treacle).
[NS] $P > 0.05$; ***$P \leq 0.001$.

At present, there are attempts to use the somatic cell count (SCC) as the criterion for establishing milk prices, but there is currently no legislation defining the limits of SCC,

as this depends on a variety of factors (Vivar-Quintana *et al.* 2006). The average concentration of SCC in milk samples was 3.63×10^5 cells/g and the average concentration for the mesophilic aerobic microbiota counts was 4.90 $\log_{10}$ cfu/g. In accordance with the Ministerial Order of 27 June 1985, the samples could be classified as class 2, as established by this legislation, since they presented < 6 $\log_{10}$ cfu/g.

Descriptive statistics

Table 2 shows the average proportions of the 32 FAs and seven groups of FAs for the 120 bulk tank sheep milk samples studied. The variation coefficients were between 52.54 and 7.46%; the average proportions of SFA (66.65%), MUFA (26.57%) and PUFA (6.78%) were different to those described in sheep milk by De La Fuente *et al.* (2009). These authors reported finding 71.35% SFA, 22.10% MUFA and 6.54% PUFA, obtaining a lower UFA/SFA ratio (0.40) than in the present study (0.50).

In the present study, the average proportion of α-linolenic acid in the sheep milk samples was 0.99 g/100g total FAs, coinciding with the values obtained by Zhang *et al.* (2006). However, Pellattiero *et al.* (2015) found a lower α-linolenic acid content (0.30 g/100 g total FAs) in their study of sheep milk samples.

In the bulk tank milk samples, there was a higher average proportion of CLA isomer $C_{18:2}$ *cis*-9,*trans*-11 (0.79 g/100 g total FAs) than of $C_{18:2}$ *trans*-10,*cis*-12 (0.12 g/100 g total FAs). Renobales *et al.* (2012) noted that the CLA isomer $C_{18:2}$ *cis*-9,*trans*-11 accounts for between 70 and 90% of the total CLA content in milk fat. The proportion of both isomers in the milk samples studied coincided with the range of values reported by Tsiplakou *et al.* (2006) and De La Fuente *et al.* (2009).

The percentage of variance explained by the sources of variation studied for the 12 FAs, seven groups of FAs and the five FA indexes selected is shown in **Table 3**.

Feeding regimen effect

The feeding regimen was the most important source of variation for $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratio and DI. This factor also explained a high percentage of variance for *omega*-6/*omega*-3 ratio, $C_{18:2}$ *cis*-9,*trans*-11 (CLA) and $C_{18:2}$ *trans*-10,*cis*-12 (CLA) proportions (**Table 3**).

Table 4 shows that significant differences were found ($P \leq 0.05$) between the three groups studied with respect to the proportions of $C_{18:2}$ *cis*-9,*cis*-12, $C_{18:3}$ *cis*-9,*cis*-12,*cis*-15, $C_{18:2}$ *cis*-9,*trans*-11 (CLA), $C_{18:2}$ *trans*-10,*cis*-12 (CLA), the *omega*-6/*omega*-3 ratio, AI, $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 and DI. However, no significant differences ($P > 0.05$) in proportion were found for most of the SFA and MUFA. However, bulk tank milk from the G1 presented the lowest proportion of $C_{18:2}$ *cis*-9,*cis*-12 but had the highest mean proportion of α-linolenic acid and total CLA, and the most beneficial values for the *omega*-6/*omega*-3 ratio, AI, $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratio and DI. The lower $C_{18:2}$ *cis*-9,*cis*-12 proportion and higher total CLA proportion presented by milk from G1 with respect to those from the other two groups studied may be due to greater bioconversion of $C_{18:2}$ *cis*-9,*cis*-12 in the rumen and mammary gland. These differences could be partially explained by the variation in Δ-9 desaturase activity (in the mammary gland) which can be calculated by means of specific FA indexes as indicated by Arnould and Soyeurt (2009).

Table 2. Descriptive statistics for composition of the 32 FAs, seven groups of FAs, and five FA indexes for 120 sheep bulk tank milk samples from the 30 flocks studied.

Variable*	Mean (g/100 g total FA)	Range		SD	CV (%)
		Minimum	Maximum		
C4:0	2.95	0.85	6.11	1.55	52.54
C6:0	4.98	3.15	6.90	0.63	12.65
C8:0	3.68	2.30	5.43	0.50	13.59
C10:0	5.58	3.93	6.97	0.68	12.19
C11:0	0.27	0.15	0.44	0.06	22.22
C12:0	4.20	2.92	5.46	0.53	12.62
C13:0	0.29	0.19	0.52	0.06	20.69
C14:0	8.38	6.93	9.90	0.62	7.39
C14:1 *c*-9	0.98	0.70	1.36	0.12	12.24
C15:0	1.51	1.10	2.10	0.19	12.58
C15:1 *c*-10	0.45	0.30	0.81	0.08	17.78
C16:0	18.78	15.83	25.63	1.29	6.87
C16:1 *c*-9	3.14	1.44	4.24	0.41	13.06
C17:0	1.23	0.79	1.92	0.18	14.63
C17:1 *c*-10	0.64	0.44	0.97	0.11	17.19
C18:0	13.50	9.14	20.41	2.18	16.15
C18:1 *c*-9 + *t*-11	21.15	13.23	29.10	1.38	7.63
C18:2 *t*-9,*t*-12	0.37	0.09	1.17	0.16	43.24
C18:2 *c*-9,*c*-12	3.65	2.52	5.52	0.57	15.62
C20:0	0.62	0.37	1.05	0.12	19.34
C18:3 *c*-9,*c*-12,*c*-15	0.99	0.44	1.94	0.29	29.29
C18:2 *c*- 9,*t*-11 (CLA)	0.78	0.32	2.04	0.25	32.05
C18:2 *t*- 10,*c*-12 (CLA)	0.12	0.04	0.35	0.04	33.33
C20:2 *c*-11,*c*-19	0.06	0.04	0.10	0.01	16.67
C22:0	0.23	0.16	0.37	0.04	17.39
C20:4 *c*-5,*c*-8,*c*-11,*c*-14	0.22	0.15	0.32	0.02	9.09

*CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.

Table 2 (continuation). Descriptive statistics for composition of the 32 FAs, seven groups of FAs, and five FA indexes for 120 sheep bulk tank milk samples from the 30 flocks studied.

Variable*	Mean (g/100 g total FA)	Range		SD	CV (%)
		Minimum	Maximum		
C23:0	0.25	0.18	0.39	0.04	16.00
C22:2 *c*-13,*c*-16	0.18	0.11	0.37	0.05	27.78
C20:5 *c*-5,*c*-8,*c*-11,*c*-14,*c*-17	0.12	0.01	0.22	0.04	33.33
C24:0	0.20	0.14	0.30	0.03	15.00
C24:1 *c*-15	0.22	0.13	0.38	0.04	18.18
C22:6 *c*-4,*c*-7,*c*-10,*c*-13,*c*-16,*c*-19	0.28	0.17	0.43	0.05	17.86
SCFA	17.20	10.89	21.88	2.03	11.80
MCFA	16.08	12.77	19.46	1.38	8.58
LCFA	66.72	61.45	75.16	2.65	3.97
MUFA	26.57	21.78	30.74	1.41	5.31
PUFA	6.78	5.00	8.87	0.76	11.21
Omega-6	3.94	2.75	5.92	0.59	14.97
Omega-3	1.39	0.74	2.55	0.34	24.46
UFA/SFA	0.50	0.40	0.60	0.04	7.46
Omega-6/*omega*-3	3.52	1.82	6.75	0.96	27.27
AI	1.70	1.30	2.15	0.16	9.41
C18:2 *c*- 9,*t*-11/ C18:2 *c*-9,*c*-12	0.22	0.10	0.77	0.08	36.36
DI	0.12	0.08	0.17	0.01	8.33

*CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.

The Δ-9 desaturase enzyme adds a double bond at position 9 and configuration *cis* to MUFA and PUFA with a carbon chain length from 10 to 18 atoms. The most reliable of the ratios used to determine Δ-9 desaturase enzyme activity in milk is the $C_{14:1}/C_{14:0}$ ratio. This is because the myristoleic acid present in milk is generated almost exclusively (over 95%) by endogenous synthesis from myristic acid, in contrast with the other MUFA, which can also come from the diet (Griinari *et al.* 2000; Renna *et al.* 2012). The results obtained in the present study confirm this, as G1 presented a higher value (0.13) for the $C_{14:1}/C_{14:0}$ index than G3 (0.10) or G2 (0.10). Lock and Garnsworthy (2003) observed that sheep fed under a grazing system showed an increase in Δ-9 desaturase activity in the mammary gland. This same trend in Δ-9 desaturase activity was observed by Renna *et al.* (2012) in their study of the FA profile in milk from goats fed with different levels of fresh forages. The CLA proportion observed by these authors and by Couvreur *et al.* (2006) in the study of the CLA proportion in dairy cow milk was higher than that found in our study. This fact can be justified by differences in the botanical composition of fresh pastures as well as that the flocks studied in this work were commercial and not experimental.

Table 3. Percentage of variance explained by each fixed effect for the 12 FAs, seven groups of FAs, and five FA indexes considered.

Variable*	Feeding regimen	Lactation stage	Flock	Sampling month
C8:0	0.00	4.18	17.65	38.75
C10:0	0.00	3.35	27.68	36.76
C12:0	0.00	3.81	28.97	33.15
C14:0	0.00	3.80	18.10	34.97
C16:0	1.03	2.85	13.22	36.75
C18:0	0.00	1.33	21.37	29.52
C18:1	0.00	2.20	7.10	43.12
C18:2 *c*-9,*c*-12	14.21	3.22	24.25	25.20
C18:3 *c*-9,*c*-12,*c*-15	18.36	1.94	24.96	23.35
C18:2 *c*-9,*t*-11 (CLA)	21.58	0.98	15.26	30.25
C18:2 *t*-10,*c*-12 (CLA)	22.46	1.86	8.70	25.50
C20:4 *c*-5,*c*-8,*c*-11,*c*-14	0.00	0.40	18.23	21.81
SCFA	0.00	4.01	18.29	31.38
MCFA	0.00	3.98	21.82	35.11
LCFA	0.00	2.27	8.35	38.60
MUFA	0.00	3.05	5.58	42.24
PUFA	11.26	3.27	4.08	42.43
Omega-6	6.75	3.64	19.45	34.29
Omega-3	17.22	3.20	18.80	32.40
UFA/SFA	0.00	1.84	45.68	8.50
Omega-6/*omega*-3	30.42	3.66	41.95	3.27
AI	12.03	0.05	47.36	4.85
C18:2 *c*-9,*t*-11/ C18:2 *c*-9,*c*-12	56.29	0.14	20.93	3.77
DI	58.75	0.10	19.72	2.34

*CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.

Milk form the G1 farms had a lower *omega*-6/*omega*-3 ratio than the G2 and the G3 farms which may be explained by high $C_{18:3}$ intakes from pasture herbage. However, the changes described in the milk FA profile are not only due to the supply of grass in the animal diet. Preserved forages and concentrate in the sheep diet also influence the FA milk

profile. As shown in **Table 4**, G3 presented the lowest concentration of CLA and $C_{18:3}$ *cis*-9,*cis*-12,*cis*-15 with respect to the other two groups. This is because it was the only one fed on forage and concentrate. As indicated by Chillard *et al.* (2007), this type of diet does not contribute to elevated CLA proportions in milk, as it does not provide sufficient amounts of CLA precursors. The G1 diet had a lower forage proportion than G2, but more grazing and higher CLA proportion. Therefore, the factors that may affect the proportions of these beneficial FAs in milk could be the level of precursor intake and the extent of biohydrogenation in the rumen. Incomplete biohydrogenation occurs when grazing yields more $C_{18:1}$ *trans*-11 that can be desaturated in the mammary gland. The rumen environment may be affected by diet, which may be translated into a change in the FA profile of sheep milk (Tsiplakou *et al.* 2006).

Table 4. Least squares means and standard errors for FA content (g/100 g total FA) by feeding regimen in commercial dairy sheep flocks.

Variable [x]	Feeding regimen[+]			SE	*P*-value
	Group 1 (n=40)	Group 2 (n=40)	Group 3 (n=40)		
C8:0	3.67	3.52	3.55	0.07	NS
C10:0	5.33	5.32	5.46	0.09	NS
C12:0	11.46	10.44	11.83	0.07	NS
C14:0	8.15	8.13	8.29	0.08	NS
C16:0	17.67[b]	18.21[ab]	18.65[a]	0.17	**
C18:0	13.93	14.59	14.63	0.23	NS
C18:1	20.94	21.60	21.62	0.21	NS
C18:2 *c*-9, *c*-12	3.03[c]	3.60[b]	4.00[a]	0.07	***
C18:3 *c*-9, *c*-12, *c*-15	1.25[a]	0.91[b]	0.95[b]	0.04	***
C18:2 *c*-9, *t*-11 (CLA)	0.99[a]	0.79[b]	0.71[c]	0.03	***
C18:2 *t*-10, *c*-12 (CLA)	0.17[a]	0.11[b]	0.08[c]	0.01	***
C20:4 *c*-5, *c*-8, *c*-11, *c*-14	0.22	0.23	0.22	0.00	NS
SCFA	17.04	17.11	17.10	0.14	NS
MCFA	15.46	15.43	15.44	0.19	NS
LCFA	67.50	67.46	67.46	0.31	NS
MUFA	26.89	26.65	26.84	0.25	NS
PUFA	6.92[a]	6.47[b]	6.99[a]	0.11	**
Omega-6	3.31[c]	3.38[b]	4.30[a]	0.07	***
Omega-3	1.74[a]	1.27[b]	1.26[b]	0.04	***
UFA/SFA	0.51	0.47	0.50	0.01	NS
Omega-6/*omega*-3	1.90[c]	2.66[b]	3.41[a]	0.09	***
AI	1.59[b]	1.84[a]	1.88[a]	0.02	***
C18:2 *c*-9, *t*-11/ C18:2 *c*-9, *c*-12	0.33[a]	0.22[b]	0.18[b]	0.01	***
DI	0.13[a]	0.10[b]	0.10[b]	0.09	***

[a-c] Same row with different superscripts differ ($P \leq 0.05$).

[x] CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.

[+]Diet G1 consisting of 50% grazing + alfalfa silage (200 g of dry matter/animal and day) + barley grains (250 g of dry matter/animal and day); Diet G2 consisting of 25% grazing + alfalfa silage (600 g of dry matter/animal and day) + hay (600 g of dry matter/animal and day); Diet G3 consisting of 0% grazing, alfalfa silage (600 g of dry matter/animal and day) + hay (500 g of dry matter/animal and day) + commercial concentrate mix (2 kg of dry matter/animal and day; granulated feed, corn grain, granulated dehydrated alfalfa, cotton seed, beet pulp, treacle).

[NS] $P > 0.05$; [**] $P \leq 0.01$; [***] $P \leq 0.001$.

Lactation stage effect

Lactation stage accounted for < 4.18% of variance in the 12 FAs, seven groups of FAs and five FA indexes and was therefore much less important as regards variation than the other factors (**Table 3**). This is in agreement with other authors (De La Fuente *et al.* 2009; Peterson *et al.* 2002). Lactation stage had a significant effect ($P \leq 0.05$) on the proportions of $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{18:0}$, PUFA, *omega*-6 and *omega*-3 (**Table 5**). Bulk tank milk obtained in the final lactation stage showed higher proportion of these FAs than bulk tank milk obtained in the initial lactation stage. As Nogalski *et al.* (2012) have indicated, the differences observed in the proportions of FAs between the initial lactation stage and the final lactation stage could be due to more intense fat reserve mobilisation in the early lactation stage.

Table 5. Least squares means and standard errors for FA content (g/100 g total FA) by lactation stage in commercial dairy sheep flocks.

Variable [x]	Lactation stage[+]			SE	*P*-value
	Initial (n=37)	Middle (n=48)	Final (n=35)		
C8:0	3.43	3.51	3.76	0.09	NS
C10:0	5.08^{b}	5.36^{ab}	5.51^{a}	0.08	*
C12:0	3.84^{b}	3.99^{b}	4.22^{a}	0.07	*
C14:0	8.24^{b}	8.26^{b}	8.52^{a}	0.08	*
C16:0	18.13	18.20	18.50	0.20	NS
C18:0	13.59^{b}	14.50^{a}	15.27^{a}	0.16	*
C18:1	21.08	21.48	21.61	0.21	NS
C18:2 *c*-9,*c*-12	3.51	3.56	3.58	0.09	NS
C18:3 *c*-9,*c*-12,*c*-15	0.87^{b}	0.95^{ab}	1.19^{a}	0.05	**
C18:2 *c*-9,*t*-11 (CLA)	0.87	0.85	0.85	0.01	NS
C18:2 *t*-10,*c*-12 (CLA)	0.13	0.12	0.12	0.01	NS
C20:4 *c*-5,*c*-8,*c*-11,*c*-14	0.22	0.22	0.22	0.00	NS
SCFA	17.29	17.12	17.09	0.26	NS
MCFA	15.21	15.38	15.37	0.20	NS
LCFA	67.50	67.50	67.54	0.36	NS
MUFA	26.67	26.90	26.95	0.30	NS
PUFA	6.53^{b}	6.76^{ab}	6.92^{a}	0.08	*
Omega-6	3.65^{b}	3.69^{b}	3.87^{a}	0.02	*
Omega-3	1.25^{b}	1.34^{ab}	1.65^{a}	0.06	*
UFA/SFA	0.50	0.50	0.51	0.02	NS
Omega-6/*omega*-3	2.92^{a}	2.75^{ab}	2.36^{b}	0.10	**
AI	1.65	1.64	1.67	0.02	NS
C18:2 *c*-9,*t*-11/ C18:2 *c*-9,*c*-12	0.25	0.24	0.24	0.01	NS
DI	0.10	0.10	0.10	0.11	NS

[a-c] Same row with different superscripts differ ($P \leq 0.05$).
[x] CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.
[+] Initial: 20-60 days postpartum; Middle: 60-110 days postpartum; Final: 110-160 days postpartum.
[NS] $P > 0.05$; [*] $P \leq 0.05$; [**] $P \leq 0.01$.

The proportions of CLA isomers and DI were not affected ($P > 0.05$) by the lactation stage. However, several recent studies have shown that in dairy cattle, the lactation stage effect on CLA proportion and DI was significant (Kelsey *et al.* 2003; Craninx *et al.* 2008; Bilal *et al.* 2014). This may be due to the fact that the management system in dairy cattle is different from dairy sheep and to the physiological differences between both species.

Sampling month effect

According to the percentage of variance analysis (**Table 3**), sampling month was the most important source of variation for the majority of individual FA, SFA, MUFA, PUFA, the two CLA isomers and α-linolenic acid, in agreement with the results reported by Cabiddu *et al.* (2005). This may be because the composition of the fresh herbage forming the groups' diet may have varied according to the month, and these variations have in turn been found to affect the FA profile of sheep milk (Cabiddu *et al.* 2005).

As can be seen in **Table 6**, the highest average proportions for the majority of the FAs studied in bulk tank milk were observed in April, except for $C_{14:0}$, $C_{16:0}$, $C_{20:4,}$ SFA and *omega*-6. Furthermore, samples collected in April and May yielded better UFA/SFA and $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratios and lower AI than samples collected in June and July. The values obtained for the AI were consistent with those reported by Chillard *et al.* (2003). The only variable for which no significant differences ($P > 0.05$) were found between sampling months was the DI.

Table 6. Least squares means and standard errors for FA content (g/100g total FA) in sheep milk from commercial dairy flocks by sampling month.

Variable[x]	Sampling month				SE	*P*-value[+]	
	April (n=30)	May (n=30)	June (n=30)	July (n=30)		M	M*R
C8:0	3.77[a]	3.57[b]	3.52[b]	3.45[c]	0.08	***	NS
C10:0	5.54[a]	5.43[a]	5.28[b]	5.16[b]	0.11	***	NS
C12:0	4.19[a]	4.13[a]	3.98[b]	3.81[c]	0.09	***	NS
C14:0	8.26[b]	7.93[c]	8.13[b]	8.45[a]	0.11	***	NS
C16:0	17.68[b]	18.05[ab]	18.64[ab]	18.80[a]	0.23	***	NS
C18:0	14.25[ab]	14.72[a]	14.52[ab]	14.03[b]	0.35	***	NS
C18:1	21.78[a]	21.44[ab]	21.28[ab]	21.03[b]	0.26	***	NS
C18:2 *c*-9,*c*-12	3.48[a]	3.36[b]	3.43[c]	3.47[c]	0.09	***	NS
C18:3 *c*-9,*c*-12,*c*-15	1.10[a]	1.07[ab]	0.94[ab]	0.92[b]	0.06	***	NS
C18:2 *c*-9,*t*-11 (CLA)	0.98[a]	0.83[b]	0.77[bc]	0.69[c]	0.07	***	**
C18:2 *t*-10,*c*-12 (CLA)	0.15[a]	0.12[b]	0.11[b]	0.11[b]	0.05	**	**
C20:4 *c*-5,*c*-8,*c*-11,*c*-14	0.21[b]	0.21[b]	0.23[a]	0.23[a]	0.01	**	NS
SCFA	16.17[c]	16.15[c]	16.70[b]	17.30[a]	0.28	***	NS
MCFA	16.09[a]	16.05[a]	15.59[b]	15.62[b]	0.35	**	NS
LCFA	67.74[ab]	67.80[a]	67.75[ab]	67.08[b]	0.33	**	NS
MUFA	27.18[a]	26.73[ab]	26.73[ab]	26.68[b]	0.21	***	NS
PUFA	7.17[a]	6.67[ab]	6.59[b]	6.41[b]	0.15	***	NS
Omega-6	3.75[b]	3.63[c]	3.76[b]	3.94[a]	0.09	***	NS
Omega-3	1.56[a]	1.46[b]	1.35[c]	1.31[c]	0.06	***	NS
UFA / SFA	0.51[a]	0.49[ab]	0.48[b]	0.48[b]	0.03	**	NS
Omega-6 / *omega*-3	2.40[b]	2.49[b]	2.79[a]	3.01[a]	0.12	**	NS
AI	1.60[b]	1.61[b]	1.65[ab]	1.70[a]	0.02	***	NS
C18:2 *c*-9,*t*-11/ C18:2 *c*-9,*c*-12	0.28[a]	0.25[ab]	0.22[bc]	0.20[c]	0.01	***	**
DI	0.11	0.11	0.11	0.10	0.01	NS	NS

[a-c] Same row with different superscripts differ ($P \leq 0.05$).
[x] CLA, conjugated linoleic acid; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; DI, desaturase index.
[+]M: sampling month fixed effect; M*R: interaction effect between sampling month and feeding regimen.
[NS] $P > 0.05$; [**] $P \leq 0.01$; [***] $P \leq 0.001$.

Notably, the highest average proportion of total CLA was obtained in April (1.13 g/100g total FAs), followed by May (0.95 g/100 g total FAs), June (0.88 g/100 g total FAs)

and July (0.80 g/100 g total FAs). Our results are consistent with those reported by Tsiplakou *et al.* (2006), who found that the highest values for total CLA in sheep milk were obtained in April and May, whereas the proportion was lowest in January, March and June. The CLA values detected by these authors were higher than the CLA concentration observed in the present study. However, as in our study, they found higher levels of CLA in spring, when fresh herbage is rich in α-linolenic acid (Tsiplakou *et al.* 2008). The proportion of this FA steadily decreases as the herbage matures and develops higher fibre proportion (Dewhurst *et al.* 2001). Accordingly, intake of mature herbage deficient in α-linolenic acid leads to a reduction in the levels of vaccenic acid, an intermediate generated during biohydrogenation in the rumen. This in turn results in a reduction in CLA synthesised from vaccenic acid in the mammary gland (Nudda *et al.* 2005). In the present study, it can be observed that the interaction effect between the sampling month and the feeding regimen was significant ($P \leq 0.01$) for the proportions of CLA and for the $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratio (**Table 6**). The importance of the grazing time on the proportions of CLA in bulk tank milk can be observed because the milk collected from the G1 and G2 dietary groups in April showed higher proportions of CLA (1.15 and 0.91 g/100 g total FAs, respectively) and $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 (0.41 and 0.22, respectively) than the milk collected from the same groups in July which presented 0.82 and 0.65 g CLA/100g of total FA for G1 and G2, respectively, and values of 0.25 (G1) and 0.17 (G2) for the $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 ratio. However, there were not significant differences ($P > 0.05$) between the values for the proportions of CLA (0.79 g/100 g total FAs) and $C_{18:2}$ *cis*-9,*trans*-11/$C_{18:2}$ *cis*-9,*cis*-12 (0.19) observed in the milk from G3 group collected in April, May, June or July.

Flock effect

Table 3 shows that flock was the main source of variation for $C_{18:3}$ *cis*-9,*cis*-12,*cis*-15, the UFA/SFA ratio, the *omega*-6/*omega*-3 ratio and AI. Similarly, flock explained a large percentage of variance for SCFA and the CLA isomer *cis*-9,*trans*-11, in agreement with results obtained by Stoop *et al.* (2008), who found that flock explained a high percentage of variance for 16 FAs, including the CLA isomer *cis*-9,*trans*-11.

As shown in **Figure 1**, there were considerable differences among flocks with regard to FA proportion. In the statistical analysis of flocks, taking into account the average values of the milk FA profile throughout the experimental period, two clusters were formed. This could be because each flock belonged to different farm and animal population. Cluster 1 was formed by the flocks belonging to the dietary group G3 and by some flocks of the G2 group. However, cluster 2 was made up of flocks belonging to the G1 and some flocks of the G2 groups (**Figure 1**). This last cluster showed higher average proportions of CLA and UFA/SFA ratio than cluster 1, whereas this presented higher average proportions for the $C_{18:3}$ *cis*-9,*cis*-12,*cis*-15 and SCFA than cluster 2. Likewise, cluster 2 showed the lowest average values for the *omega*-6/*omega*-3 ratio and AI in comparison with cluster 1. These results were similar to those previously observed when the effect of the feeding regimen was analyzed.

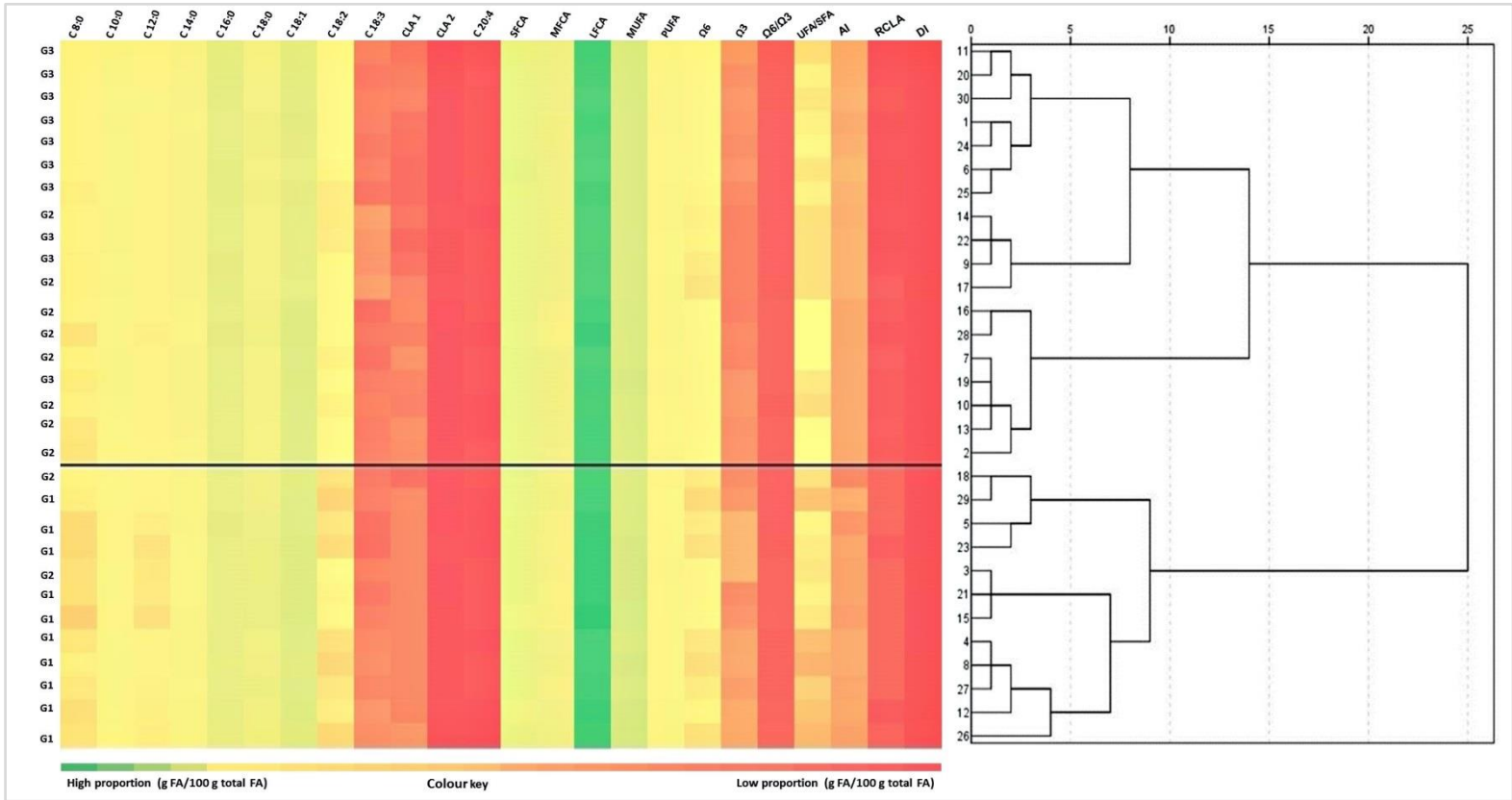

Figure 1. Heatmap with hierarchical clustering of the commercial dairy sheep flocks studied according to the mean values of the 12 FAs, seven groups of FAs and five FA indexes studied during the experimental period.
CLA, conjugated linoleic acid; CLA 1, *c*-9,*t*-11 $C_{18:2}$; CLA 2, *t*-10,*c*-12 $C_{18:2}$; SCFA, sum of short-chain saturated fatty acids; MCFA, sum of medium-chain saturated fatty acids; LCFA, sum of long-chain saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; UFA, sum of unsaturated fatty acids; SFA, sum of saturated fatty acids; AI, atherogenicity index; RCLA, *c*-9,*t*-11 $C_{18:2}$/*c*-9,*c*-12 $C_{18:2}$; DI, desaturase index.

CONCLUSIONS

This study showed that the proportions of beneficial FAs, such as CLA and α-linolenic acid, decreased in bulk tank milk when sheep grazed less and ate more concentrates. Lactation stage had no effect on MUFA, CLA, linoleic acid or arachidonic acid. At present, sheep milk production is being intensified giving less importance to the nutritional value of the milk. However, less intensive sheep milk production systems have been shown to have a beneficial effect on the nutritional profile of milks. This study provides information that the dairy industry could take into account to foment animal production practices that lead to an improvement in sheep milk quality and therefore in the nutritional value of cheese.

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CAPÍTULO 2

Production of conjugated linoleic acid and gamma-aminobutyric acid by autochthonous lactic acid bacteria and detection of the genes involved

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Abstract

In this study, 85 strains of lactic acid bacteria isolated from artisanal cheeses were screened to determine their capacity to synthesise bioactive compounds with important beneficial properties to human health, such as conjugated linoleic acid (CLA) and gamma-amino butyric acid (GABA).

Four *Lactobacillus plantarum* and two *Lactobacillus casei* subsp. *casei* strains were found to be capable of synthetizing CLA. The highest level of CLA formed in the media after 48 h incubation was 19.26 μg/mL. Six *Lactobacillus brevis* and four *Lactococcus lactis* subp. *lactis* strains were able to produce GABA and the highest concentration (2524.05 μg/mL) was found after 72 h incubation.

The detection of genes encoding linoleate isomerase could be suitable for use as screening method of CLA–producer strains but many strain-dependent factors affect their expression and/or activity. The detection of genes encoding the glutamate decarboxylase system could be a method for screening *Lactococcus lactis* GABA-producing strains, although this should be studied in a larger number of strains.

Our findings suggest that the above strains are potential candidates for the design of starter cultures with the capacity to generate bioactive compounds, offering new possibilities for the manufacture of functional dairy products.

INTRODUCTION

The relationship between food and consumer health and well-being has become a priority concern in food production. Fermented dairy products, especially cheese, contain compounds that exert beneficial effects on human health, such as conjugated linoleic acid (CLA) and gamma-aminobutyric acid (GABA) (Diana, Quílez, & Rafecas, 2014; Sofi et al., 2010; Zlatanos, Laskaridis, Feist, & Sagredos, 2002).

CLA is a group of positional and geometric isomers of octadecadienoic acid with a system of conjugated double bonds which have been attributed with several functional properties, including anti-carcinogenic, antiatherogenic, antiobesity effects and modulation of the immune system (Koba & Yanagita, 2014; Tanaka, 2005).

CLA is naturally found in foods derived from ruminants because it occurs as an intermediary in biohydrogenation of polyunsaturated fatty acids, specifically of linoleic acid and α-linolenic acid, by the action of various anaerobic bacteria enzymes in the rumen. It is also synthesised in the mammary gland by the action of the Δ-9 desaturase enzyme on vaccenic acid, which is another intermediary in ruminal biohydrogenation (Rodríguez-Castañedas, Peña-Egido, García-Marino, & García-Moreno, 2011). This has prompted research to determine whether other bacteria involved in the fermentation of dairy products, are also capable of synthesising CLA. In recent years, several studies have shown that some lactic acid bacteria (LAB) and bifidobacteria strains can efficiently convert LA to CLA due to activity of the linoleate isomerase enzyme which has been observed to be strain-dependent (Coakley et al., 2003; Gorissen et al., 2011; Nieuwenhove, Oliszewski, González, & Pérez Chaia, 2007a; Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011).

GABA is a non-protein amino acid with numerous physiological functions, including neurotransmission, blood pressure regulation and insulin secretion (Adeghate & Ponery, 2002; Diana et al., 2014; Okada et al., 2000). GABA is synthesised by glutamate decarboxylase (GAD), an enzyme dependent on pyridoxal 5'-phosphate, which catalyses α-decarboxylation of L-glutamate or its salts to GABA (Narayan & Nair, 1990). This enzyme has been found in LAB (Cotter & Hill, 2003; Komatsuzi, Nakamura, Kimura, & Shima, 2008), and thus a study of the capacity of LAB strains to produce GABA is of particular interest with a view to using them as starter cultures in the manufacture of fermented products. As Siragusa et al. (2007) have indicated, cheese is a good vehicle for GABA because milk caseins have a high content in L-glutamate (17.5% of the total amino acid content) which is released during cheese ripening and can be metabolised to GABA by the action of LAB (Hejtmánková, Pivec, Trnková, & Dragounová, 2012).

However, the concentration of these compounds in cheese is generally under the minimum level required to play a beneficial role on human health. Information is lacking on CLA and GABA levels required by humans, but it is estimated that a daily intake of 3 g per day for a 70 kg person may be effective to achieve the beneficial effects of CLA and it has been pointed out that a daily oral administration of 26.4 mg GABA is required to be effective in treating neurological disorders (Okada et al., 2000; Pariza, 2004).

It is therefore of interest to design natural strategies for enriching bioactive compound content in the final product. Several studies have indicated the possibility of modifying cheese composition by acting on the cheese manufacture process, and more particularly by using starter cultures with the capacity to synthesise CLA and GABA from

linoleic acid (LA) and monosodium glutamate (MSG), respectively (Diana et al., 2014; Mohan, Anand, Kalscheur, Hassan, & Hippen, 2013; Nieuwenhove, Oliszewski, González, & Pérez Chaia, 2007b).

The objectives of this study were to: (i) screen 85 LAB strains isolated from artisanal cheeses for their capacity to synthesise CLA and GABA; (ii) test the biosynthesis of CLA and GABA under different incubation times; and (iii) detect the genes encoding linoleate isomerase and glutamate decarboxylase as a possible screening method. Therefore, this study was aimed to identify LAB strains capable of efficient production of CLA and GABA, for use in the design of starter cultures with the capacity to generate bioactive compounds, offering new possibilities for the manufacture of functional dairy products.

MATERIALS AND METHODS

Strains and culture media

For this study, 85 autochthonous LAB strains were selected for their adequate technological aptitude observed in previous studies and could potentially be considered as starter cultures for dairy products manufacture (González et al., 2007; González, Cuadrillero, Castro, Bernardo, & Tornadijo, 2015; González, Sacristán, Arenas, Fresno, & Tornadijo, 2010; Herreros, Fresno, González Prieto, & Tornadijo, 2003). These strains were obtained from the collection held in the Department of Food Hygiene and Technology at the University of León (**Table 1**). All strains were screened for CLA and GABA production, and all experiments were carried out in duplicate. Before experimental use, the strains were subcultured twice in Elliker broth (BD Difco, New Jersey, USA) for *Lactococcus lactis* or in MRS broth (Oxoid, Hampshire, UK) for the remaining strains at 30ºC during 24 h. At this time of incubation, the growth phase of the strains studied was the initial stationary phase.

After subculture, the cultures were centrifuged at 20,800 × *g* for 5 min at 4ºC and the pellets were resuspended in a sterile physiological solution (0.85% NaCl) until reaching an absorbance value between 1.0 - 1.3 at a 550 nm wavelength, which corresponded to a cell density of 9.0 to 9.2 $\log_{10}$ CFU/mL.

Screening for CLA producers

Screening for strains capable of converting free LA to CLA was carried out following the method described by Barrett, Ross, Fitzgerald, and Stanton (2007), which is based on spectrophotometric detection of CLA, with some modifications. Briefly, the activated cultures were inoculated (5% v/v) to the corresponding culture medium (Elliker or MRS) containing 0.5 mg/mL LA (99% purity; 0.902 g/mL density; Sigma-Aldrich, St. Louis, MO, USA). The LA was added as a 30 mg/mL stock solution containing 2% (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate; Merck-Schuchardt, Hohenbrunn, Germany) and was previously filter sterilized through a 0.45 μm Minisart filter (Sartorius AG, Goettingen, Germany) and stored in the dark at -20ºC until use. The strains were incubated for 24 h and 48 h at 30ºC to determine the effect of the incubation time for CLA production. Following incubation, the cultures were centrifuged at 20,800 × *g* for 1 min at 4ºC, and the supernatants were mixed with 2 mL isopropanol and allowed to stand for 3 min. Then, 1.5 mL of hexane was added and again allowed to stand for 3 min.

Table 1. List of the 85 autochthonous lactic acid bacteria strains screened in the present study isolated from Armada (TAUL), Genestoso (GE) and San Simón Da Costa (SS) cheeses and effect of the linoleic acid (LA) on their growth.

Species	Number of strains	Growth inhibition by LA*
Lactococcus lactis subsp. *lactis* GE 61, 118, 102, 103; TAUL 32, 238, 262, 250, 266; SS 194, 193	11	1 strain ++
Lactococcus lactis subsp. *cremoris* TAUL 1239, TAUL 216	2	-
Leuconostoc pseudomesenteroides GE 2068; GE 2070	2	1 strain +
Leuconostoc mesenteroides GE 2002; SS 1435, 1437, 1664	4	4 strains +
Lactobacillus brevis TAUL 198, 1267, 141, 174, 67, 69, 70, 179, 195, 205	10	-
Lactobacillus casei TAUL 171, 173, 175, 177, 180, 185, 190	7	-
Lactobacillus paracasei GE 2036, 2071	2	-
Lactobacillus casei subsp. *casei* TAUL 1506, 1508, 1699; SS 1614, 1615, 263, 1644, 1661, 1694, 1689, 1770, 1778, 1785	14	-
Lactobacillus casei subsp. *rhamnosus* SS 1684	1	-
Lactobacillus plantarum GE 2077; TAUL 1736, 1765, 1521, 1522, 101, 1539, 1588, 1795, 38, 39, 52, 55, 122, 125, 194	16	-
Enterococcus italicus TAUL 250; SS 194	2	-
Enterococcus faecalis GE 26, 35, 2320, 2371, 2381; TAUL 117, 32, 262, 198; SS 193, 1378, 191, 1449	13	1 strain +
Enterococcus raffinosus TAUL 1351	1	-

*The occurrence of growth inhibition of the cultures after addition of 0.5 mg/ml of LA. (−) growth was not inhibited, (+) growth was retarded; (++) strains were unable to continue growth after addition of LA to the cultures.

The amount of CLA synthetized was determined by measuring the absorbance at 233 nm using a 96-well plate spectrophotometer (BioTek Synergy HT spectrophotometer, Winooski, Vermont, USA).

To calculate the concentration of CLA produced by the LAB studied, a calibration curve was constructed for absorbance at 233 nm versus CLA (C18:2 c9,t11) (≥96% purity; Sigma-Aldrich, St. Louis, MO, USA) concentration (0-30 μg/mL), obtaining the equation: $y=0.0654x+0.0085$ ($R^2 = 0.9994$).

Growth was monitored by measuring optical density at 600 nm (OD600) and cultures without LA were used as control to determine whether this compound inhibited growth of the bacteria studied.

The pH value of the culture medium was determined using a pH meter (Mettler Toledo, Columbus, Ohio, USA) equipped with a combined electrode at room temperature (20 ± 2°C).

Screening for GABA producers

The 85 strains were inoculated at 5% (v/v) into the appropriate culture medium containing 5 mg/mL MSG (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 72 h at 30ºC. Then, the strains identified as GABA producers were re-inoculated at 5% (v/v) into the appropriate culture medium containing 5 mg/mL MSG and incubated for 24, 48 and 72 h at 30ºC to determine the effect of incubation time on GABA production by the selected LAB. Detection of GABA-producing strains and quantification of GABA production at different incubation times was performed according to the method described by Barrett, Ross, O'Toole, Fitzgerald, and Stanton (2012).

Linoleate isomerase, glutamate decarboxylase and glutamate-GABA antiporter gene

Detection of genes encoding linoleate isomerase, glutamate decarboxylase and glutamate transporter was performed as follows: first, a search was conducted in the GenBank database for all genes of the same species encoding the corresponding enzyme. The sequences available for each species were aligned to identify highly conserved regions, which enabled the design of the primers (Sigma-Aldrich, St. Louis, MO, USA) (**Table 2**). The primers (0.4 µM each) were used to amplify the respective target sequences from the genomic DNA (0.1 ng) of the selected bacteria. The amplification reaction (50 µL) was performed using BioMix RedTM (Bioline) in an Applied Biosystems 2720 Thermal Cycler (Life Technologies). PCR cycles (95°C, 1 min; 55°C, 60 s; 72°C, 90 s) were preceded by a denaturation step (95°C, 5 min) and concluded with a final elongation step (70°C, 7 min).

Table 2. Primers used in this study for the detection of the genes encoding the enzymes linoleate isomerase (*lis*), glutamate decarboxylase (*gadB*) and glutamate transporter (*gadC*) involved in CLA and GABA biosynthesis.

Primer	Sequence (5'-3')	Gene	Expected size
Lbcasf	GATCAAGCATAAGGCGATCATGATCGG	*lis* (*L. casei*)	600 bp
Lbcasr	TAAATCATCATGTGCATGTACCGCCG		
Lbplf	GCAATTATGATTGGTGCCGG	*lis* (*L. plantarum*)	450 bp
Lbplr	CTTCGTTTCTTCACTATCTGGCATC		
lacBf	TCTGTCAAACTTATATGGAACCTGAAGC	*gadB* (*L. lactis*)	1100 bp
lacBr	CTTGAACATAGTTAAATGCCATATTCATCCC		
lacCf	GCCACGTCAAAATTACATTTGGTGTTCT	*gadC* (*L. lactis*)	1000 bp
lacCr	CCTCCTCCTCCAAAAGTTAATACAGCGCCCC		
brevB1f	AGGCAGTGTCGAAGCCGGGCAA	*gadB1* (*L. brevis*)	1300 bp
brevB1r	CATGGATGGGCGTACCACGATCC		
brevB2f	CTCGCCACGTTCTGTCAGACTTACATGG	*gadB2* (*L. brevis*)	1100 bp
brevB2r	TCATCAATAAAGTCGTGGGCCATACTCATACC		
brevCf	CCATGACGACTTCCATGGTCATGACGGTT	*gadC* (*L. brevis*)	1300 bp
brevCr	GCATGACCACAAAGGCACTCACTAACAACA		

Next, PCR products were separated by electrophoresis on 1% (wt/vol) agarose gel (Thermo Fisher Scientific, Waltham, Massachusetts). A DNA molecular marker was loaded

in parallel in the gel to predict the estimated size of the PCR products. Gels were stained with 0.5 µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and then imaged under ultraviolet light in an Alpha Imager™ 3400 (Alpha Innotech, Kasendorf, GmbH).

Statistical analysis

All experimental data obtained in the study of CLA and GABA production by LAB at different incubation times were statistically analysed using SPSS v.21 (SPSS, Chicago, IL, USA). The variables studied were tested for the assumptions of normality and homoscedasticity. Subsequently, we performed a one-way analysis of variance (ANOVA) and then applied Tukey's HSD *post hoc* test at a 5% significance level.

RESULTS AND DISCUSSION

In this study, first of all the tolerance of the strains to LA was tested in MRS or Elliker broth, since several studies have reported that LA can retard or inhibit the growth of some bacteria (Gorissen et al., 2010; Jiang, Björck, & Fondén, 1998; Nieuwenhove et al., 2007a). The concentration of LA used in this study was based on the concentration (0.5 mg/mL) of this fatty acid in sheep's milk (De La Fuente et al., 2009), which would mean that the CLA-producing strains could grow in sheep milk.

Of the 85 strains tested, only 6 strains showed inhibited growth and one did not grow after 48 h incubation (**Table 1**), which was in agreement with the antimicrobial effect of free LA on LAB (Alonso, Cuesta, & Gilliland, 2003). These results confirm that inhibition of LAB growth by LA is strain- and not species-dependent, as has also been reported by Gorissen et al. (2010) in their study of *Bifidobacterium* species.

Determination of CLA production by bacteria is usually performed by fat extraction and methylation of fatty acids followed by gas chromatography analysis. El-Salam, El-Shafei, Sharaf, Effat, and El-Aasar (2010) have reported that although this method gives good results, it is time consuming and requires expensive equipment. Consequently, we decided to use a method based on spectrophotometric measurement of the conjugated double bond in the fatty acids, which is a less expensive and time consuming technique. This method offers advantages when screening a large number of samples, as was the case in this study.

Of all the strains tested, only 2 *L. casei* subsp. *casei* and 4 *L. plantarum* strains were capable of synthesising CLA from free LA (**Table 3**). *Lactobacillus* are non-starter lactic acid bacteria (NSLAB) which play a role in cheese ripening, and thus their study as a potential co-culture is of particular interest.

Several studies have reported that the CLA concentration produced by LAB strains can be affected by various factors, including incubation time and pH (El-Salam et al., 2010; Rodríguez-Alcalá et al., 2011). Mean CLA production by bacteria at different incubation times was calculated using the calibration curve described above. All strains showed a statistically significant increase ($P \leq 0.001$) in CLA synthesis from 24 h to 48 h incubation, indicating that incubation time is an important factor to consider in CLA production by *Lactobacillus* strains (**Table 3**). *L. plantarum* TAUL 1588 strain produced the most CLA after 24 h and 48 h incubation, yielding values similar to those reported by Rodríguez-Alcalá et al. (2011) for a strain of the same species, using the same method and

by Terán et al. (2015) using the gas chromatography analysis. This strain is therefore of great interest due to its high rate of CLA production which opens the opportunity to study this autochthonous strain as a co-culture for the possible development of functional dairy products.

Table 3. CLA strains producers in this study and CLA concentration[a] detected in the culture medium[b] for 24 and 48 hours of incubation.

Strain	24	48	Time effect
Lactobacillus casei casei SS 1644	2.43 (5.05)	6.51 (3.14)	***
Lactobacillus casei casei SS 1614	3.01 (2.63)	6.40 (1.42)	***
Lactobacillus plantarum TAUL 1522	4.88 (2.13)	6.74 (2.73)	***
Lactobacillus plantarum TAUL 1539	7.52 (1.28)	13.52 (2.31)	***
Lactobacillus plantarum TAUL 1588	10.50 (2.80)	19.26 (2.07)	***
Lactobacillus plantarum TAUL 1795	3.89 (2.00)	6.83 (2.93)	***

[a]Results as mean values of duplicate determination expressed as µg/mL (coefficient of variation, RSD).
[b]CLA concentration in µg/ml of MRS medium with free LA (0.5 mg/mL) for 24 h and for 48 h calculated spectrophotometrically at wavelength of 233 nm from the linear trend of the standard curve.
***$P \leq 0.001$.

L. casei subsp. *casei* SS 1644 was the strain that produced the lowest concentration of CLA at 24 h incubation, while *L. casei* subsp. *casei* SS 1614 produced the lowest concentration at 48 h. In general, *L. casei* subsp. *casei* was less efficient at producing CLA than *L. plantarum*. Although some authors have indicated that the ability of LAB to produce CLA is strain-dependent, these results suggest the need to continue studying the pathway and mechanism of CLA production by LAB.

The initial pH of the culture medium (5.92 - 6.12) decreased to values of 3.85 - 4.00 at 24 h and remained at that level or decreased slightly after 48 h incubation. The *L. plantarum* TAUL 1539 and TAUL 1588 strains produced the greatest drop in the pH medium after 24 h and 48 h, and also produced the highest CLA concentration at these incubation times.

It should be borne in mind that the spectrophotometric method employed does not detect the different CLA isomers (Barrett et al., 2007), which would be of interest since the properties beneficial to human health are mainly related to two CLA isomers: *cis*-9,*trans*-11 $C_{18:2}$ and *trans*-10,*cis*-12 $C_{18:2}$. In addition, the *trans*-9,*trans*-11 $C_{18:2}$ isomer is being studied because of its potential anti-carcinogenic effects on human colon cancer cell lines (Coakley et al., 2006), and its capacity to induce the expression of genes involved in lipid metabolism (Ecker, Liebisch, Patsch, & Schmitz, 2009). For this reason and for the good capacity of the studied *Lactobacillus* strains to produce CLA, it would be of interest to conduct further and more specific studies on these autochthonous LAB strains.

The presence of the gene encoding linoleate isomerase in the CLA-producing strains was identified in this study (**Figure 1**). Linoleate isomerase is the enzyme responsible for the production of CLA from LA as a substrate in LAB (Chen, 2012; Gorissen et al., 2011). PCR products were detected as a single band. In the case of the *L. plantarum* strains, the approximate size of the detected band was 500 bp, while in case of the two *L. casei* subp. *casei* strains, it was 600 bp. This gene was also detected in some of the strains that did not produce CLA (**Figure 2**), indicating that the presence of a gene does not imply the

production of the relative compound. Therefore, strains should be genetically characterized at first and then evaluated for the production. The non-production of CLA in strains containing the gene can be explained according to Gorissen et al. (2011), because the linoleate isomerase gene may be present in the strains, but its expression can be affected by various factors, such as pH or temperature, and these effects are in turn strain-dependent.

Figure 1. Linoleate isomerase gene detection of the strains selected as CLA producers.

Figure 2. Linoleate isomerase gene detection of some no CLA producing-strains.

None of the CLA-producing strains produced GABA, and *vice versa*. This fact opens new perspectives for the study of the existence of possible mechanisms that may interfere in the joint production of CLA and GABA by LAB.

Ten of the 85 strains studied were identified as GABA producers (**Table 4**). These strains were: *L. lactis* subp. *lactis* (GE 61, GE 118, GE 102, GE 103) and *L. brevis* (TAUL 141, TAUL 174, TAUL 69, TAUL 70, TAUL 179, TAUL 195). These results indicate the variability between strains of the same species and confirm that the capacity to synthesise GABA is strain-dependent, as indicated by Siragusa et al. (2007).

Dhakal, Bajpai, and Baek (2012)have reported that different fermentation factors, including incubation time, can affect GABA synthesis by microorganisms. **Table 4** shows

the evolution of GABA production by the strains studied over various incubation times in culture medium supplemented with MSG. All strains showed a statistically significant increase in GABA synthesis ($P \leq 0.05$) from 24 h to 72 h incubation. This is in agreement with a study by Wu and Shah (2015), who reported an increase in GABA production by 9 LAB strains after 72 h incubation in MRS medium supplemented with 50 mg/mL of MSG as substrate.

Table 4. GABA strains producers in this study and GABA concentration[a] detected in the culture medium[b] for 24, 48 and 72 hours of incubation.

Strain	24	48	72	Time effect
Lactococcus lactis lactis GE 61	280.51 (3.20)	468.66 (1.07)	668.10 (1.48)	***
Lactococcus lactis lactis GE 118	245.05 (2.43)	282.02 (3.74)	516.87 (1.97)	***
Lactococcus lactis lactis GE 102	95.44 (4.62)	279.04 (1.71)	573.74 (1.49)	***
Lactococcus lactis lactis GE 103	267.16 (7.44)	678.33 (1.68)	766.38 (2.78)	***
Lactobacillus brevis TAUL 141	1350.36 (2.32)	1378.04 (0.31)	2492.51 (2.54)	***
Lactobacillus brevis TAUL 174	1046.78 (2.43)	1161.06 (3.69)	1820.25 (1.64)	***
Lactobacillus brevis TAUL 69	927.52 (1.22)	1024.15 (2.29)	1306.85 (6.42)	**
Lactobacillus brevis TAUL 70	1949.82 (3.05)	2068.50 (0.65)	2442.74 (0.90)	**
Lactobacillus brevis TAUL 179	1819.85 (4.77)	2112.66 (4.98)	2310.63 (7.69)	**
Lactobacillus brevis TAUL 195	2003.71 (2.19)	2333.07 (3.80)	2524.05 (8.83)	**

[a]Results as mean values of duplicate determination expressed as µg/mL (coefficient of variation, RSD).
[b]GABA concentration in µg/ml of MRS or Elliker broth with monosodium glutamate (5 mg/mL) for 24, 48 and 72 h.
$^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

The strain that produced the highest GABA concentration was *L. brevis* TAUL 195 after 72 h incubation, synthesizing 2524.05 µg/mL, which makes it a very interesting autochthonous strain to be used as a co-culture.

These findings indicate that the *L. brevis* strains were more efficient at producing GABA than *L. lactis* strains, coinciding with the results reported in other studies (Barrett et al., 2012; Zhang et al., 2012).

The glutamate decarboxylase system, which is composed of GAD and the glutamate – GABA antiporter, is responsible for the production of GABA in LAB (Li, Li, Liu, & Cao, 2013). In this study, we also tested the presence of the genes encoding the GAD enzyme and the glutamate – GABA antiporter in GABA-producing and in GABA-not producing strains (**Figures 3 and 4**).

The presence of both genes was detected only in the four GABA-producers *L. lactis* strains (**Figure 3**). These results highlight the possibility of using this technique as a method for screening of *L. lactis* GABA- producing strains based on the presence of these genes, although this should be studied in a larger number of strains.

Nomura et al. (1999) noted that *L. lactis* contains only one glutamate decarboxylase gene, as was observed in our study. However, in *L. brevis,* two different GAD encoding genes were detected as Li et al. (2013) indicated. The three genes encoding the glutamate decarboxylase system were detected in all the GABA- producer *L. brevis* (**Figure 4**).

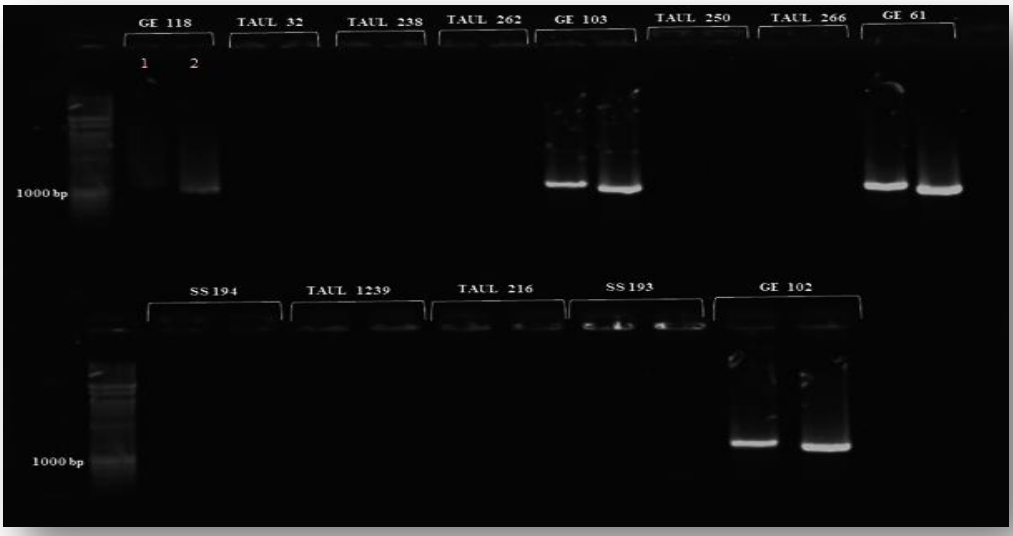

Figure 3. Glutamate decarboxylase and glutamate-GABA antiporter genes detection of *Lactococcus lactis* strains.(1) gene 1 encoding glutamate decarboxylase; (2) gene encoding glutamate-GABA antiporter.

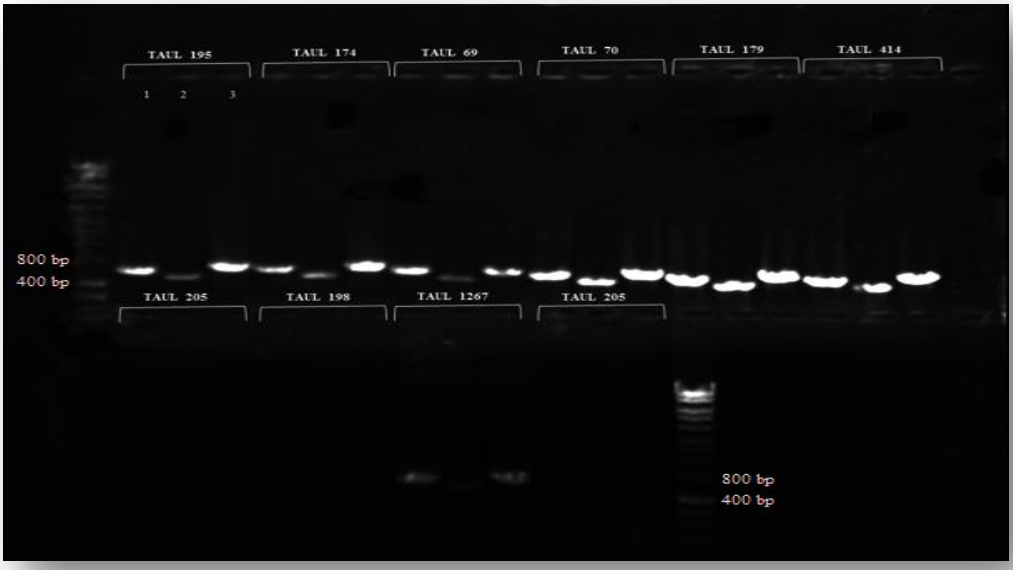

Figure 4. Glutamate decarboxylase and glutamate-GABA antiporter genes detection of *Lactobacillus brevis* strains.(1) gene 1 encoding glutamate decarboxylase; (2) gene 2 encoding glutamate decarboxylase; (3) gene encoding glutamate-GABA antiporter.

CONCLUSIONS

Considering the health benefits of CLA and GABA, the good ability of the studied autochthonous strains (*L. plantarum* TAUL 1588 and *L. brevis* TAUL 195) to produce these

bioactive compounds, could contribute to the development of starters that can be used in the manufacture of functional dairy products.

To the best of our knowledge, this is the first study to report the importance of the presence of genes to screen CLA and GABA producer-strains. This fact also opens the way for further studies to understand the mechanisms involved in the production of these bioactive compounds by LAB.

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CAPÍTULO 3

Study of the conjugated linoleic acid synthesis by *Lactobacillus* strains and by different co-cultures designed for this ability

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Abstract

Consumer concern about the relationship between food and health has generated a growing interest in functional foods. Conjugated linoleic acid (CLA) has been associated with several beneficial health properties. Therefore, this study reports the design of co-cultures containing CLA-producing strains and their performance in MRS and in skim milk.

The four designed co-cultures were tested under different conditions. In MRS, co-culture 2 (containing autochthonous *Lactococcus lactis* strains and *Lactobacillus plantarum* TAUL 1588) was the highest CLA-producer (98.01 μg/mL) after 48 h incubation. In milk, the highest total CLA production (56.51 μg/mL) was reached with co-culture 4 (containing autochthonous *Lactococcus lactis*, *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* SS 1644) after 72 h.

These results open the way for the use of co-cultures containing CLA-producing strains to manufacture cheese and obtain functional dairy products rich in bioactive fatty acids.

INTRODUCTION

Conjugated linoleic acid (CLA) consists of a group of positional and geometric isomers of octadecadienoic acid with a system of conjugated double bonds which have been associated with several properties that are beneficial for human health, such as: anti-carcinogenic, antiatherogenic and antiobesity effects and modulation of the immune system (Coakley et al., 2006; Ecker, Liebisch, Patsch, & Schmitz, 2009; Kim et al., 2016; Yang et al., 2015). The main CLA isomers attributed with these beneficial properties are *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ and *trans*-9,*trans*-11 $C_{18:2}$.

CLA is naturally found in foods derived from ruminants because it is produced as an intermediary in biohydrogenation of polyunsaturated fatty acids, by the action of the enzymes of various anaerobic bacteria in the rumen. It is also synthesised in the mammary gland by the action of the Δ-9 desaturase enzyme on vaccenic acid, which is another intermediary in ruminal biohydrogenation (Rodríguez-Castañedas, Peña-Egido, García-Marino, & García-Moreno, 2011). CLA content in milk and dairy products ranges between 0.68% and 0.12% of total fat (Luna, Juárez, & de la Fuente, 2007). At present, the estimated human intake of CLA from food sources is insufficient to obtain the potential effects against cancer, atherosclerosis and obesity reported in studies on animal models (Watkins & Li, 2003). Such effects would require a much higher consumption of milk and dairy products than the normal intake provided in a standard diet. Consequently, various methods are being explored to increase the content of this beneficial compound in fermented dairy products, being the use of starter cultures containing CLA-producing strains during manufacture as a the possible strategies (Andrade et al., 2012). Some studies have examined the capacity of certain lactic acid bacteria (LAB) and bifidobacteria to synthesise CLA from linoleic acid (LA) as substrate, finding that various strains are capable of producing significant amounts of CLA (Alonso, Cuesta, & Gilliland, 2003; Gorissen et al., 2010). However, there is no knowledge about the design of co-cultures which could produce CLA in culture medium or skim milk supplemented with LA during different incubation times, in order to know the optimal conditions for CLA production.

The objectives of this study were to: (i) quantify CLA production by LAB in culture medium and in skim milk supplemented with free LA at different incubation times; (ii) design co-cultures containing CLA-producing strains; and (iii) quantify CLA production by those co-cultures in the synthetic medium and in skim milk supplemented with free LA at different incubation times. Consequently, the overall aim of the present study was to obtain co-cultures that not only contributed actively to cheese ripening, but were also capable of generating bioactive compounds, thus increasing CLA content in cheese and could obtain functional dairy foods.

MATERIALS AND METHODS

Strains, co-cultures, media and growth conditions for CLA production

In a previous study, of the 85 strains isolated from three traditional Spanish cheeses (Armada cheese, Genestoso cheese and San Simon da Costa cheese) and technologically characterized (González, Cuadrillero, Castro, Bernardo, & Tornadijo, 2015; Herreros, Fresno, González Prieto, & Tornadijo, 2003) only six autochthonous LAB strains

showed the capacity to synthesize CLA. In this previous study, the screening of the strains capable of converting free LA to CLA was carried out following the method described by Barrett, Ross, Fitzgerald, and Stanton (2007), which is based on spectrophotometric detection of CLA. These six strains were selected for this study and were: *Lactobacillus plantarum* TAUL 1539, TAUL 1588, TAUL 1795 and TAUL 1522, and *Lactobacillus casei* subsp. *casei* SS 1614 and SS 1644. Prior to examination of CLA production, each strain was subcultured twice at 30ºC for 24 h in MRS broth (Oxoid, Hampshire, UK) for *Lactobacillus* or in M17 broth (BD Difco, New Jersey, USA) for *Lactococcus lactis*. These activated cultures were transferred at 5% (v/v) to MRS broth and reconstituted skim milk at 10% (w/v) in deionised water containing 0.5 mg/mL of LA (Sigma, St. Louis, MO, USA). The LA was added as a 30 mg/mL stock solution containing 2% (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate; Merck-Schuchardt, Hohenbrunn, Germany) and was previously filter sterilized through a 0.45 µm Minisart filter (Sartorius AG, Goettingen, Germany) and stored in the dark at -20ºC until use. The cultures were incubated at 30ºC for 24, 48 and 72 h to determine optimal conditions for CLA production. Total viable counts (CFU) were determined by plating serial dilutions on MRS agar (Oxoid, Hampshire, UK) and the pH value of the cultured media was determined using a pH meter (Mettler Toledo, Columbus, Ohio, USA) equipped with a combined electrode.

Based on the CLA production results obtained with the strains studied, four co-cultures (**Figure 1**) were designed consisting of *Lactococcus lactis* (60%, v/v) and *Lactobacillus* (40%, v/v) strains, having previously verified compatibility between strains by means of the agar spot test (Schillinger & Lücke, 1989). The activated co-cultures were transferred at 5% (v/v) to the MRS broth or the skim milk containing 0.5 mg/mL of LA and incubated at 30ºC for 24, 48 and 72 h. All experiments were carried out in duplicate.

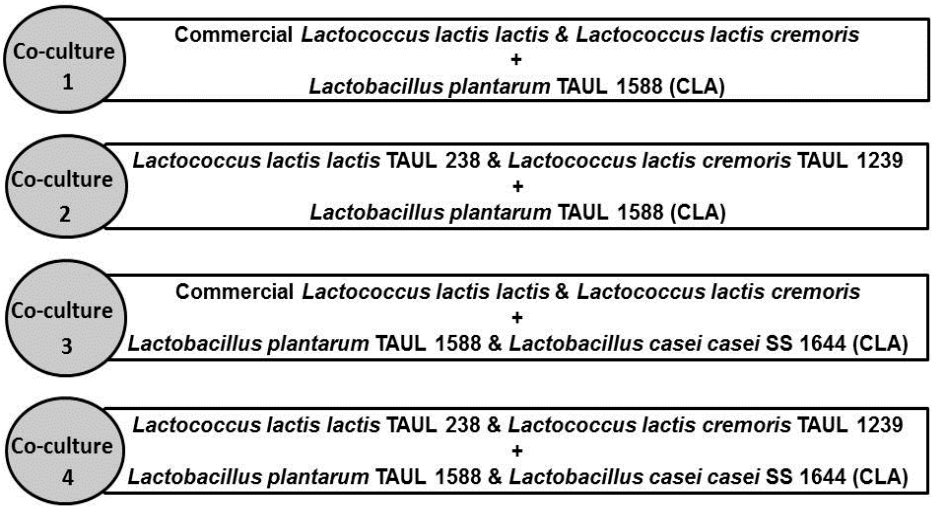

Figure 1. Description of each co-culture designed with *Lactococcus lactis* (commercial or autochthonous) strains and CLA-producing *Lactobacillus* strains.

Quantification of CLA production by gas chromatographic analysis

Lipids were extracted from samples using the method described by Coakley et al. (2003) and fatty acids methyl esters (FAMES) were prepared by acid-catalysed methanolysis of glycerides (12% methanolic HCl) (Supelco, Bellefonte, PA, USA) following the above-mentioned method. Heptadecanoic acid (C 17:0) was used as internal standard. FAMES were analysed by gas chromatography (GC) and the chromatographic system consisted of an Agilent 7890 B Chromatography System (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 80 Series Injector (Agilent Technologies, Santa Clara, CA, USA) and fitted with a flame ionisation detector (FID).

FAMES separation was carried out using a Chrompak CP - Sil 88 capillary column (0.2 μm particle size, 100 m x 0.25 mm I.D.) (Chrompack, Middelburg, Netherlands). Helium was used as a carrier gas at a flow rate of 1 mL/s. The injector temperature was held isothermally at 225ºC for 10 min and the detector temperature was 250ºC. The column oven was held at an initial temperature of 140ºC for 8 min and then programmed to increase at a rate of 8.5ºC/min to a final temperature of 200ºC, which was held for 41 min. Samples (1 μL) were injected by splitless injection.

In order to determine the time of elution of each of the methyl esters of the CLA isomers and linoleic acid, and thus be able to identify and quantify them from the chromatograms, we prepared a series of dilutions from individual standard solutions of the three CLA isomers of interest in this study (*cis*-9,*trans*-11 $C_{18:2}$; *trans*-10,*cis*-12 $C_{18:2}$; *trans*-9,*trans*-11 $C_{18:2}$) (Larodan Fine Chemicals AB, Malmö, Sweden) and from a standard solution of linoleic acid (Sigma-Aldrich Co., St. Louis, MO, USA). This enabled us to obtain calibration curves for each of the FAMES under study and to quantify those present in the culture medium and in skim milk for the different strains studied. The CLA concentration naturally present in the skim milk media used in this study was subtracted from the analysed samples. The individual fatty acid content in the samples was expressed as μg/mL of broth or skim milk.

Statistical analysis

All experimental data obtained on CLA production by LAB and co-cultures at different incubation times were statistically analysed using SPSS v.21 (SPSS, Chicago, IL, USA). The variables studied were tested for the assumptions of normality and homoscedasticity. Subsequently, we performed a one-way analysis of variance (ANOVA) and then applied Tukey's HSD *post hoc* test at a 5% significance level.

RESULTS AND DISCUSSION

CLA production by strains

In the present study, 4 strains of *Lactobacillus plantarum* (TAUL 1539, TAUL 1588, TAUL 1795 and TAUL 1522) and 2 strains of *Lactobacillus casei* subsp. *casei* (SS 1614 and SS 1644), previously identified as CLA producers, were assayed for their capacity to transform LA into CLA in MRS or skim milk medium supplemented with 0.5 mg/mL of LA. Different incubation times (24, 48 and 72 h) were simultaneously assayed by GC to investigate these effects on CLA formation. This conjugated fatty acid has attracted much attention as a novel type of biologically beneficial functional lipid (Ogawa et al., 2005).

The results presented in this paper showed that the optimal incubation time for total CLA production by all strains in MRS and skim milk was 48 h, as described by Rodríguez-Alcalá, Braga, Malcata, Gomes, and Fontecha (2011), although Alonso et al. (2003) found no significant differences in CLA production by *Lactobacillus* strains after 24 h of incubation. Nieuwenhove, Oliszewski, González, and Chaia (2007) have reported a relationship between the growth phase of the bacteria and the concentration of total CLA detected in the culture medium. The highest concentration of total CLA at 48 h may be related to the increase of the viable cell count compared to 24 h (**Figure 2**). Total CLA levels in the growth medium decreased significantly ($P \leq 0.05$) after 72 h incubation. This could be due to oxidation reactions and oxidation metabolism carried out by the corresponding strain, which would favour a reaction towards catabolism of this compound (Ogawa, Matsumura, Kishino, Omura, & Shimizu, 2001; Rodríguez-Alcalá et al., 2011; Wang, Lv, Chu, Cui, & Ren, 2007).

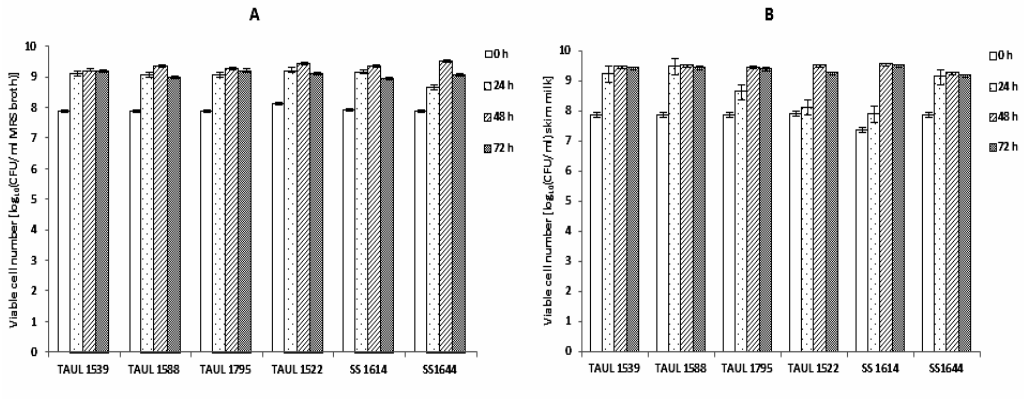

Figure 2. Viable cell count of each strain studied (A) in MRS broth and (B) in reconstituted skim milk (RSM) after 24, 48 and 72 h of incubation at 30ºC.

The formation of total CLA was affected by the different media used. The capacity of *Lactobacillus plantarum* strains to produce total CLA was greatly reduced when they were grown in skim milk. However, in the case of *Lactobacillus casei* subsp. *casei* strains, CLA biosynthesis was more efficient in skim milk, reaching total CLA values at 48 h of incubation similar to those produced by *Lactobacillus plantarum* TAUL 1588 in MRS over the same time period. After 24 h incubation in skim milk, *Lactobacillus plantarum* TAUL 1588, TAUL 1795 and TAUL 1522 were unable to synthesise CLA, and *Lactobacillus plantarum* TAUL 1539 synthesised only 1.43 µg/mL of total CLA. However, *Lactobacillus casei* subsp. *casei* SS 1614 and SS 1644 produced 27.16 and 26.52 µg/mL of total CLA, respectively (data not shown). The reason for this is still unclear, but might be due to the fact that as Gorissen et al. (2011) have indicated, different environmental factors such as

pH and temperature can affect the activity of linoleate isomerase, the enzyme involved in CLA synthesis from LA by LAB.

The GC analysis performed in the present study showed that under the experimental conditions, the six *Lactobacillus* strains produced three CLA isomers; *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ and *trans*-9,*trans*-11 $C_{18:2}$, except when grown in skim milk, when they only produced two CLA isomers; *cis*-9,*trans*-11 $C_{18:2}$ and *trans*-9,*trans*-11 $C_{18:2}$. Thus, the isomer *trans*-10,*cis*-12 $C_{18:2}$ was biosynthesized specifically in MRS. Nevertheless, even in MRS this was the least abundant isomer (around 10% of total CLA in *Lactobacillus plantarum*, and around 20% of total CLA in *Lactobacillus casei* supernatants) confirming the results obtained by Rodríguez-Alcalá et al. (2011). Accordingly, the type of medium employed affected production of the different CLA isomers synthesised by the different strains. The importance of CLA isomers resides in the fact that several studies have reported that each isomer can have different effects on metabolism and cell functions, and each one acts through different cell signalling pathways. To date, *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ and *trans*-9,*trans*-11 $C_{18:2}$ have attracted particular research attention due to their marked biological activities (Coakley et al., 2006; Ogawa et al., 2005).

The highest amount of *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ and *trans*-9,*trans*-11 $C_{18:2}$ in MRS broth supplemented with LA after 48 h was produced by *Lactobacillus plantarum* TAUL 1588, with values of 23.73, 3.37, and 27.97 µg/mL, respectively, and 55.07 µg/mL total CLA (**Table 1**). *Lactobacillus casei* subsp. *casei* SS 1644 was the strain that produced the highest concentration of the CLA isomers *cis-9,trans-11* $C_{18:2}$, and *trans-9,trans-11* $C_{18:2}$ in skim milk broth supplemented with LA after 48 h, with values of 18.33 and 35.05 µg/mL, respectively, and 53.38 µg/mL total CLA (**Table 2**). Although all 6 strains produced CLA from free LA as substrate, we found significant differences ($P \leq 0.05$) in the concentration of the different CLA isomers synthesised by strains of the same species, suggesting that the capacity to produce this compound is dependent on strain and not on species.

Time of incubation affected the proportion of isomers synthesised by the *Lactobacillus* strains studied, all of which presented a common pattern. In MRS medium, after 24 and 48 h incubation, the main isomer detected was *cis*-9,*trans*-11 $C_{18:2}$, while after 72 h incubation it was *trans*-9,*trans*-11 $C_{18:2}$, in agreement with various authors (Ando, Ogawa, Kishino, & Shimizu, 2004) which have reported that as incubation time increases, the concentration of the isomer *trans*-9,*trans*-11 $C_{18:2}$ tends to increase with respect to that of the isomer *cis*-9,*trans*-11 $C_{18:2}$. However, when the culture medium was skim milk, *trans*-9,*trans*-11 $C_{18:2}$ was the main isomer detected at all incubation times. Ogawa et al. (2005) have pointed out that the isomer *cis*-9,*trans*-11 $C_{18:2}$ can be interconverted towards the isomer *trans*-9,*trans*-11 $C_{18:2}$. At present, the mechanisms of production and interconversion among isomers are unclear and require further study. We speculate that some of these interconversion reactions specific for a particular isomer could require specific enzymatic activities that could be affected by the concentration of the other isomers or even the evolution of the pH.

Table 1. Production of individual isomers and total conjugated linoleic acid (CLA) by *Lactobacillus plantarum* and *Lactobacillus casei* subp. *casei* in MRS broth supplemented with 0.5 mg/mL of linoleic acid after 48 h of incubation at 30°C.

Culture	pH	Log_{10} CFU/mL	Concentration μg/mL[1]			
			***c*-9, *t*11**	***t*-10,*c*-12**	***t*-9,*t*-11**	**Total CLA**
L. plantarum TAUL 1539	3.90	9.22 (0.35)	23.43 (0.14)[a]	3.20 (0.09)[a]	17.58 (0.27)[a]	44.20 (0.32)[a]
L. plantarum TAUL 1588	3.89	9.36 (0.14)	23.73 (0.06)[a]	3.37 (0.12)[a]	27.97 (0.28)[b]	55.07 (0.21)[b]
L. plantarum TAUL 1795	3.89	9.27 (0.14)	5.79 (0.40)[b]	3.08 (0.16)[a]	11.19 (0.40)[c]	20.06 (0.95)[c]
L. plantarum TAUL 1522	3.87	9.43 (0.07)	8.31 (0.35)[c]	4.50 (0.34)[b]	14.90 (1.47)[a]	27.71 (2.17)[d]
L. casei SS 1614	3.85	9.36 (0.18)	10.87 (0.59)[d]	5.06 (0.21)[bc]	10.70 (0.75)[c]	26.63 (0.37)[d]
L. casei SS 1644	3.98	9.52 (0.14)	13.43 (0.37)[e]	5.35 (0.04)[c]	11.33 (0.11)[c]	30.10 (0.32)[d]

[a-e] Means in a column with different letter are significantly different ($P \leq 0.05$).
[1]Results as mean values of duplicate determination expressed as μg/mL (standard deviations).

Table 2. Production of individual isomers and total conjugated linoleic acid (CLA) by *Lactobacillus plantarum* and *Lactobacillus casei* subp. *casei* in skim milk supplemented with 0.5 mg/mL of linoleic acid after 48 h of incubation at 30°C.

Culture	pH	Log_{10} CFU/mL	Concentration μg/mL[1]		
			***c*-9,*t*11**	***t*-9,*t*-11**	**Total CLA**
L. plantarum TAUL 1539	4.01	9.48 (0.03)	6.32 (0.03)[a]	4.57 (0.16)[a]	10.90 (0.19)[a]
L. plantarum TAUL 1588	3.96	9.47 (0.11)	8.51 (0.49)[a]	9.27 (0.13)[b]	17.78 (0.62)[b]
L. plantarum TAUL 1795	4.28	9.40 (0.06)	1.43 (0.45)[b]	5.28 (0.25)[a]	6.71 (0.70)[c]
L. plantarum TAUL 1522	4.05	9.60 (0.35)	0.88 (0.25)[b]	4.87 (0.08)[a]	5.74 (0.33)[c]
L. casei SS 1614	4.02	9.56 (0.36)	17.78 (1.28)[c]	33.91 (0.675)[c]	51.69 (1.95)[d]
L. casei SS 1644	4.01	9.17 (0.09)	18.33 (0.63)[c]	35.05 (0.30)[c]	53.38 (0.33)[d]

[a-d] Means in a column with different letter are significantly different ($P \leq 0.05$).
[1]Results as mean values of duplicate determination expressed as μg/mL (standard deviations).

Lactobacillus species are non-starter LAB which dominate cheese microbiota during ripening. They tolerate the hostile environment well and strongly influence the biochemistry of curd ripening, contributing to the development of the final characteristics of cheese (Settanni & Moschetti, 2010). The amounts of CLA synthesised by the strains *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS 1644 at 48 h incubation in MRS culture medium and skim milk supplemented with 0.5 mg/mL of LA, respectively, were comparable with the results obtained in other studies of the same species (Alonso et al., 2003; Rodríguez-Alcalá et al., 2011; Terán et al., 2015). Consequently, the study of their potential use as a co-culture is of particular interest, since these findings suggest that they could be employed to manufacture functional fermented dairy products with elevated levels of CLA.

CLA production by co-cultures

Dairy products are the most important source of CLA in the human diet (Andrade et al., 2012). Based on the results reported above, we explored the possibility of designing co-cultures that could increase CLA content in sheep milk cheese to reach the levels necessary to obtain the potential health-promoting effects of CLA on human health. Bathazar et al. (2017) indicate that the nutritional value of sheep milk is higher than those of goat and cow milks. Therefore, sheep milk is a good food matrix for the manufacture of functional cheese.

Since industrial starters generally include a strain of *Lactococcus lactis* as an acidifier, we therefore designed 4 co-cultures containing strains of this species combined with CLA-producing strains of *Lactobacillus* (**Figure 1**). Two of these contained *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, of commercial origin (CHOOZIT™ LYO MA 011), while the other two contained *Lactococcus lactis* subsp. *lactis* TAUL 239 and *Lactococcus lactis* subsp. *cremoris* TAUL 1239, autochthonous isolates of Armada cheese (Herreros et al., 2003). None of the *Lactococcus lactis* strains included in this study were capable of producing CLA. These *Lactococcus* strains were selected for the design of the co-cultures because these strains showed good technological aptitude (Herreros et al., 2003) and in the compatibility test were the ones that presented better behavior with the CLA-producing strains and between them. The objective of this approach was to determine whether the co-culture with the commercial or autochthonous *Lactococcus lactis* strains affected CLA synthesis by CLA-producing strains. In the case of co-cultures 3 and 4 (**Figure 1**), our aim was to assess whether CLA production increased when using two CLA-producing strains in conjunction rather than individually. Therefore, we combined the *Lactobacillus plantarum* TAUL 1588 strain, which produced most CLA in MRS medium, with the *Lactobacillus casei* subsp. *casei* SS 1644 strain, which produced most CLA in skim milk.

The results obtained for total CLA content and each of the isomers produced by the four co-cultures designed, at different incubation times in MRS medium and in skim milk, are shown in **figures 3 and 4**, respectively. The incubation times presenting significant differences in CLA production between co-cultures are shown with different lowercase letters.

In MRS, the content of the isomer *cis*-9,*trans*-11 $C_{18:2}$ presented statistically significant differences ($P \leq 0.05$) between co-cultures at 24, 48 and 72 h incubation. In all

cases, the highest concentration of this isomer was produced at 48 h and the co-cultures that showed greatest production of the isomer *cis*-9,*trans*-11 $C_{18:2}$ were co-cultures 1 and 2, with no statistically significant differences ($P > 0.05$) between them. In the case of the isomer *trans*-10,*cis*-12 $C_{18:2}$, no statistically significant differences were observed ($P > 0.05$) between the different co-cultures throughout incubation. Content of the isomer *trans*-9,*trans*-11 $C_{18:2}$ presented statistically significant differences ($P \leq 0.01$) at 72 h incubation, and the highest concentration was produced by co-culture 2. With respect to the total CLA produced by the co-cultures, as observed in the co-cultures with individual *Lactobacillus* strains, CLA production was greatest at 48 h incubation, after which the concentration of CLA began to decline significantly ($P \leq 0.05$). The highest total CLA concentration at 48 h incubation was produced by co-culture 2, consisting of autochthonous strains of *Lactococcus lactis* subsp. *lactis* and the CLA-producing *Lactobacillus plantarum* TAUL 1588 strain, and co-culture 1, containing commercial strains of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* TAUL 1588. In MRS, these co-cultures produced almost double the concentration of CLA as that produced individually by the *Lactobacillus plantarum* TAUL 1588 strain in the same medium. Andrade et al. (2012) have indicated that environmental factors can affect CLA biosynthesis by LAB, explaining why the presence of *Lactococcus* species in this study enhanced conditions for CLA production by the *Lactobacillus plantarum* TAUL 1588 strain. As shown in **Figure 3**, the *Lactococcus lactis* strains (autochthonous or commercial) did not significantly affect ($P > 0.05$) CLA production by the co-culture, except at 72 h incubation. This was because in the case of co-culture 2, consisting of autochthonous strains of *Lactococcus lactis*, the reduction in CLA concentration was less marked than in the other co-cultures.

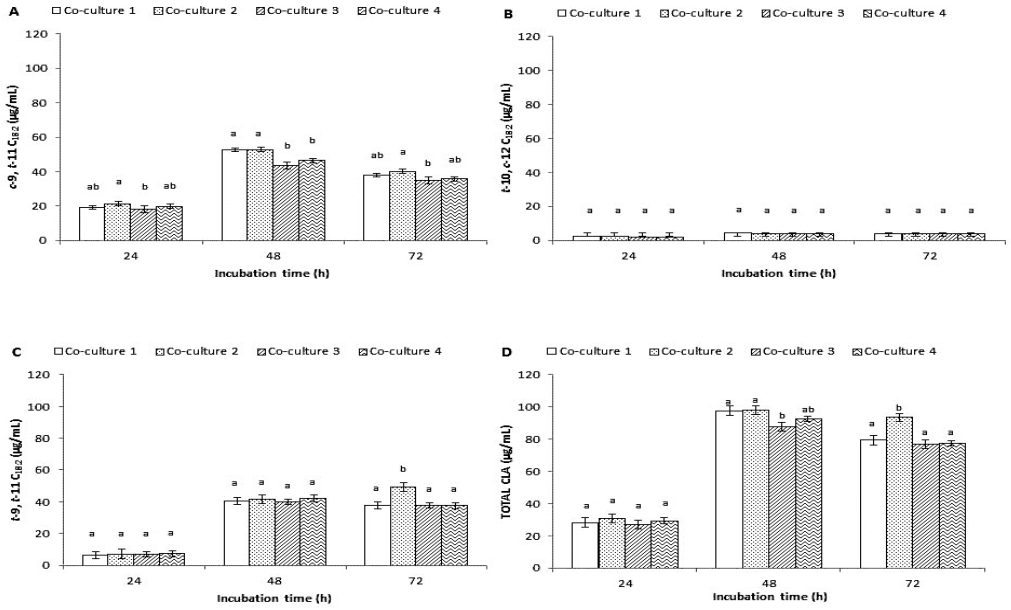

Figure 3. Co-cultures production of (A) *cis*-9,*trans*-11 CLA isomer; (B) *trans*-10,*cis*-12 CLA isomer; (C) *trans*-9,*trans*-11 CLA isomer; (D) total CLA in MRS broth after 24, 48 and 72 h of incubation at 30°C. Data represent mean ± SD, n=2. Means for the same incubation time with different lowercase letters are significant at $P \leq 0.05$.

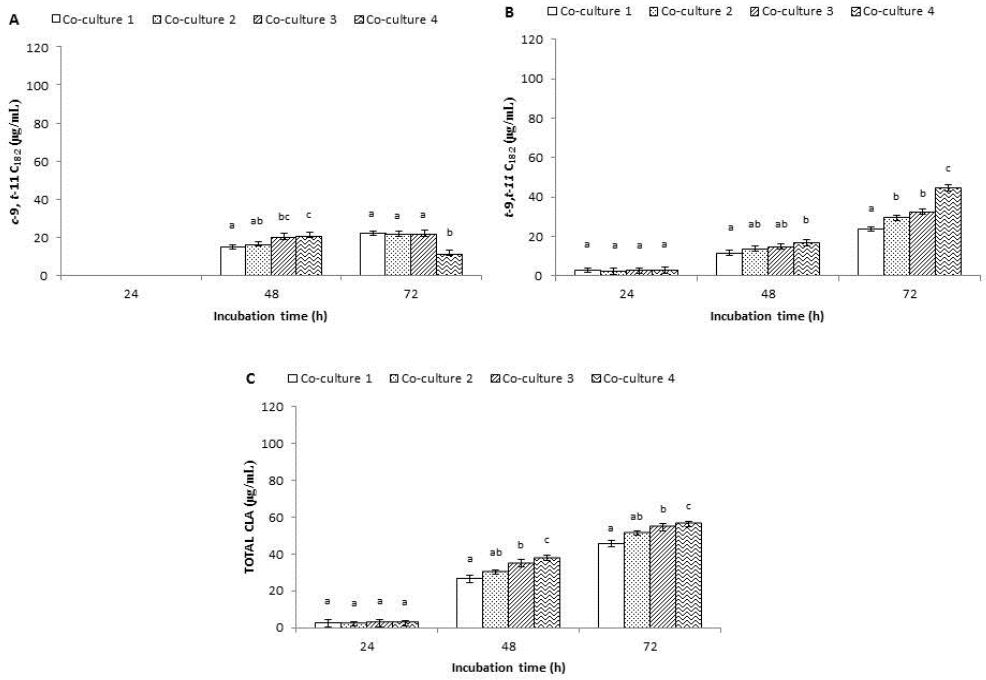

Figure 4. Starters production of (A) *cis*-9,*trans*-11 CLA isomer; (B) *trans*-9,*trans*-11 CLA isomer; (C) total CLA in reconstituted skim milk (RSM) after 24, 48 and 72 h of incubation at 30°C. Data represent mean ± SD, n=2. Means for the same incubation time with different lowercase letters are significant at $P \leq 0.05$.

In MRS medium, the combination of CLA-producing strains from different species did not increase CLA concentrations at any incubation interval.

In skim milk, however, total CLA content and content of each of the isomers produced in skim milk by the four co-cultures at different incubation times (**Figure 4**) was different to that observed in MRS medium. Content of the isomer *cis*-9,*trans*-11 $C_{18:2}$ presented statistically significant differences ($P \leq 0.05$) between co-cultures at 48 and 72 h incubation. For the latter incubation time, co-cultures 1, 2 and 3 produced a higher concentration of this isomer, whereas with co-culture 4 it dropped by 9.40 µg/mL with respect to levels at 48 h. The higher concentration of the isomer *trans*-9,*trans*-11 $C_{18:2}$ produced by co-culture 4 at 72 h incubation might be related both to its synthesis from LA and to a greater interconversion of the isomer *cis*-9,*trans*-11 $C_{18:2}$ towards this, in agreement with Ogawa et al. (2005). In turn, this would explain the reduction observed earlier in the concentration of the isomer *cis*-9,*trans*-11 $C_{18:2}$ produced by co-culture 4.

As was the case when we studied CLA production by *Lactobacillus* strains in skim milk medium, none of the co-cultures was capable of synthesising the isomer *trans*-10,*cis*-12 $C_{18:2}$.

Highest total CLA production occurred at 72 h incubation. The highest CLA concentration was produced by co-culture 4, which consisted of autochthonous strains of *Lactococcus lactis* and the CLA-producing strains *Lactobacillus plantarum* TAUL 1588 and

Lactobacillus casei subsp. *casei* SS 1644. Co-cultures 1 and 2 synthesised the lowest concentrations of CLA, neither of which included the *Lactobacillus casei* subsp. casei strain; as observed in the study of strains, this species synthesised CLA more efficiently in skim milk than *Lactobacillus plantarum*. Accordingly, the presence of *Lactobacillus casei* subsp. casei SS 1644 in skim milk yielded a greater increase in CLA production in this medium than when using the *Lactobacillus plantarum* TAUL 1588 strain in isolation. The presence of autochthonous *Lactococcus lactis* strains significantly improved ($P < 0.05$) CLA production in skim milk medium in the case of co-culture 4 with respect to co-culture 3 and of co-culture 2 with respect to co-culture 1.

CONCLUSIONS

In this study, we found that the strains *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS 1644 presented a good capacity to produce CLA, could be used as co-cultures together with *Lactococcus lactis* strains, and were more effective in combination with autochthonous strains of this species. The results reported here open the way for the use of co-cultures containing CLA-producing strains to manufacture cheese and thus obtain functional dairy products rich in bioactive fatty acids.

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CAPÍTULO 4

CLA-producing adjunct cultures improve the nutritional value of sheep cheese fat

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Abstract

The influence of the autochthonous non-starter *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS 1644 strains, previously identified as conjugated linoleic acid (CLA) producers *in vitro*, and the ripening time on the fatty acid (FA) content and sensory characteristics of sheep cheese were investigated. Three batches with different cultures and the control batch were produced in duplicate and ripened for 8 months. 86 individual FA were determined by gas chromatography. Ripening time (2, 90, 180 and 240 days) did not have a significant effect ($P > 0.05$) on the FA content. However, the presence of both *Lactobacillus* CLA-producing strains led to a decrease of the total saturated FA content and to 1.30, 1.19 and 1.27 times higher levels of vaccenic acid, CLA and *omega*-3, respectively, when compared to the control batch. This combination resulted in sheep milk cheeses with a healthier FA content, without appreciable changes on sensory characteristics. This work could be a promising approach to increase the bioactive fatty acid content of cheeses.

INTRODUCTION

The development of foods with beneficial effects on human health is an aspect of great interest to the food industry and especially to the dairy industry (Balthazar et al., 2017). In relation to healthy food trends, milk and fermented dairy products contain lipid compounds that may exert beneficial effects on human health, such as conjugated linoleic acid (CLA) (Koba & Yanagita, 2014; Lock, Kraft, Rice, & Bauman, 2009; Tanaka, 2005). In contrast, dairy fat is a poor source in other bioactive lipids as *omega*-3 fatty acids (Parodi, 2004).

CLA is a group of positional and geometric isomers of octadecadienoic acid which are naturally found in foods derived from ruminants (Shingfield, Bonnet, & Scollan, 2013). They are mainly biohydrogenation intermediates of dietary polyunsaturated fatty acids (PUFA). Rumenic acid or *cis*-9,*trans*-11 $C_{18:2}$, the major CLA isomer in dairy foods, is also synthesized in the ruminant mammary gland by the action of the Δ-9 desaturase enzyme on vaccenic acid (*trans*-11 $C_{18:1}$) (Bichi et al., 2012), which is another intermediary in the ruminal biohydrogenation.

Balthazar et al. (2017) indicated that the nutritional value of sheep milk, mostly used for cheese manufacturing, could be higher than milk from other ruminants. In addition, various functional dairy products have been developed from sheep milk (Bárcenas et al., 2007). In the Mediterranean region, most sheep-milk production systems are semi-extensive, and grass plays an important role in the flocks' diet. As Gómez-Cortés et al. (2009) pointed out, the supply of grass and thus *omega*-3 PUFA in the livestock diet represents one of the strategies to enhance the content of these fatty acids and CLA in milk, and subsequently also in cheese (Shingfield et al., 2013).

Several studies have shown that strains of lactic acid bacteria (LAB) are able to synthesize CLA from linoleic acid in culture medium or milk (Gorissen et al., 2010; Ogawa et al., 2005; Renes et al., 2017a). This fact has opened new research lines aimed at increasing the CLA content of cheese by the use of starters or adjunct cultures able to biosynthesize CLA *in vitro*. The design of autochthonous CLA-producing cultures with application for the production of functional cheeses could increase the diversity of cultures available to the dairy industry but, research in this topic remains still very limited. Taboada, Van Niewenhove, Alzogaray, and Medina (2015) have shown that the CLA content of goat cheese could be increased by the use of autochthonous LAB strains and the same was observed by Mohan, Anand, Kalscheur, Hassan, and Hippen (2013) in Cheddar cheese. To the best of our knowledge, there is no information about the effect of the combination of different autochthonous CLA-producing strains used as a mixed adjunct culture on the fatty acid content of sheep milk cheese.

The objective of this study was to investigate the effect of two autochthonous *Lactobacillus* strains used as adjunct cultures, previously identified and characterized *in vitro* as CLA producers (Renes et al., 2017a, 2017b), to improve the nutritional value of sheep cheese fat without modifying the sensory properties of the cheeses.

MATERIAL AND METHODS

Preparation of cultures

The autochthonous *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lactococcus lactis* subsp. *cremoris* TAUL 1239 strains were selected for their good technological aptitude showed in previous studies (Renes et al., 2017b) and both *Lactococcus* strains were the starter culture. The autochthonous *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS 1644 strains were selected for their good technological characteristics as well as for their ability to synthesize CLA in skim milk (Renes et al., 2017b). These *Lactobacillus* strains, individually or in combination, were used as adjunct cultures.

First, each LAB strain was cultured in MRS broth (Oxoid, Hampshire, UK) for *Lactobacillus* or Elliker broth (BD Difco, New Jersey, USA) for *Lactococcus lactis* at 30ºC for 24 h. Then, each strain was cultured in sterilised reconstituted skim milk (10%, w/v) at 30ºC for 24 h. Total viable counts (CFU/g) were determined by plating serial dilutions on MRS or Elliker agar (Oxoid, Hampshire, UK) and no significant differences ($P > 0.05$) were observed (data not shown). The strain composition of the designed cultures for cheese manufacture is shown in **Table 1**. Finally, each activated autochthonous cultures were transferred at 1% (v/v) to sterilized sheep milk and incubated for 48 h at 30ºC.

Milk and cheese manufacture

Milk was obtained from a farm of *Castellana* breed sheep fed *ad libitum* with natural pasture.

Four sheep cheese batches were produced in duplicate at pilot scale (Institute of Food Science and Technology (ICTAL), University of León, Spain) according to the following method: milk was pasteurized at 72ºC for 15 s and after cooling at 31ºC, calcium chloride (0.2 g/L) and starter culture or starter culture plus adjunct (1%, v/v) were added. Batch 1 was the control and it was produced with the first autochthonous culture (non CLA-producing) indicated in **Table 1**. Batches 2, 3, and 4 were manufactured with the autochthonous cultures 2, 3, and 4 (CLA-producing), respectively (**Table 1**). After 30 min, chymosin (CHY-MAX Extra, 100 % chymosin; 600 IMCU/mL; Chr. Hansen SL, Madrid, Spain) was added at a rate of 0.05 mL/L of milk (diluted in 1:20 with deionized water).

After 40-45 min, the curd was cut to rice grain size and the whey was drained off. The curd was transferred to cylindrical moulds (15 cm height, 21 cm diameter) which were pressed for 2 h.

Table 1. Strain composition (%, v/v)[1] of autochthonous cultures.

Strains	Cultures			
	1	2	3	4
Lc. lactis spp. *lactis* TAUL 238 (non CLA-producing)	50	30	30	30
Lc. lactis spp. *cremoris* TAUL 1239 (non CLA-producing)	50	30	30	30
Lb. plantarum TAUL 1588 (CLA-producing)	-	40	-	20
Lb. casei spp. *casei* SS1644 (CLA-producing)	-	-	40	20

[1]volume of each individual strain activated in milk respect to the final volume of the mixed culture in sheep milk.

Then, cheeses were salted by immersion (18ºBaume, 8ºC and pH 5.4) for 17 h. Finally, the cheeses were taken to a ripening chamber where they remained at a temperature of 10ºC and at 80 - 85% relative humidity for 240 days.

Samples (each sample corresponded to a whole cheese of 2.5 kg) were taken from each case after 2, 90, 180 and 240 days of ripening. Part of the fresh samples was used to carry out the colour, texture and sensory analyses and another portion of the samples was vacuum packed and stored in a freezer (-30ºC) until fatty acid analysis.

Determination of the fatty acids content in sheep cheese

Cheese fat extraction was carried out using n-pentane after grind the sample with a mixture of sand and sodium sulfate (Bodas et al., 2010). Fatty acids were derivatized to methyl esters (FAME) by base-catalyzed methanolysis of glycerides with KOH in methanol (Bichi et al., 2012). FAME were analysed by gas chromatography with two different columns, CP-Sil 88 (100 m × 0.25 mm i.d., Varian) and SLB-IL111 capillary column (100 m × 0.25 mm i.d., Supelco). Detailed gas chromatography methods, identification of unknown FAME and quantification are described in a previous study (de la Fuente, Rodríguez-Pino, & Juárez, 2015). Sheep milk cheeses were analysed for fatty acid composition after 2, 90, 180 and 240 days of ripening. The health indexes considered were: the *omega*-6/*omega*-3 ratio and the atherogenicity index (AI) defined as $[(C_{12:0} + 4\times C_{14:0} + C_{16:0})/(\Sigma \text{ unsaturated FA})]$ (Ulbricht & Southgate, 1991).

Texture profile analysis

Texture analysis was performed on eight cube-shaped (1.9 cm^3) samples obtained from each cheese batch through the ripening time (2, 90, 180, 240 days) at room temperature (20ºC ± 2ºC). The cheese samples were kept at room temperature for approximately 3 h before analysis and a 0.5 cm layer from the surface of the cheese was removed. Texture properties of the batches were determined in two successive cycles of 80% compression with a cross-head constant speed of 0.5 mm/s using a TZ-XT2 texture analyser (Stable Micro Systems, Godalming, UK). The following textural parameters were determined from the resultant force-time curve using the Texture Expert software (Stable Micro Systems, Godalming, UK): hardness (N), springiness, cohesiveness and chewiness (Pinho, Mendes, Alves, & Ferreira, 2004).

Colour instrumental measurement

Colour analyses of sheep milk cheeses were performed throughout the ripening time (2, 90, 180, 240 days) using a reflectance colorimeter spectrophotometer CM- 700 d (Konica Minolta, Osaka, Japan) provided with a measuring glass head of 8 mm of diameter, an illuminant D65 and a 10° observer. The L*, a*, and b* colour measurements were determined according to the CIELab colour space, were L* corresponds to light/dark chromaticity (0% dark to 100% light), a* to green/red chromaticity (−60% green to 60% red), and b* to blue/yellow chromaticity (−60% blue to 60% yellow) (Pinho et al., 2004). The determination by triplicate of colour parameters was carried out measuring 12 different places on the longitudinal cheese sample surface (1 cm thick).

Sensory analysis

The sensory evaluation of the 4 cheese batches at 2 time points (180 and 240 days of ripening) was carried out by 20 panellists recruited from the Food Hygiene and Technology Department of the University of León, in gender proportion of 12 female and 8 male with ages between 22 and 60 years. Prior to the sensory evaluation of the cheese batches, the panel's members were trained in five training sessions of 1 h with commercial sheep milk cheeses. In this training, odour, flavour and texture attributes were defined and quantified according to the methodology previously described by Bárcenas et al. (2007) and Fresno and Álvarez (2012) for semi-hard and hard cheeses.

Cheese pieces of the same dimension (4 cm × 1.5 cm × 0.5 cm) of each batch were presented to the panel at ambient temperature (20ºC ± 2ºC) and identified with a randomly 3-digit code in one session for cheese batches at 180 d of ripening and another session for cheese batches at 240 d of ripening. Panel assessed a total of 15 sensory parameters, divided into 4 main groups: appearance (colour intensity ranging from white to yellow, holes size and homogenous distribution of the holes), odour (odour intensity, butyric, pungent and mouldy), taste (saltiness, bitterness, sweetness and acidity), and texture (elasticity, adhesiveness, firmness and solubility). These attributes were recorded on a 7-point intensity scale containing the following descriptors: (1) non-existent, (2) very weak, (3) weak, (4) moderate, (5) strong, (6) very strong and (7) extremely strong.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS v.21 (SPSS, Chicago, IL, USA). The variables studied were tested for the assumption of normality using the Shapiro-Wilk test and for homoscedasticity using the Levene test. Subsequently, a two-way Analysis of Variance (ANOVA) was performed in order to evaluate the effect of the factors culture and ripening time (as fixed factors) and the interaction between them. Tukey's HSD *post hoc* test was applied at a 5% significance level in order to compare sheep cheeses manufactured with different autochthonous cultures throughout the ripening period.

The chromatographic data obtained were also analysed by multi-variant statistical techniques (Principal Component Analysis and Euclidean distance squared) by means of the statistical package Minitab 16 (Minitab Incorporated, 2010).

RESULTS AND DISCUSSION

Cheese fatty acid content

The study of the fatty acid composition of four sheep cheese batches produced with different autochthonous cultures was carried out throughout the ripening (at time points 2, 90, 180 and 240 days). In all cheese batches, no statistically significant differences ($P > 0.05$) were observed for the 86 fatty acids contents analysed during the different ripening time points. As the ripening time effect and the interaction between cultures and ripening time effects were no significant ($P > 0.05$) , only means for the cultures effect are presented in **tables 2 and 3**. Similarly to this study, several authors have confirmed that ripening time does not affect the fatty acid content of cheeses (Bodas et al., 2010; dos Santos et al., 2012; Luna, Juárez, & de la Fuente, 2007).

On the contrary, the type of culture used in sheep cheese-making, had a significant effect ($P \leq 0.05$) on the fatty acid composition. Saturated fatty acid (SFA) contents of the four batches are shown in **Table 2**. SFA were the predominant fatty acids in the cheeses accounting for 73% of total FAME in the control batch, 72% in batches 2 and 3, and 70% in the batch 4. Palmitic acid ($C_{16:0}$) was found in major proportion, followed by myristic acid ($C_{14:0}$). These results were similar to those described by Bodas et al. (2010) in their study of the fatty acid content of sheep cheeses produced with milk from flocks fed with different diets.

Table 2. Saturated, including odd and branched-chain, fatty acid composition (g/100 g of total fatty acids methyl esters) of the four sheep cheese batches produced with different cultures.

Variable[1]	Batch[2]				SE[3]	*P- value*
	1	2	3	4		
Total SFA	73.08^{a}	71.55^{b}	71.55^{b}	70.22^{c}	0.19	***
Σ Non-BCFA	71.27^{a}	69.89^{b}	69.89^{b}	68.75^{c}	0.18	***
4:0	4.20^{a}	4.30^{a}	4.34^{a}	4.26^{a}	0.03	NS
5:0	0.03^{a}	0.03^{a}	0.03^{a}	0.05^{b}	0.00	***
6:0	3.24^{ab}	3.20^{a}	3.39^{bc}	3.54^{c}	0.03	***
7:0	0.04^{a}	0.03^{a}	0.04^{a}	0.06^{b}	0.00	***
8:0	2.85^{a}	2.73^{a}	3.00^{b}	3.33^{c}	0.04	***
9:0	0.06^{a}	0.05^{b}	0.06^{a}	0.10^{c}	0.00	***
10:0	7.98^{a}	7.33^{b}	7.97^{a}	8.64^{c}	0.09	***
11:0	0.06^{a}	0.05^{b}	0.06^{a}	0.10^{c}	0.00	***
12:0	4.39^{a}	3.89^{b}	4.12^{c}	4.11^{c}	0.04	***
13:0	0.08^{a}	0.07^{b}	0.08^{a}	0.09^{c}	0.00	***
14:0	10.71^{a}	10.15^{b}	9.83^{c}	9.15^{d}	0.10	***
15:0	0.98^{a}	0.82^{b}	0.85^{b}	0.88^{c}	0.01	***
16:0	26.08^{a}	25.79^{a}	24.57^{b}	22.44^{c}	0.27	***
17:0	0.63^{a}	0.55^{b}	0.55^{b}	0.49^{c}	0.01	***
18:0	9.37^{a}	10.36^{b}	10.46^{b}	10.77^{c}	0.10	***
20:0	0.26^{a}	0.29^{b}	0.27^{a}	0.24^{c}	0.01	***
21:0	0.05^{a}	0.05^{a}	0.05^{a}	0.05^{a}	0.00	NS
22:0	0.11^{a}	0.10^{ab}	0.09^{bc}	0.08^{c}	0.00	**
23:0	0.06^{a}	0.05^{ab}	0.04^{b}	0.03^{b}	0.00	**
24:0	0.06^{a}	0.05^{ab}	0.05^{ab}	0.04^{b}	0.00	**
Σ BCFA	1.82^{a}	1.67^{b}	1.66^{b}	1.58^{c}	0.02	***
13:0 *iso*	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.00	NS
13:0 *anteiso*	0.04^{a}	0.04^{a}	0.04^{a}	0.03^{b}	0.00	*
14:0 *iso*	0.09^{a}	0.07^{b}	0.07^{b}	0.06^{c}	0.00	***
15:0 *iso*	0.22^{a}	0.22^{a}	0.21^{b}	0.18^{c}	0.00	***
15:0 *anteiso*	0.42^{a}	0.37^{b}	0.39^{c}	0.39^{c}	0.00	***
16:0 *iso*	0.21^{a}	0.21^{a}	0.20^{a}	0.17^{b}	0.00	***
17:0 *iso*	0.23^{a}	0.24^{a}	0.23^{a}	0.23^{a}	0.00	NS
17:0 *anteiso*	0.52^{a}	0.44^{b}	0.45^{b}	0.44^{b}	0.01	***
18:0 *iso*	0.06^{a}	0.06^{a}	0.06^{a}	0.05^{b}	0.00	**

[1]BCFA=branched-chain fatty acids; SFA= saturated fatty acids.
[2]1: control cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; 2: cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; 3: cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; 4: cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.
[3]SE=standard error. Results as mean values of duplicate determination of each fatty acid at 2, 90, 180 and 240 days of ripening.
[a-d]Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
[NS]$P > 0.05$; [*]$P \leq 0.05$; [**]$P \leq 0.01$; [***]$P \leq 0.001$.

From a nutritional point of view, decreases of approximately 1.17 points on $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ contents in batch 4 were important and positive. This is because all these

fatty acids are preferentially stored as body fat and are considered as hypercholesterolemic (Parodi, 2004).

Furthermore, one of the most important results of this study was that the combination of two strains of *Lactobacillus* in the culture used for cheese-making (batch 4) led to a significant ($P \leq 0.001$) higher concentration of $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$ (1.09, 1.17 and 1.08 more times, respectively), which have been associated with positive effects on human health (Nagao & Yanagita, 2010; Parodi, 2004). These fatty acids are easily digestible, show a low tendency to be stored in the adipose tissue, are preferentially hydrolysed from the triglycerides molecules and are transferred directly from the intestine to the bloodstream. Afterwards, they are transported as free fatty acids to the liver where they are metabolized via mitochondrial β-oxidation without triglycerides resynthesize, acting as a quick energy source for cells. Furthermore, these short-chain fatty acids also play an interesting role on the sensory characteristics of cheeses due to their lower perception thresholds in comparison to longer-chain fatty acids (Laskaridis et al., 2013).

Cheese produced with the combination of both *Lactobacillus* strains showed the lowest concentration of $C_{17:0}$ and branched-chain fatty acids (BCFA) in comparison to cheeses produced with individual strains. This fact showed that the metabolism of the cultures used for cheese-making played an important role in the content of these minor FA in cheese, confirming the relationship between the production of odd and BCFA and the type of microorganism involved in the fermentative process.

The total amount of monounsaturated fatty acids (MUFA) was significantly different ($P \leq 0.05$) increasing from 22.68% in the batch 1, up to 24.64% of total fatty acids in batch 4 (**Table 3**). Batches 2 y 3 presented an intermediate proportion (23.96% and 23.68%, respectively) of MUFA between the other two batches.

Oleic acid ($C_{18:1}$ *cis*-9) was the fatty acid of the MUFA group that was detected in the highest proportion for the four sheep cheese batches, ranging between 78-80% of the total MUFA analysed. Differently, Mohan et al. (2013) detected that Cheddar cheese produced with a CLA-producing strain had a lower content of oleic acid compared to the control cheese. In the present study, it was observed that the use of a combination of cultures including autochthonous CLA-producing strains generated increases of *cis*-$C_{18:1}$ isomers, mainly *cis*-9 (**Table 3**). This trend was also observed in the case of the *trans*-MUFA, being vaccenic acid the major isomer of *trans*-$C_{18:1}$ content in sheep cheese, which represented approximately 44% of total *trans*-$C_{18:1}$. Similarly, dos Santos et al. (2012) and Taboada et al. (2015) detected 1.49% - 2.01% of vaccenic acid in goat cheese using CLA-producing strains. High vaccenic acid content in cheese would be desirable since it can be used by humans for the endogenous synthesis of rumenic acid (through the stearoyl Co-A enzyme) providing the beneficial effects on human health that have been attributed to this CLA isomer (Turpeinen et al., 2002).

The type of culture affected the PUFA content of sheep cheeses in this study (**Table 3**). Control batch presented 4.22% of total PUFA followed by batch 2 with 4.47 %. The PUFA proportion of the batches 3 and 4 was 4.75 % and 4.99%, respectively. Within this fatty acids group it is important to highlight the great importance of CLA and *omega*-3 fatty acids because they have been associated with beneficial effects on human health and the intake of these fatty acids through food is essential to achieve these beneficial effects (Swanson, Block, & Mousa, 2012; Yang et al., 2015). Batch 4 produced using the

combination of *Lb. plantarum* TAUL 1588 and *Lb. casei* subsp. *casei* SS 1644 in the culture contained 1.19 times higher content of total CLA than the control cheese and batches 2 and 3.

Table 3. Monounsaturated and polyunsaturated fatty acid composition (g/100 g of total fatty acids methyl esters) and fatty acid indexes of the four sheep cheese batches produced with different cultures.

Variable[1]	Batch[2]				SE[3]	*P- value*
	1	2	3	4		
Total MUFA	22.68^{a}	23.96^{b}	23.68^{b}	24.64^{c}	0.116	***
Σ *cis*-MUFA	19.79^{a}	20.93^{b}	20.63^{b}	20.83^{b}	0.105	***
10:1	0.26^{a}	0.26^{a}	0.26^{a}	0.26^{a}	0.003	NS
12:1 *c*-11	0.06^{a}	0.05^{b}	0.05^{b}	0.06^{a}	0.001	***
14:1 *c*-9	0.16^{a}	0.15^{ab}	0.14^{b}	0.12^{c}	0.004	***
Σ *cis*-16:1	0.96^{a}	0.92^{bc}	0.91^{c}	0.94^{ab}	0.007	***
16:1 *c*-7	0.21^{a}	0.21^{a}	0.23^{b}	0.25^{c}	0.004	***
16:1 *c*-8	0.02^{a}	0.02^{a}	0.02^{a}	0.03^{b}	0.002	**
16:1 *c*-9	0.62^{a}	0.59^{b}	0.56^{c}	0.53^{d}	0.005	***
16:1 *c*-10	0.07^{a}	0.06^{ab}	0.06^{ab}	0.05^{b}	0.003	*
16:1 *c*-11	0.03^{a}	0.03^{a}	0.03^{a}	0.05^{b}	0.003	***
16:1 *c*-13	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.002	NS
17:1 *c*-9	0.21^{a}	0.18^{b}	0.17^{bc}	0.16^{c}	0.005	***
Σ *cis*-18:1	18.11^{a}	19.31^{b}	19.05^{b}	19.23^{b}	0.102	***
18:1 *c*-9	17.00^{a}	18.14^{b}	17.81^{b}	17.88^{b}	0.100	***
18:1 *c*-11	0.65^{a}	0.67^{a}	0.69^{a}	0.76^{b}	0.014	***
18:1 *c*-12	0.30^{a}	0.33^{b}	0.33^{b}	0.32^{b}	0.003	***
18:1 *c*-13	0.03^{a}	0.03^{a}	0.04^{b}	0.05^{c}	0.002	***
18:1 *c*-15	0.07^{a}	0.06^{a}	0.10^{b}	0.12^{c}	0.004	***
18:1 *c*-16	0.07^{a}	0.08^{ab}	0.09^{bc}	0.10^{c}	0.003	***
20:1 *c*-11	0.04^{a}	0.04^{a}	0.04^{a}	0.04^{a}	0.002	NS
Other 20:1	0.00^{a}	0.01^{b}	0.01^{b}	0.01^{b}	0.001	***
Σ *trans*-MUFA	2.89^{a}	3.04^{b}	3.05^{b}	3.81^{c}	0.020	***
15:1	0.05^{a}	0.04^{b}	0.04^{b}	0.04^{b}	0.002	**
Σ *trans*-16:1	0.22^{a}	0.21^{a}	0.22^{a}	0.24^{b}	0.005	***
16:1 *t*-4	0.01^{a}	0.01^{a}	0.01^{a}	0.01^{a}	0.001	NS
16:1 *t*-5	0.01^{a}	0.01^{a}	0.01^{a}	0.01^{a}	0.001	NS
16:1 *t*-6	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.002	NS
16:1 *t*-7+ *t*-8	0.03^{a}	0.03^{a}	0.03^{a}	0.03^{a}	0.002	NS
16:1 *t*-9	0.12^{a}	0.12^{a}	0.12^{a}	0.14^{b}	0.002	***
16:1 *t*-10	0.02^{a}	0.03^{ab}	0.03^{ab}	0.04^{b}	0.002	**
Σ *trans*-18:1	2.62^{a}	2.80^{b}	2.79^{b}	3.53^{c}	0.019	***
18:1 *t*-4	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.001	NS
18:1 *t*-5	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.002	NS
18:1 *t*-6+ *t*-7+ *t*-8	0.21^{a}	0.22^{a}	0.22^{a}	0.24^{b}	0.003	***
18:1 *t*-9	0.23^{a}	0.26^{b}	0.23^{a}	0.26^{b}	0.006	**
18:1 *t*-10	0.29^{a}	0.35^{b}	0.36^{b}	0.47^{c}	0.009	***
18:1 *t*-11 (VA)	1.19^{a}	1.17^{ab}	1.13^{b}	1.55^{c}	0.013	***
18:1 *t*-12	0.41^{a}	0.46^{b}	0.46^{b}	0.54^{c}	0.005	***
18:1 *t*-16+*c*-14	0.26^{a}	0.31^{b}	0.36^{c}	0.44^{d}	0.003	***

[1]AI = atherogenicity index, calculated according to Ulbricht and Southgate (1991); CLA= conjugated linoleic acid; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; RA= rumenic acid; VA= vaccenic acid.

[2]1: control cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; 2: cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; 3: cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; 4: cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.

[3]SE=standard error. Results as mean values of duplicate determination of each fatty acid at 2, 90, 180 and 240 days of ripening.

[a-d]Means in the same row with different superscripts are significantly different ($P \leq 0.05$).

[NS]$P > 0.05$; [*]$P \leq 0.05$; [**]$P \leq 0.01$; [***]$P \leq 0.001$.

Table 3 (continuation). Monounsaturated and polyunsaturated fatty acid composition (g/100 g of total fatty acids methyl esters) and fatty acid indexes of the four sheep cheese batches produced with different cultures.

Variable[1]	Batch[2]				SE[3]	*P- value*
	1	2	3	4		
Total PUFA	4.22[a]	4.47[b]	4.75[c]	4.99[d]	0.036	***
Σ non-conjugated 18:2	2.78[a]	3.06[b]	3.20[c]	3.31[d]	0.020	***
18:2 *c*-9,*t*-13 + *t*-8,*c*-12	0.27[a]	0.31[b]	0.35[c]	0.41[d]	0.005	***
18:2 *c*-9,*t*-12 + *t*-8,*c*-13	0.13[a]	0.15[b]	0.16[b]	0.18[c]	0.002	***
18:2 *t*-9,*c*-12	0.03[a]	0.03[a]	0.03[a]	0.02[a]	0.002	NS
18:2 *t*-11,*c*-15	0.05[a]	0.04[a]	0.11[b]	0.18[c]	0.006	***
18:2 other *t,t*	0.17[a]	0.18[a]	0.18[a]	0.18[a]	0.004	NS
18:2 *c*-9,*c*-12	2.11[a]	2.34[bc]	2.37[b]	2.32[c]	0.013	***
18:2 *c*-9,*c*-15	0.01[a]	0.01[a]	0.02[b]	0.02[b]	0.001	***
Σ conjugated 18:2 (CLA)	0.58[a]	0.56[b]	0.54[c]	0.69[d]	0.004	***
18:2 *t*-7,*c*-9	0.05[a]	0.07[a]	0.05[a]	0.06[a]	0.005	NS
18:2 *c*-9,*t*-11 (RA)	0.48[a]	0.44[b]	0.44[b]	0.58[c]	0.006	***
18:2 *t*-11,*c*-13	0.01[a]	0.01[a]	0.01[a]	0.01[a]	0.001	NS
18:2 *t*-11,*t*-13	0.01[a]	0.01[a]	0.01[a]	0.01[a]	0.001	NS
18:2 other *t,t*	0.03[a]	0.03[a]	0.03[a]	0.03[a]	0.002	NS
Total 18:2	3.36[a]	3.62[b]	3.74[c]	3.99[d]	0.021	***
18:3 *c*-9,*t*-11,*c*-15	0.05[a]	0.06[a]	0.06[a]	0.08[b]	0.004	**
Σ omega-6	2.40[a]	2.63[bc]	2.68[b]	2.58[c]	0.019	***
18:3 n-6	0.04[a]	0.04[a]	0.04[a]	0.04[a]	0.002	NS
20:2 n-6	0.03[a]	0.02[a]	0.02[a]	0.02[a]	0.002	NS
20:3 n-6	0.02[a]	0.02[a]	0.02[a]	0.02[a]	0.001	NS
20:4 n-6	0.18[a]	0.17[a]	0.19[a]	0.14[b]	0.014	**
22:4 n-6	0.03[a]	0.03[a]	0.04[a]	0.03[a]	0.003	NS
Σ omega-3	0.52[a]	0.50[a]	0.63[b]	0.66[b]	0.010	***
18:3 n-3	0.37[a]	0.36[a]	0.48[b]	0.49[b]	0.008	***
20:5 n-3	0.04[a]	0.03[a]	0.03[a]	0.04[a]	0.002	NS
22:5 n-3	0.09[a]	0.09[a]	0.10[a]	0.12[b]	0.005	**
22:6 n-3	0.03[a]	0.02[a]	0.03[a]	0.02[a]	0.002	NS
omega-6/omega-3	4.63[a]	4.23[b]	4.24[b]	3.89[c]	0.074	***
AI	2.73[a]	2.47[b]	2.39[b]	2.14[c]	0.040	***

[1]AI = atherogenicity index, calculated according to Ulbricht and Southgate (1991); CLA= conjugated linoleic acid; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; RA= rumenic acid; VA= vaccenic acid.

[2]1: control cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; 2: cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; 3: cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; 4: cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.

[3]SE=standard error. Results as mean values of duplicate determination of each fatty acid at 2, 90, 180 and 240 days of ripening.

[a-d]Means in the same row with different superscripts are significantly different ($P \leq 0.05$).

$^{NS}P > 0.05$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

The combination of the two strains could generate more favourable conditions so that linoleate isomerase activity of both strains was optimal. This fact could explain the highest concentration of total CLA observed in batch 4 with respect to the control batch and batches 2 and 3. Gorissen et al. (2011) indicated that linoleate isomerase activity in LAB depends on environmental conditions such as pH or temperature and it is also strain dependent. Unfortunately, the mechanisms and factors that affect CLA synthesis by LAB remain unknown and it is necessary to carry out further studies.

The chromatographic method used allowed to detect different CLA isomers. Rumenic acid was the major isomer detected in all cheese batches representing more than 80% of total CLA (**Table 3**). This fact was in accordance with that described by other authors in sheep cheese (Mele et al., 2011; Zlatanos, Laskaridis, Feist, & Sagredos, 2002). These values were in agreement with those compiled by El-Salam and El-Shibiny (2014), highlighting the great interest of the combination of CLA-producing strains for cheese-making, since it generated higher concentration of this bioactive fatty acid.

α-linolenic acid was the major *omega*-3 fatty acid in all sheep cheeses (**Table 3**). Batches including the *Lb. casei* subsp. *casei* SS 1644 strain showed higher concentration of this fatty acid. On the contrary, batch 2 containing the *Lb. plantarum* TAUL 1588 strain showed a similar α-linolenic acid content as the control batch. Consequently, *Lb. casei* strain could be responsible for the higher α-linolenic acid contents in sheep cheeses.

Overall, changes in the fatty acid content of the cheeses gave rise to significant differences ($P \leq 0.001$) in the indexes related to human health (**Table 3**). High *omega*-6/*omega*-3 ratio and atherogenicity index (AI) in foods do not imply that the consumption of these foods should be eliminated, but must be kept to a moderate level (Taboada et al., 2015). The *omega*-6/*omega*-3 values obtained for control batch (4.63) were significantly higher ($P \leq 0.001$) than those observed in batches 2, 3 and 4 (4.23, 4.24 and 3.89, respectively). The same trend was observed for the AI values, where the control batch showed the highest value (2.73) for this index. Batches 2 and 3 presented lower ($P \leq 0.001$) AI values (2.47 and 2.39) than the control batch. Finally, the batch that showed the lowest AI values (2.14) was the batch 4.

As Astrup et al. (2016) described, current dietary recommendations indicate there is strong evidence that replacing SFA with MUFA and PUFA, reduces plasma LDL-cholesterol and cardiovascular disease risk. These authors also highlighted that according to the report of the Dietary Guidelines Advisory Committee (2015), strong and consistent evidence indicates that replacing SFA with PUFA reduces the risk of cardiovascular disease events and coronary mortality. However, this occurred when SFA is replaced by an adequate *omega*-6/*omega*-3 ratio (approximately 5:1), because replacement with only *omega*-6 fatty acids may increase the risk of cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of *omega*-3 (a low *omega*-6/*omega*-3 ratio) exert suppressive effects (Simopoulos, 2002). Therefore, the results of the present study showed that the use of the autochthonous CLA-producing *Lactobacillus plantarum* TAUL 1588 and *Lb. casei* subsp. *casei* SS1644 strains as adjuncts for cheese-making enhanced the content of the fatty acids with beneficial properties on human health. This effect was higher when both strains were included in the culture (batch 4) compared to the culture that did not include CLA-producing strains (control batch).

Additionally, a principal component analysis (PCA) was performed in order to get a better picture of the fatty acid content showed by the four sheep batches throughout ripening (**Figure 1**). In the PCA plot, 3 main groups were identified along the first two components, which explained 91% of the total variation. Cheeses produced with the combination of the two strains were located on the right side of the plot and correlated with *cis*-, *trans*-, and total MUFA, non-conjugated and total $C_{18:2}$, total CLA, PUFA, *omega*-6, and *omega*-3. On the contrary, control cheeses were located on the left side of the plot

and associated with the content of total SFA, *omega*-6/*omega*-3 ratio and AI. Cheeses that were part of batches 2 and 3 constituted a single group that presented intermediate values between batches 1 and 4. Therefore, these results confirm that there is almost no variation related to the cheese-making process itself, and that the type of culture used in cheese-making is the predominant factor influencing cheese fatty acid content **(Tables 2 and 3)**. These results also confirmed that the autochthonous cultures previously detected as CLA-producers *in vitro* presented this ability when cheeses were produced (Renes et al., 2017b). Therefore, the results of the present study are of great interest in obtaining dairy products with improved fatty acid content from sheep milk.

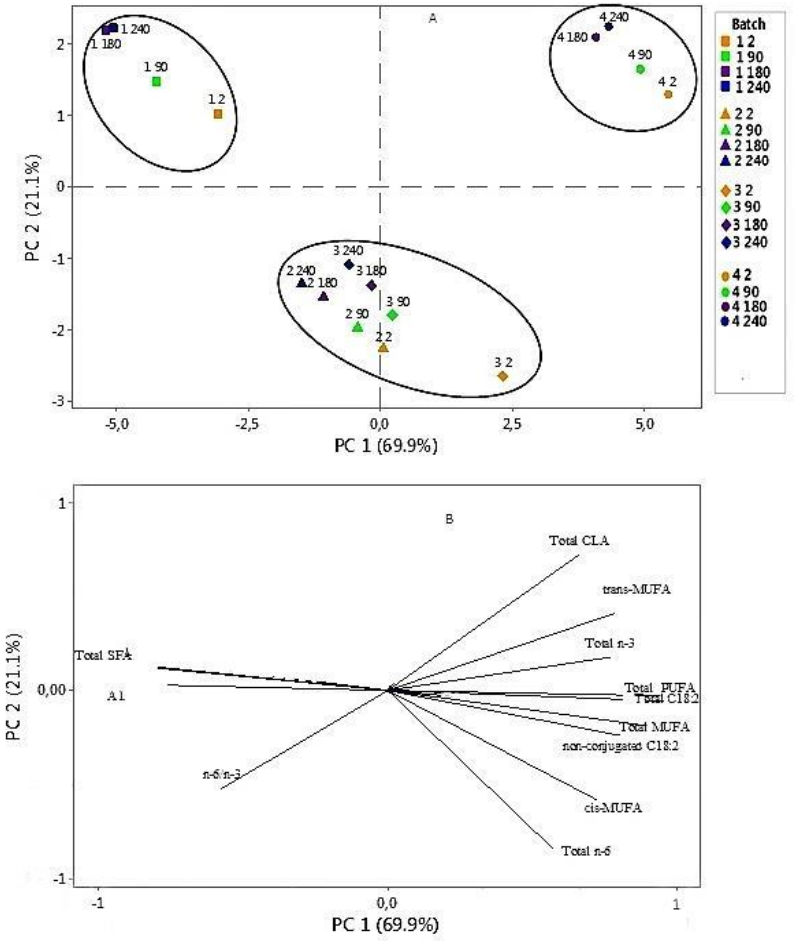

Figure 1. Score plot (A) and loading vectors (B) of the variables after principal component analysis of the fatty acid composition of the four different batches of sheep cheese throughout ripening (2, 90, 180 and 240 days). CLA= conjugated linoleic acid; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; BCFA= branched-chain fatty acids; SFA= saturated fatty acids.

Texture, colour and sensory analysis

In general, texture, colour and sensory data revealed that the use of different adjunct cultures for cheese-making did not lead to important changes on the analysed parameters.

The mean values obtained for the parameters of the texture profile analysis (TPA) and the colour analysis of the four cheese cases during ripening are presented in **Table 4**. Hardness increased up to 240 days of ripening in all the studied batches. At this ripening time, batches 1 and 2 showed the highest ($P \leq 0.01$) hardness values. In general, cohesiveness and chewiness decreased slightly during the first 90 days of ripening, after which remained constant or increased slightly. However, springiness decreased gradually during all the ripening period, reaching after 240 days a final value 50% lower than the initial at 2 days. At 240 days of ripening, no significant differences ($P > 0.05$) were observed for cohesiveness, springiness and chewiness between the four cheese batches studied. In fact, important changes in texture occur during the first days of cheese ripening because initially the rubbery texture of young cheese is converted to a more homogeneous product and then a gradual change in texture occurs due to hydrolysis of caseins (Pinho et al., 2004).

No significant differences ($P > 0.05$) were observed for the colour parameters studied between the batches throughout 240 days of ripening. In all the cheese batches, a decrease in lightness (L*) and an increase in redness (a*) and yellowness (b*) during cheese ripening was detected.

The mean scores allocated by the panellists for the attributes evaluated in the four cheese batches after 180 and 240 days of ripening are shown in **Figure 2**. The use of the selected strains as cultures did not cause any changes ($P > 0.05$) on the majority of the attributes studied in cheeses after 180 days of ripening. The control batch showed higher values of colour and odour intensity (**Figure 2A**). Regarding the colour attribute, no correlation was found with the results obtained in the instrumental analysis. For this ripening period L*, a* and b* values were similar in the four cheese batches studied. None of the batches showed anomalous odour. At 240 days of ripening, significant differences were detected ($P \leq 0.05$) for the attributes of odour and flavour intensity (**Figure 2 B**). Batches 2 and 3 presented the lowest score for odour and taste intensity. No significant correlation was observed between the values obtained for the texture attributes and those resulting from the TPA for neither of the two ripening times. This was observed due to the fact that panellists reported no noteworthy effect of the culture used for cheese-making on the texture attributes of the cheeses. In any case, even for significantly different attributes, the differences were small, always lower than 1 point in the perception scale.

CONCLUSION

The combination of the autochthonous *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS 1644 strains in the culture 4 led to healthier fatty acid content in the sheep cheese batch produced with it in comparison to the control batch. The sheep cheese batch produced with the culture including both *Lactobacillus* strains as adjunct cultures showed higher vaccenic acid, CLA and *omega*-3 levels, as well as lower $C_{14:0}$ and $C_{16:0}$ content, *omega*-6/*omega*-3 ratio and AI than the control batch.

Table 4. Texture profile analysis and colour measurement of the four cheese batches during 240 days of ripening time.

Ripening time	Batch[1]	Hardness (N)	Cohesiveness	Springiness	Chewiness	L*	a*	b*
2 days	1	101.48 ± 4.76^{aA}	0.19 ± 0.02^{aA}	0.69 ± 0.03^{aA}	12.21 ± 1.02^{aA}	88.53 ± 1.67^{aA}	-1.95 ± 0.10^{aA}	14.58 ± 0.51^{aA}
	2	103.34 ± 6.31^{aA}	0.18 ± 0.01^{aA}	0.69 ± 0.04^{aA}	11.59 ± 0.46^{aA}	88.92 ± 1.00^{aA}	-1.97 ± 0.12^{aA}	14.05 ± 0.46^{aA}
	3	166.85 ± 3.18^{bA}	0.21 ± 0.01^{aA}	0.74 ± 0.04^{aA}	25.86 ± 2.63^{bA}	89.44 ± 0.46^{aA}	-1.90 ± 0.01^{aA}	14.64 ± 0.46^{aA}
	4	162.21 ± 4.37^{bA}	0.18 ± 0.02^{aA}	0.69 ± 0.03^{aA}	19.55 ± 2.76^{cA}	87.32 ± 1.82^{aA}	-1.90 ± 0.10^{aA}	14.33 ± 0.52^{aA}
90 days	1	148.44 ± 1.27^{aB}	0.13 ± 0.01^{aB}	0.53 ± 0.03^{aB}	7.65 ± 5.11^{aAB}	75.94 ± 1.27^{aB}	-2.69 ± 0.13^{aB}	18.81 ± 0.78^{aB}
	2	133.45 ± 8.88^{bB}	0.13 ± 0.01^{aB}	0.36 ± 0.04^{bB}	5.98 ± 0.64^{aC}	75.75 ± 2.054^{aB}	-3.03 ± 0.11^{bB}	18.67 ± 0.42^{aB}
	3	180.84 ± 7.35^{cB}	0.13 ± 0.01^{aB}	0.49 ± 0.05^{aB}	12.42 ± 1.72^{aB}	77.74 ± 1.84^{aB}	-2.25 ± 0.18^{cB}	18.78 ± 0.78^{aB}
	4	174.98 ± 7.43^{cB}	0.13 ± 0.02^{aB}	0.42 ± 0.04^{abB}	9.17 ± 1.20^{aB}	76.53 ± 1.47^{aB}	-2.49 ± 0.22^{acB}	21.06 ± 0.36^{bB}
180 days	1	293.42 ± 7.95^{aC}	0.14 ± 0.01^{aB}	0.38 ± 0.02^{abC}	16.11 ± 1.84^{aC}	75.75 ± 2.45^{aB}	-1.93 ± 0.26^{aA}	18.30 ± 0.77^{aB}
	2	230.37 ± 2.12^{bC}	0.13 ± 0.01^{aB}	0.30 ± 0.03^{aB}	8.62 ± 0.30^{bCB}	75.12 ± 2.42^{aB}	-2.23 ± 0.21^{aA}	19.36 ± 0.88^{aB}
	3	265.65 ± 3.15^{abC}	0.13 ± 0.01^{aB}	0.42 ± 0.05^{bB}	13.64 ± 2.11^{acB}	75.44 ± 1.88^{aC}	-2.25 ± 0.24^{aB}	18.59 ± 0.76^{aB}
	4	252.00 ± 1.73^{abC}	0.13 ± 0.01^{aB}	0.33 ± 0.05^{aC}	10.82 ± 1.35^{bcB}	74.64 ± 0.86^{aB}	-2.29 ± 0.19^{aB}	18.00 ± 0.48^{aB}
240 days	1	324.00 ± 1.52^{aD}	0.14 ± 0.01^{aB}	0.31 ± 0.03^{aC}	13.89 ± 0.93^{aBC}	75.00 ± 1.60^{aB}	-1.89 ± 0.13^{aA}	19.43 ± 0.56^{aB}
	2	289.42 ± 2.81^{abD}	0.13 ± 0.01^{aB}	0.30 ± 0.03^{aB}	11.22 ± 2.10^{aAB}	74.99 ± 1.80^{aB}	-2.22 ± 0.20^{aA}	19.48 ± 0.93^{aB}
	3	267.31 ± 2.09^{bC}	0.13 ± 0.01^{aB}	0.35 ± 0.03^{aC}	11.81 ± 1.82^{aB}	74.84 ± 0.86^{aC}	-2.29 ± 0.19^{aB}	19.39 ± 0.85^{aB}
	4	266.03 ± 2.71^{bD}	0.13 ± 0.01^{aB}	0.32 ± 0.04^{aC}	12.10 ± 1.93^{aB}	74.61 ± 2.48^{aB}	-2.22 ± 0.27^{aAB}	19.87 ± 0.54^{aB}

[1]1: control cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; 2: cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; 3: cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; 4: cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.

[a-d]Means ± standard deviation in the same column with different superscripts (lowercase for differences between batches in the same time of ripening and uppercase for differences between ripening times in each batch) are significantly different ($P \leq 0.05$).

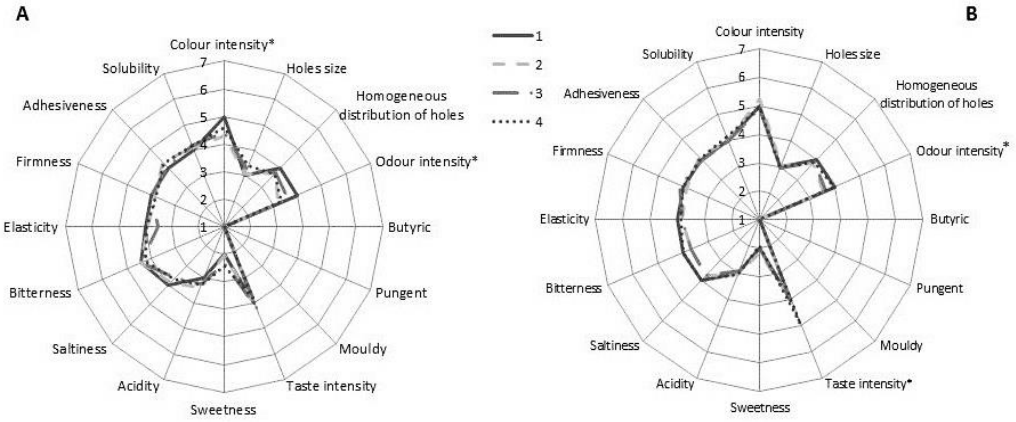

Figure 2. Sensory analysis of the four sheep cheese batches after 180 days (A) and 240 days (B) of ripening. 1: control cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; 2: cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; 3: cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; 4: cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4. *Denotes attributes significantly different (P < 0.05) between batches.

In addition, these fatty acid content changes in sheep cheeses did not generate differences on their sensory characteristics. Overall, the use of these autochthonous CLA-producing cultures could be a promising approach to improve the nutritional quality of cheese fat with special emphasis on bioactive fatty acids, which would be of special interest to the dairy industry in order to meet consumer demands.

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CAPÍTULO 5

Production of sheep milk cheese with high γ-aminobutyric acid and ornithine concentration and with reduced biogenic amines level using autochthonous lactic acid bacteria strains

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Abstract

Consumer demand for health-promoting foods is generating the need to develop biofunctional dairy products. Lactic acid bacteria are employed in cheese-making and some of them are able to produce beneficial compounds on human health such as γ-aminobutyric acid (GABA) and ornithine but also to synthetize biogenic amines. The aim was to investigate the effect of four selected autochthonous cultures on the free amino acid profile, with special emphasis on GABA and ornithine, and on the biogenic amine content of pasteurized sheep milk cheese during ripening. High average concentrations of GABA (1296.75 mg/kg cheese) and ornithine (2355.76 mg/kg cheese) were found in all the cheese batches at 240 days of ripening. Batch 2, manufactured with the culture containing autochthonous *Lactococcus lactis* strains as starter and *Lactobacillus plantarum* TAUL1588 as adjunct, showed 2.37 fold reduced biogenic amines concentration with respect to the batch 1 made with the starter during the ripening time. The microstructure and microbiological counts of cheeses were affected ($P \leq 0.001$) by the ripening time, without appreciating differences ($P > 0.05$) in the physico-chemical composition between batches. This study could be a good approach to the development of functional sheep milk cheese.

INTRODUCTION

The relationship between food and consumer health has become a priority concern in food production. Balthazar et al. (2017) have stated that sheep milk, which is mainly used for cheese production, is an excellent source of nutrients. At present, the hygienic quality of cheese is guaranteed by the pasteurization of milk. This thermal treatment causes the elimination of part of the milk`s microbiota and this fact makes necessary the use starters and adjunct cultures to guaranteeing the appropriate sensory characteristics of each cheese variety (Minervini et al., 2009). Lactic acid bacteria (LAB) are usually employed as cultures for cheese-making and play a very important role in the proteolysis that takes place during cheese ripening since they contain proteinases and peptidases that can lead to the production of free amino acids (Fox et al., 2016). Several studies have been carried out to study the effect of different autochthonous LAB strains on the content of free amino acids in cheese (Madrau et al., 2006; Mangia et al., 2008; Poveda et al., 2015, 2004). These free amino acids can act as substrates for secondary catabolic reactions by LAB, leading to the formation of compounds such as gamma-aminobutyric acid (GABA) and ornithine (Manca et al., 2015). These two compounds have recently attracted the attention of the food industry since GABA and ornithine have numerous beneficial physiological functions on human health (Adeghate and Ponery, 2002; Diana et al., 2014a; Sugino et al., 2008).

It has been observed that various strains of LAB, such as *Lactobacillus brevis* DPC6108, *Lb. brevis* PM17, *Lactobacillus plantarum* C48, *Lactobacillus paracasei* PF6 and *Lactococcus lactis* PU1, were able to synthesize GABA when they are grown in culture medium supplemented with monosodium glutamate (Barrett et al., 2012; Siragusa et al., 2007). It has been also observed that the capacity to synthesize GABA by LAB is dependent on the strain and not on the specie (Dhakal et al., 2012). Several studies have been carried out in order to know the concentration of this bioactive compound in commercial cheeses (Diana et al., 2014b; Manca et al., 2015; Poveda et al., 2016). However, as Diana et al. (2014b) have indicated little attention has been given to ornithine. Likewise, few studies have been focused on testing the ability of autochthonous LAB cultures to produce GABA and ornithine during cheese making (Poveda et al., 2004; Poveda, Chicón, & Cabezas, 2015).

However, it must be taken into account that some decarboxylation reactions can lead to toxic compounds such as biogenic amines that can also be synthesized during cheese ripening. Biogenic amines are organic, basic, nitrogenous compounds with biological activity. The consumption of foods containing large amounts of these amines can provoke toxicological effects and these problems can be more severe in consumers in whom detoxification is less efficient because of their genetic constitution or if they are under some treatments (Linares et al., 2011). Cheese is one of the most prevalent foods associated with amine poisoning and the consumption of cheese that has a high level of tyramine may result in a dangerous intoxication characterized by an increase in blood pressure (Ladero et al., 2010). For this reason, when cultures are designed to be employed in the production of cheese with an amino acid profile beneficial on human health, it is also necessary to ensure that these cultures do not generate high concentrations of biogenic amines in the final product.

The objective of this study was to investigate the effect of the use of four different autochthonous cultures on the free amino acid profile, with special emphasis on GABA and ornithine, on the biogenic amine content, microstructure, physico-chemical and microbiological parameters of pasteurized sheep milk cheese during ripening.

MATERIAL AND METHODS

Preparation of cultures

The autochthonous cultures used in sheep cheese manufacture were selected based on their good technological aptitude showed in previous studies (González et al., 2015; Herreros et al., 2003).

Before culturing, each LAB strain was activated in either MRS broth (Oxoid, Hampshire, UK) for *Lactobacillus* or Elliker broth (BD Difco, New Jersey, USA) for *Lactococcus lactis*, and then in reconstituted skim milk (10%, w/v) at 30ºC for 24 h. The strain composition of the designed cultures for cheese manufacture is shown in **Figure 1**. Finally, each activated autochthonous cultures were transferred at 1% (v/v) to sterilized sheep milk and incubated for 48 h at 30ºC.

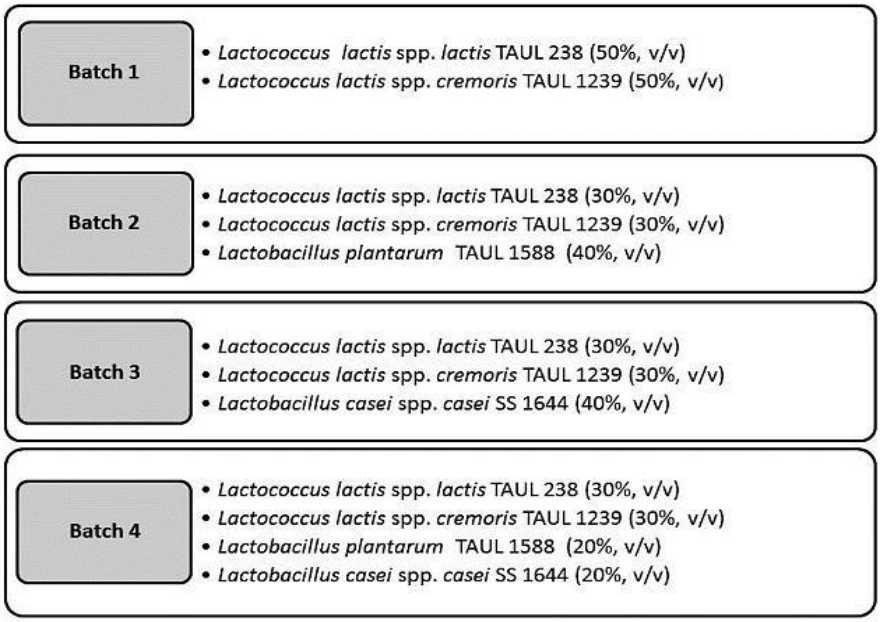

Figure 1. Strain composition of the autochthonous cultures. Volume of each individual strain cultured in milk respect to the final volume of the mixed culture in sheep milk.

Milk and cheese manufacture

Sheep milk was obtained over four consecutive days from a local farm which belongs to the Consortium for Ovine Promotion (Villalpando, Zamora, Castilla-León, Spain).

Four sheep cheese batches were manufactured in duplicate at pilot scale (Institute of Food Science and Technology (ICTAL), University of León, Spain) according to the following method: 75 L of sheep milk (for each batch and replicate) was pasteurized at 72ºC for 15 s and after cooling at 31ºC, calcium chloride (0.2 g/L) and starter culture or starter culture plus adjunct (1%, v/v) were added. The composition of the cultures used for each cheese batch is indicated in **Figure 1**. After 30 min, chymosin (CHY-MAX Extra, 100 % chymosin; 600 IMCU/mL; Chr. Hansen SL, Madrid, Spain) was added at a rate of 0.05 mL/L of milk (diluted in 1:20 with deionized water). After 40-45 min, the curd was cut to rice grain size and the whey was drained off. The curd was transferred to cylindrical molds (15 cm height, 21 cm diameter) which were pressed for 2 h. Then, cheeses were salted by immersion (18ºBaume, 8ºC and pH 5.4) for 17 h. Finally, the cheeses without being packaged were taken to a ripening chamber where they remained at a temperature of 10ºC and at 80 - 85% relative humidity for 240 d.

Samples were taken from each batch after 2, 90, 180 and 240 days of ripening. The samples (each sample corresponded to a whole cheese of 2.5 kg) were grinded, packed and stored in a freezer (-30ºC) until analysis. Microstructure, physico-chemical and microbiological analyses were carried out on fresh samples.

Physico-chemical analysis

The pH and titratable acidity of cheese batches were determined according to standard 14.022 (AOAC, 1980a, 1980b). Water activity (A_w) was analyzed instrumentally using an Aqua Lab Dew Point Analyzer CX-2 (Decagon Devices, Pullman, WA, USA). Total solids, NaCl, fat and protein contents were determined according to standards 004 (FIL-IDF, 2004), 935.43 (AOAC, 1990), 221 (FIL-IDF, 2008), 20-1 (FIL-IDF, 2001), respectively. All samples were carried out in duplicate.

Microbiological analysis

Fifty grams of milk or cheese samples were homogenized with 200 mL of a 2% (w/v) sodium citrate solution (Panreac, Barcelona, Spain) in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Decimal dilutions were prepared by mixing 10 mL of this homogenate with 90 mL of sterile peptone water (Oxoid, Unipath, Ltd., Basingstoke, UK) at 0.1% (w/v) according to standard 122B (FIL-IDF, 1992).

Aerobic mesophilic bacteria were enumerated on standard Plate Count Agar (PCA; Oxoid) after incubation at 30ºC for 48 h. LAB were determined on De Man-Rogosa-Sharpe (MRS) agar (Oxoid) after incubation at 30ºC for 72 h. Lactobacilli were enumerated on Rogosa agar (Oxoid) incubated at 30ºC during 5 days. *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA; Oxoid) after incubation at 37ºC for 18-24h.

Determination of free amino acids and biogenic amines by ultra-high performance liquid chromatography

Free amino acids and biogenic amines were determined following the method described by Redruello et al. (2013). Briefly, amino acids and biogenic amines were extracted by the homogenization of one gram of cheese in 10 mL of 0.1 M HCl-0.2% 3,3´thiodipropionic acid (Sigma-Aldrich, Madrid, Spain). This mixture was kept in an ultrasonic bath Bransonic 221 (Branson Ultrasonics S.A, Danbury, USA) for 30 min and then centrifuged at 5000 × g for 20 min. The supernatant was deproteinised by passing through ultrafiltration inserts (Amicon Biomax 5K; Millipore, MA, USA) by centrifugation at 3500 × *g* for 1h. 20 µL of this diluted sample were derivatised with diethyl ethoxymethylenemalonate (DEEMM; Sigma-Aldrich). Duplicate samples were freshly derivatised before injection in the chromatograph system. L-2-aminoadipicacid (Sigma-Aldrich) was used as internal standard. The chromatograph system consisted of an H-Class Acquity UPLC™ system (Waters, Milford, MA, USA) coupled to a photodiode array detector. Free amino acids and biogenic amines separation was carried out using a Waters Acquity UPLC™ BEH C18 column (1.7 µm particle size, 100 mm × 2.1 mm I.D.) held at 35ºC. The mobile phase consisted of 25 mM acetate buffer pH 6.7 plus 0.02% sodium azide (eluent A), methanol (eluent B) and acetonitrile (eluent C). Samples (1 µL) were applied to the column and eluted at a flow rate of 0.45 mL/min according to the linear gradient used by Redruello et al. (2013). The target compounds were identified by their retention times and their spectral characteristics at 280 nm, and were quantified using the internal standard method. Data were acquired and analyzed using the software Empower 2 (Waters, Milford, MA, USA).

Confocal scanning laser microscopy (CSLM)

The four sheep cheese batches were examined using CSLM at 2, 90, 180 and 240 days of ripening, as described by Auty et al. (2001), in order to visualize the changes that took place in the distributions and microstructures of the fat and protein. Cheese sample measuring 10 mm × 10 mm × 5 mm was stained with 50 L of a probe mixture constituted by Nile Red (Sigma-Aldrich) 0.02 g/L and Fast Green FCF (Sigma-Aldrich) 0.1 g/L and examined using a Zeiss LSM310 confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City, Herts, UK). Dual excitation using 488 nm/633 nm for Nile Red/Fast Green FCF was used.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS v.21 (SPSS, Chicago, IL, USA). The free amino acids, biogenic amines, physico-chemical and microbiological variables were tested for the assumption of normality using the Shapiro-Wilk test and for homoscedasticity using the Levene test. Subsequently, a two-way Analysis of Variance (ANOVA) was performed in order to evaluate the effect of the factors culture and ripening time (as fixed factors) and the interaction between them. Tukey's HSD *post hoc* test was applied at a 5% significance level in order to compare sheep cheeses manufactured with different autochthonous cultures throughout the different ripening times. The Spearman's rank correlation coefficient (ρ) was applied to estimate the

relationship between the physico-chemical parameters and microbiological populations of cheese samples.

RESULTS AND DISCUSSION

Physico-chemical composition of sheep milk cheeses during ripening

The changes in the physico-chemical parameters of the four sheep milk cheeses throughout ripening are shown in **Table 1**. These results demonstrated that the different cultures used for cheese-making did not have a statistically significant effect ($P > 0.05$) on most of the analyzed parameters at the same ripening time. Nevertheless, the titratable acidity values for the 3 and 4 batches manufactured with cultures containing the *Lactobacillus casei* subsp. *casei* SS1644 were higher than the other two cheese batches at 90 and 180 days of ripening. The greater acidifying capacity of this strain with respect to the *Lb. plantarum* TAUL 1588 strain was previously reported in other studies (González et al., 2015; Herreros et al., 2003).

It is well known that during ripening a very complex set of interrelated biochemical processes happen and they are responsible of cheese characteristics (Fox et al., 2016).

The evolution of the physico-chemical parameters analyzed during the ripening time enabled an understanding that the process of cheese-making for the 4 batches of cheese was similar. At the beginning of ripening (2 days), there was an increase in pH until 90 days to subsequently remain constant or decrease slightly. The increase observed in the values of this parameter could be due mainly to the buffer capacity that can present the cheese curd during the ripening process (Salaün et al., 2005). This fact prevents obtaining information on microbial growth, however, a clear indicator of the metabolic activity of the cultures used in the cheese-making was the increase of the titratable acidity values until 180 days of ripening to later descend. Moreover, the acidification that took place during the ripening process of the cheese batches contributed to the internal drying of the cheese. A significant negative correlation was found between acidity and moisture values ($\rho = -0.84$; $P \leq 0.01$). Moisture decreased significantly ($P \leq 0.001$) throughout ripening, reaching values lower than 30%. A_w followed a similar trend, the mean values decreased as ripening progressed, from 0.989 to 0.915 at the end of the ripening period. It was observed that as the salt/moisture ratio increased, A_w values decreased ($\rho = -0.87$; $P \leq 0.01$). Guinee (2004) has described that salt is a major determinant of the A_w parameter, and thereby exerts control over microbial growth, enzyme activity, and biochemical changes during cheese ripening. The salt/moisture ratio initially increased up to 180 days of ripening to finally remain constant, reaching a value of around 5% in all cheese batches. These A_w and salt/moisture values were similar to those described by other authors for ripened sheep milk cheeses (Guinee and Fox, 2004).

During the ripening period no significant ($P > 0.05$) differences were observed in fat and protein in dry matter contents. The values obtained were comparable with those described by other authors for sheep milk cheese (Fernández et al., 2012).

Table 1. Changes in pH, titratable acidity, water activity (A_W) values and in moisture, salt/moisture, fat and protein contents of four sheep milk cheese batches.

Physico-chemical parameter	Batch[1]	Ripening time (days)				*P*-value[2]		
		2	90	180	240	R	B	R*B
pH	1	5.26±0.03[a]	5.32±0.05[a]	5.33±0.01[a]	5.30±0.02[a]	*	NS	NS
	2	5.26±0.01[a]	5.28±0.02[a]	5.31±0.01[a]	5.30±0.01[a]	*	NS	NS
	3	5.27±0.03[a]	5.34±0.03[a]	5.29±0.01[a]	5.28±0.01[a]	*	NS	NS
	4	5.24±0.02[a]	5.31±0.01[a]	5.29±0.01[a]	5.26±0.01[a]	*	NS	NS
Titratable acidity (g lactic acid/kg total solids)	1	16.51±1.30[a]	17.50±0.50[a]	22.44±0.52[a]	20.88±0.33[a]	**	*	NS
	2	16.90±0.31[a]	17.95±0.15[a]	22.83±0.52[a]	20.45±0.54[a]	***	*	NS
	3	17.01±0.50[a]	20.61±1.45[b]	23.90±0.10[b]	21.17±0.21[a]	**	*	NS
	4	16.19±0.13[a]	20.11±0.67[b]	23.42±0.42[b]	21.31±0.33[a]	***	*	NS
A_W	1	0.990±0.001[a]	0.966±0.001[a]	0.945±0.001[a]	0.913±0.001[a]	***	NS	NS
	2	0.987±0.001[a]	0.968±0.000[a]	0.944±0.001[a]	0.917±0.001[a]	***	NS	NS
	3	0.987±0.001[a]	0.965±0.001[a]	0.938±0.000[a]	0.912±0.001[a]	***	NS	NS
	4	0.991±0.001[a]	0.970±0.001[a]	0.940±0.000[a]	0.918±0.000[a]	***	NS	NS
Moisture (g/kg cheese)	1	411.01±11.70[a]	337.79±4.21[a]	286.15±0.44[a]	281.04±3.52[a]	***	NS	NS
	2	399.55±4.39[a]	341.35±1.71[a]	296.58±1.41[a]	278.46±1.72[a]	***	NS	NS
	3	409.43±8.36[a]	338.87±1.57[a]	288.03±4.89[a]	279.73±0.30[a]	***	NS	NS
	4	399.09±10.61[a]	340.22±2.41[a]	297.30±5.97[a]	280.24±0.30[a]	***	NS	NS
Salt/moisture (g salt/kg moisture)	1	15.08±0.40[a]	43.38±0.11[a]	54.15±0.39[a]	53.23±0.20[a]	**	NS	NS
	2	16.20±0.17[a]	43.42±0.12[a]	54.66±1.20[a]	53.93±0.81[a]	**	NS	NS
	3	15.89±0.33[a]	43.07±0.38[a]	54.02±0.65[a]	53.34±0.10[a]	**	NS	NS
	4	16.11±0.43[a]	43.16±0.17[a]	54.50±0.70[a]	53.87±0.61[a]	**	NS	NS
Fat (g/kg total solids)	1	551.32±3.35[a]	555.20±2.70[a]	555.22±5.06[a]	555.19±2.01[a]	NS	NS	NS
	2	550.52±2.60[a]	554.86±1.14[a]	553.90±6.17[a]	552.97±1.06[a]	NS	NS	NS
	3	550.47±5.37[a]	555.40±1.71[a]	555.46±5.06[a]	555.47±2.12[a]	NS	NS	NS
	4	549.73±5.35[a]	555.05±2.99[a]	554.21±6.01[a]	554.32±3.35[a]	NS	NS	NS
Protein (g/kg total solids)	1	344.55±3.40[a]	338.23±4.53[a]	347.60±3.54[a]	348.88±3.21[a]	NS	NS	NS
	2	344.47±2.26[a]	338.02±3.41[a]	348.26±4.41[a]	348.96±5.11[a]	NS	NS	NS
	3	345.10±2.38[a]	338.75±3.41[a]	348.51±4.31[a]	349.01±4.35[a]	NS	NS	NS
	4	344.97±2.13[a]	338.33±5.30[a]	348.92±4.16[a]	349.06±5.14[a]	NS	NS	NS

[1]1: *Lactococcus lactis* subsp. *lactis* TAUL 238 + *Lactococcus lactis* subsp. *cremoris* TAUL 1239; 2: starter 1 + *Lactobacillus plantarum* TAUL 1588; 3: starter 1 + *Lactobacillus casei* subp. *casei* SS 1644; 4: starter 1 + *Lactobacillus plantarum* TAUL 1588 + *Lactobacillus casei* subp. *casei* SS 1644.
Results expressed as mean values ± standard deviation, n=4. [a-b] Different superscript letters in the same column denote significant statistical differences ($P \leq 0.05$) between cheese batches. [2]R: ripening time fixed effect; B: batch fixed effect; R*B: interaction between the fixed effects. NS $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Table 2. Changes in microbial counts (log CFU/mL) of the four sheep milk cheese batches throughout ripening.

Microbial group	Batch[1]	Milk[2]	Ripening time (days)				*P*-value[3]		
			2	90	180	240	R	B	R*B
Aerobic mesophilic microbiota	1	6.80±0.96[a]	9.55±0.04[a]	7.31±0.04[a]	7.17±0.11[a]	7.17±0.12[a]	***	***	***
	2	7.18±0.11[a]	9.68±0.53[b]	8.74±0.28[b]	7.97±0.14[b]	7.84±0.42[b]	***	***	***
	3	7.13±0.07[a]	9.19±0.10[c]	10.92±0.25[c]	9.19±0.11[c]	8.98±0.11[c]	***	***	***
	4	7.36±0.22[a]	9.59±0.27[a]	8.95±0.35[d]	8.47±0.04[d]	8.33±0.04[d]	***	***	***
Lactic acid bacteria	1	6.87±0.52[a]	7.50±0.18[a]	7.36±0.18[a]	7.03±0.18[a]	6.71±0.18[a]	***	***	**
	2	7.14±0.12[a]	9.77±0.41[b]	8.73±0.18[b]	7.74±0.07[b]	7.65±0.27[b]	***	***	**
	3	7.10±0.05[a]	9.20±0.05[c]	8.87±0.37[b]	8.89±0.07[c]	8.75±0.07[c]	***	***	**
	4	7.38±0.10[a]	9.70±0.11[b]	8.83±0.18[b]	8.36±0.28[d]	8.24±0.20[d]	***	***	**
Lactobacilli	1	-	-	-	3.40±0.20[a]	2.40±0.10[a]	***	***	***
	2	5.99±0.04[a]	7.93±0.81[a]	8.71±0.07[a]	7.95±0.78[b]	7.54±0.35[b]	***	***	***
	3	5.40±0.04[a]	9.28±0.17[b]	7.60±0.36[b]	7.22±0.13[c]	7.03±0.20[c]	***	***	***
	4	7.30±0.07[b]	7.77±0.42[c]	8.70±0.25[a]	7.80±0.22[d]	6.83±0.15[d]	***	***	***
Enterobacteriaceae	1	-	3.52±0.22[a]	-	-	-	***	***	***
	2	2.58±0.50	3.57±0.27[a]	-	-	-	***	***	***
	3	-	3.98±0.71[a]	-	-	-	***	***	***
	4	-	2.77±0.35[b]	-	-	-	***	***	***

[1]1: *Lactococcus lactis* subsp. *lactis* TAUL 238 + *Lactococcus lactis* subsp. *cremoris* TAUL 1239; 2: starter 1 + *Lactobacillus plantarum* TAUL 1588; 3: starter 1 + *Lactobacillus casei* subp. *casei* SS 1644; 4: starter 1 + *Lactobacillus plantarum* TAUL 1588 + *Lactobacillus casei* subp. *casei* SS 1644.
Results expressed as mean values ± standard deviation, n=2.
[a-d] Different superscript letters in the same column denote significant statistical differences ($P \leq 0.05$) between cheese batches.
[2]Pasteurized milk + each culture (0 h).
[3]R: ripening time fixed effect; B: batch fixed effect; R*B: interaction between the fixed effects. **$P \leq 0.01$; ***$P \leq 0.001$.

Table 3. Total free amino acid concentration (mg/kg cheese) in the sheep milk cheeses manufactured with different cultures throughout ripening.

Ripening time (days)	Batch[1]				*P*-value[2]		
	1	2	3	4	B	R	B*R
2	4924.12±53.59[a]	4530.12±49.23[b]	5910.09±53.17[c]	4760.68±46.64[a]	***	***	***
90	16877.92±170.46[a]	16687.85±158.53[a]	40320.11±450.44[b]	16978.97±187.61[a]	***	***	***
180	34563.46±307.75[a]	34763.36±360.19[a]	79662.49±771.78[b]	38414.89±414.78[a]	***	***	***
240	44671.13±478.42[a]	42076.23±451.02[a]	87623.26±928.00[b]	49083.01±473.76[c]	***	***	***

[1]1: *Lactococcus lactis* subsp. *lactis* TAUL 238 + *Lactococcus lactis* subsp. *cremoris* TAUL 1239; 2: starter 1 + *Lactobacillus plantarum* TAUL 1588; 3: starter 1 + *Lactobacillus casei* subp. *casei* SS 1644; 4: starter 1 + *Lactobacillus plantarum* TAUL 1588 + *Lactobacillus casei* subp. *casei* SS 1644.
Results as mean values ± standard deviation, n=4. [a-d] Different lowercase superscript letters in a same row denote significant statistical differences ($P \leq 0.05$) between cheese batches. [2] B: batch fixed effect; R: ripening time fixed effect; B*R: interaction between the fixed effects. ***$P \leq 0.001$.

Microbial populations of sheep milk cheeses during ripening

The changes in the microbial counts of the four cheese batches throughout the ripening time are reported in **Table 2**. The aerobic mesophilic microbiota, lactic acid bacteria and lactobacilli counts increased significantly ($P \leq 0.001$) from the pasteurized milk inoculated with each culture (0 h) until the initial stage of ripening. Diezhandino et al. (2015) indicated that this increase in counts is due to the physical retention of microorganisms in curds and to microbial multiplication during the coagulation phase of cheese-making.

The highest counts for aerobic mesophilic microbiota were observed at 2 days of ripening (10 log CFU/mL), except for batch 3, in which the maximum counts were detected at 90 days (11 log CFU/mL). From this point, aerobic mesophilic bacteria gradually decreased towards the end of the ripening with values ranging 7 (batch 1) and 9 (batch 3) log units.

The lactic acid bacteria counts throughout the ripening period were similar to those observed in the mesophilic aerobic microbiota, which reflects that the lactic acid bacteria were the predominant microorganisms in the four sheep cheese batches. There was a significant negative correlation between lactic acid counts and the salt/moisture ratio ($\rho = -0.57$; $P \leq 0.01$). Lactic acid bacteria counts increased until 2 days, after which they decreased approximately 1-2 log CFU/mL until the end of the ripening time. The large increase in salt/moisture ratio values at 90 days (**Table 1**) could exert an inhibitory effect on the lactic acid bacteria growth.

Lactobacilli are considered as non-starter lactic acid bacteria (NSLAB) which dominate cheese microbiota during ripening. They tolerate the hostile environment well and strongly influence the biochemistry of curd ripening, contributing to the development of the final characteristics of cheese (Settanni and Moschetti, 2010). In the batches 2 and 4, which were the batches that included in the culture the *Lb. plantarum* TAUL 1588 strain, the highest counts were observed at 90 days while in the batch 3 manufactured with the culture that included the *Lb.* casei subsp. *casei* SS1644 strain the highest counts of lactobacilli were detected after 2 days and then decreased slightly until the end of the ripening time. In batch 1, lactobacilli were not detected until 180 days of ripening and with values much lower than the other batches since it was the only batch in which no strains of *Lactobacillus* were included in the culture used.

The *Enterobacteriaceae* counts are an indicator of the hygienic conditions applied during the cheese-making. The good sanitary quality of the four batches was evidenced because *Enterobacteriaceae* counts were detected at 2 days of ripening with values lower than 4 log CFU/mL. It was observed a significant negative correlation between *Enterobacteriaceae* counts and acidity ($\rho = -0.73$; $P \leq 0.01$) as well as with the salt/moisture ratio ($\rho = -0.95$; $P \leq 0.01$) coinciding with that described by other authors in sheep cheese (Piras et al., 2013).

Free amino acid content and microstructure of sheep milk cheeses during ripening

The evolution of total free amino acids (TFAAs) during ripening in the four sheep milk cheeses manufactured with different cultures is shown in **Table 3**. The TFAAs concentrations in all the batches increased significantly ($P \leq 0.001$) throughout the ripening period, from 5031.25 mg/kg cheese at 2 days up to 55863.41 mg/kg cheese at

240 days of ripening. This increase in the TFAAs concentration was mainly due to the proteolytic activity of the strains which made up the cultures (González et al., 2015; Herreros et al., 2003). The large proteolytic activity shown by the strains gave rise that the TFAA values observed in the present study were higher than those reported by Poveda et al. (2004) for sheep milk cheese manufactured with different starters and analyzed during 150 days of ripening.

The proteolysis that took place during the cheese ripening can be visually evidenced by the images (**Figure 2**) obtained when the microstructure of the different batches was analyzed using confocal laser scanning microscopy. The images of the four cheese batches were very similar. For this reason, in **Figure 2** only the microstructure of batch 1 is shown at 2, 90, 180 and 240 days of ripening. At 2 days of ripening, a continuous and fibrous protein matrix containing irregularly shaped fat globules was observed (**Figure 2 A and E**). The fat and protein phases had a slight linear orientation as was also observed by Auty et al. (2001) in Cheedar cheese, attributing this fact to the pressing stage. As the ripening time progressed, changes in the microstructure of cheese could be observed. On the one hand, the protein matrix gradually presented an amorphous structure that could be due to the proteolysis produced by LAB during ripening (**Figure 2 A-D**). On the other hand, the coalescence of fat globules generated large fat particles and the lipolytic activity during ripening could contribute to the output of free fat (**Figure 2 H**). These results were consistent with those reported by other authors for the microstructure of cheese (Everett and Auty, 2008; O'Reilly et al., 2003). It was also shown the physical holes that were generated during ripening (**Figure 2 C, D, G and H**).

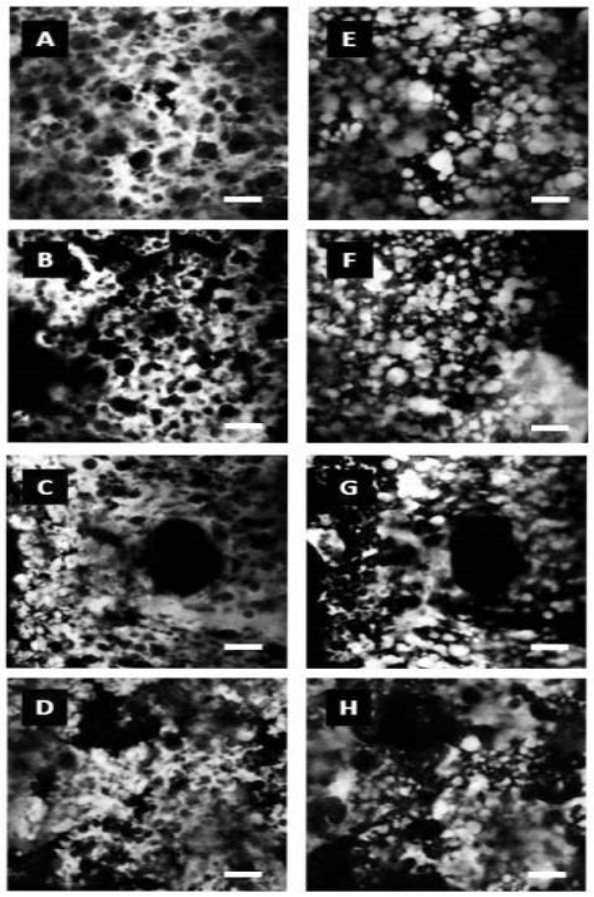

Figure 2. Confocal laser scanning images of the protein (A-D) and fat (E-H) in cheese batch 1 throughout the ripening period: 2 days (A and E), 90 days (B and F), 180 days (C and G) and 240 days (D and H). The protein and fat are shown as light areas against a dark background. Scale bar, 25 μm.

The different cultures used for cheese-making in the present study had a significant ($P \leq 0.001$) effect on the TFAAs concentrations (**Table 3**). Batch 3 manufactured with the *Lb. casei* subsp. *casei* SS1644 strain showed the highest TFAAs concentrations throughout the

ripening period. After 90 days of ripening, this batch showed a TFAAs concentration similar to that shown by the other batches at the end of ripening. Azarnia et al. (2006) have indicated that ripening is a relatively expensive process for the cheese industry. Therefore, the use of this culture could reduce the ripening time of sheep milk cheese, providing technological benefits.

Batches 1, 2 and 4 did not show significant differences ($P > 0.05$) in the TFAAs concentration until the end of ripening. At 240 days, batch 2 made with *Lc. lactis* strains plus *Lb. plantarum* TAUL1588 presented a similar concentration of TFAAs to that of batch 1 made with the *Lc. lactis* strains. However, batch 4 manufactured with the four autochthonous strains had higher TFAAs concentration than batches 1 and 2 but lower than batch 3.

The individual concentration of 21 free amino acids in sheep milk cheeses made with different cultures throughout the ripening period is shown in **Figure 3**. The concentration of most of the free amino acids studied increased significantly ($P \leq 0.001$) during the cheese ripening. In general, the most abundant free amino acids were leucine, glutamic acid, phenylalanine, proline, alanine and valine which accounted approximately the 60% of the TFAAs. Tyrosine, histidine, glycine, tryptophan and arginine were minor free amino acids representing less than the 5% of the TFAAs. The data available on the literature about the major free amino acids and their concentration in sheep milk cheese varies widely (Madrau et al., 2006; Mangia et al., 2008; Poveda et al., 2015). This fact could be mainly due to the different lactic acid bacteria (LAB) used for cheese-making since LAB present a complex proteolytic system formed by different proteinases and peptidases depending on the LAB specie (Fox et al., 2016). As Poveda et al.(2004) have indicated the composition of the caseins is different; α_{S1}-casein has a high content of leucine, phenylalanine and valine, while β-casein has high content of proline. In addition free amino acids are released by the action of peptidases that vary among different strains. Therefore, depending on the specific substrates of the enzymes present in the proteolytic system of LAB, the type and concentration of free amino acids in cheese will be different.

The results obtained in the present study are relevant since little information is available about the effect of different autochthonous cultures on the concentration of GABA and ornithine in sheep milk cheese during ripening. GABA and ornithine have been reported as non-protein amino acids with numerous physiological functions (Diana et al., 2014b). GABA could be synthesized from L-glutamate by the glutamate decarboxylase (GAD) present in some LAB (Cotter and Hill, 2003; Lacroix et al., 2013). Milk caseins do not present GABA but they have a high content in L-glutamate (17.5% of the TFAAs) which is released during cheese ripening and can be metabolized to GABA by the action of LAB (Hejtmánková et al., 2012). In **Figure 3** it is shown that GABA concentration in the four cheese batches increased more than eighty-fold from 2 days to 240 days of ripening. The major concentration of GABA was observed at 240 days of ripening in batch 1 followed by the other three cheese batches which did no present differences ($P > 0.05$) between them. The importance of this study falls on the high concentration of GABA found in all the cheese batches at the end of the ripening time. Regarding the dose of GABA which is in the health-promoting range, it has been observed that a daily intake of 50 g of experimental cheese (containing 16 mg of GABA) decreased blood pressure in humans (Pouliot-Mathieu et al., 2013). In the present study 50 g of batch 1 and 50 g of the other three batches

would suppose 90 mg and 60 mg of GABA, respectively. This fact implied that small portions of these cheeses would be necessary to achieve the physiological effect indicated above.

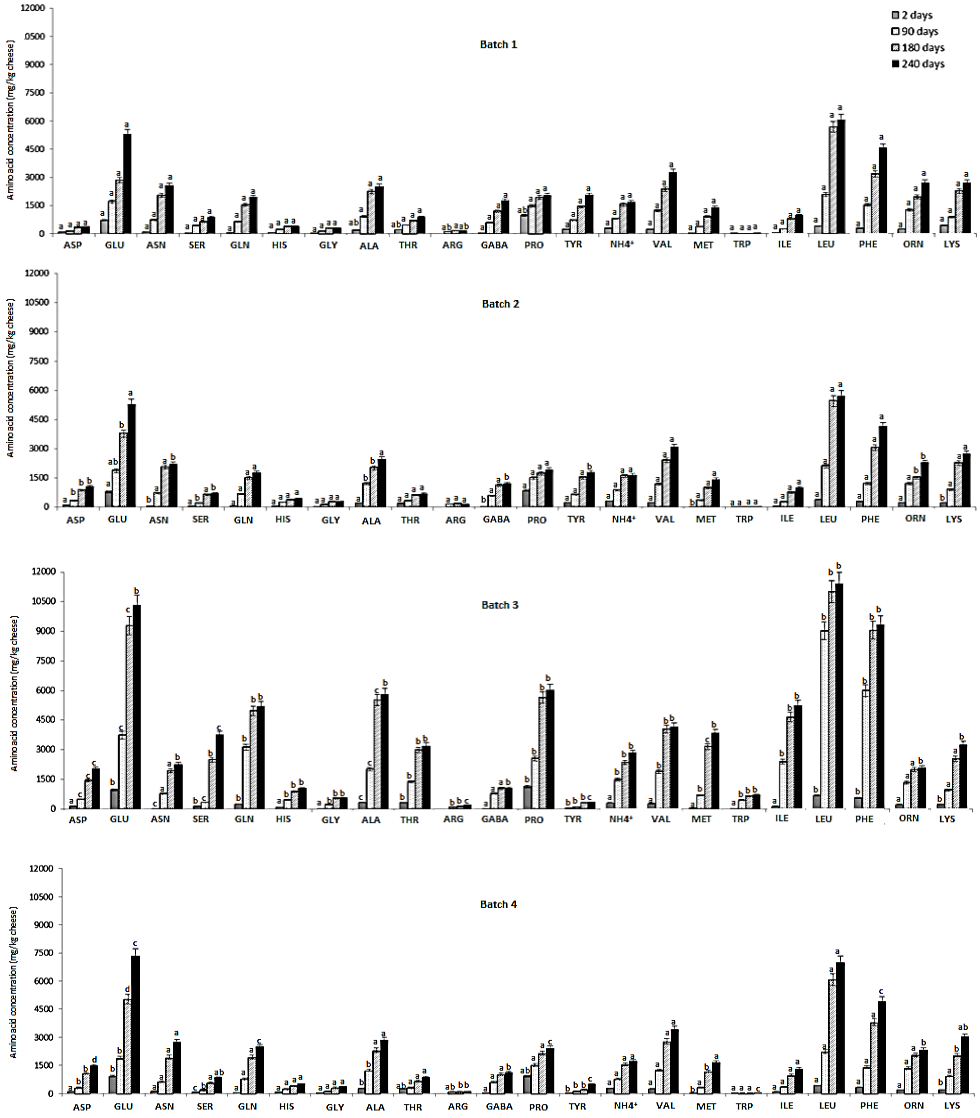

Figure 3. Evolution of the individual free amino acids during ripening of sheep milk cheese manufactured with different cultures: (Batch1) cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; (Batch 2) cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; (Batch 3) cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; (Batch 4) cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.
Data represent mean ± standard deviation (indicated by vertical error bars), n = 4. Different letters (a-d) in the same bar of the diagram for each free amino acid in the same ripening time indicate significant statistical differences ($P \leq 0.05$) between cheese batches.
ASP: aspartic acid; GLU: glutamic acid; ASN: asparagine; SER: serine; GLN: glutamine; HIS: histidine; GLY: glycine; ALA: alanine; THR: threonine; ARG: arginine; GABA: gamma-aminobutyric acid; PRO: proline; TYR: tyrosine; VAL: valine; METH: methionine; TRYP: tryptophan; ISOLEU: isoleucine; LEU: leucine; PHENYL: phenylalanine. ORNI: ornithine; LYS: lysine.

Presence of ornithine in foods is gaining attention since some studies have shown its bioactive functions on human health (Kurata et al., 2012; Sugino et al., 2008). Currently, there is no information about the effective dose of ornithine to achieve the reported physiological benefits, what is known is that ornithine can be synthetized by the enzymatic activity of LAB metabolism through the precursors arginine and citrulline during cheese ripening (Diana et al., 2014b). In the four sheep milk cheeses there was an increase ($P \leq 0.001$) in the values of ornithine throughout the ripening time. No significant ($P > 0.05$) differences were observed between the four batches analyzed until 240 days of ripening. As in the case of GABA, batch 1 was the one with the highest concentration of ornithine. Batches 2, 3 and 4 did not show differences ($P \geq 0.05$) between them. These concentrations were higher than those (ranged between 0.40 and 1.08 g/kg) described by Diana et al. (2014b) for sheep milk cheeses. These results open the possibility of carrying out further studies with these co-cultures for the possible development of functional cheeses.

Biogenic amines content of sheep milk cheeses during ripening

During cheese ripening, free amino acids were generated by the autochthonous cultures studied. Some of these free amino acids can act as substrate for secondary catabolic reactions by the decarboxylase activity of LAB. The result of these metabolic routes can be other free amino acids such as GABA and ornithine which have beneficial effects on human health or biogenic amines which consumption in elevated concentrations could have negative effects on human health when the organism in not able to degrade them though the action of monoamine and diamine oxidases (Broadley, 2010; EFSA, 2011; Manca et al., 2015). For this reason, the effect of the different cultures used and the ripening time on the biogenic amines content of the cheese batches was also analyzed.

The total biogenic amines content of the sheep milk cheeses manufactured with different cultures increased significantly ($P \leq 0.001$) during ripening, ranging between 10.17 mg/kg cheese for batch 1 at 2 days and 820.18 for batch 3 at 240 days (**Table 4**). These values were similar to those described for sheep milk cheeses (Renes et al., 2014; Schirone et al., 2013). The maximum permitted concentration of biogenic amines in dairy products has not been established yet from a legislative point of view. Most studies have concentrated on the study of histamine and tyramine, as these are the biogenic amines most often associated with food poisoning and the most abundant in sheep milk cheese (Linares et al., 2016). In the present study, histamine was not detected in all the cheese batches (the detection limit for histamine was 1.78 mg/kg cheese). Tyramine concentrations ranged between 308.65 mg/kg cheese and 585.47 mg/kg cheese at the end of ripening, being within the maximum tolerable limits (100 mg/kg – 800 mg/kg) reported by ten Brink et al. (1990). Nonetheless, it is necessary to highlight that cadaverine and putrescine which have not been associated with food poisoning, may enhance the toxicity of histamine and tyramine. In this sense, the total biogenic amines content should not exceed the amount of 900 mg/kg established by Valsamaki et al. (2000). In this regard, none of the samples exceeded the indicated limit, not representing a risk to the consumer`s health.

Table 4. Concentration (mg/kg cheese) of biogenic amines in the sheep milk cheeses manufactured with different cultures throughout ripening.

Biogenic amine	Ripening time (days)	Batch[1]				*P*-value[2]		
		1	2	3	4	B	R	B*R
Tyramine	2	nd	nd	71.47±0.17^{a}	44.04±0.14^{b}	***	***	***
	90	342.95±2.13^{a}	274.29±1.81^{b}	408.60±2.05^{c}	415.58±4.05^{c}	***	***	***
	180	356.67±3.21^{a}	288.08±2.88^{b}	451.27±2.02^{c}	436.09±8.69^{d}	***	***	***
	240	493.85±2.47^{a}	308.65±1.54^{b}	585.47±11.47^{c}	539.29±11.69^{d}	***	***	***
Putrescine	2	nd	nd	8.27±0.01	nd	***	**	***
	90	nd	nd	72.56±0.09	nd	***	**	***
	180	nd	nd	72.56±0.09	nd	***	**	***
	240	nd	nd	75.45±0.11^{a}	56.76±0.10^{b}	***	**	***
Cadaverine	2	10.17±0.01^{a}	10.20±0.01^{a}	56.20±0.06^{b}	10.20±0.01^{a}	***	**	***
	90	10.19±0.04^{a}	10.20±0.03^{a}	84.29±0.20^{b}	10.21±0.01^{a}	***	**	***
	180	10.33±0.02^{a}	10.20±0.01^{a}	84.47±0.20^{b}	10.22±0.01^{a}	***	**	***
	240	10.40±0.02^{a}	10.21±0.01^{a}	89.47±0.21^{b}	31.65±0.31^{c}	***	**	***
Phenylethylamine	2	nd	nd	11.99±0.01	nd	***	**	***
	90	nd	nd	60.54±0.26^{a}	24.23±0.02^{b}	***	**	***
	180	nd	nd	65.31±0.55^{a}	30.54±0.02^{b}	***	**	***
	240	nd	nd	69.79±0.75^{a}	45.29±0.06^{b}	***	**	***
Total	2	10.17±0.01^{a}	10.20±0.02^{a}	147.93±0.25^{b}	54.24±0.15^{c}	***	***	***
	90	353.12±2.34^{a}	284.49±1.28^{b}	625.99±1.27^{c}	450.02±1.00^{d}	***	***	***
	180	367.00±1.58^{a}	298.28±0.47^{b}	673.61±1.69^{c}	476.85±1.67^{d}	***	***	***
	240	504.25±0.71^{a}	318.86±0.47^{b}	820.18±4.00^{c}	672.99±2.94^{d}	***	***	***

[1]1: *Lactococcus lactis* subsp. *lactis* TAUL 238 + *Lactococcus lactis* subsp. *cremoris* TAUL 1239; 2: starter 1 + *Lactobacillus plantarum* TAUL 1588; 3: starter 1 + *Lactobacillus casei* subp. *casei* SS 1644; 4: starter 1 + *Lactobacillus plantarum* TAUL 1588 + *Lactobacillus casei* subp. *casei* SS 1644.
Results expressed as mean values ± standard deviation, n=4.
[2]B: batch effect; R: ripening effect; B*R: interaction batch and ripening time effect.
[a-d] Different lowercase superscript letters in a same row denote significant statistical differences ($P \leq 0.05$) between cheese batches
nd: not detected; limit of detection for tyramine, putrescine and phenylethylamine was 0.86, 0.41, 0.26 and 1.53 mg kg^{-1} cheese, respectively.
[2] B: batch fixed effect; R: ripening time fixed effect; B*R: interaction between the fixed effects. **$P \leq 0.01$; ***$P \leq 0.001$.

In **Table 4**, it can be observed that batch 3, manufactured with the culture containing the *Lb. casei* subsp. *casei* SS1644 strain, was the batch that presented the highest concentration of total biogenic amines throughout the ripening time. It was also the batch that had the highest concentration of TFAAs (**Table 3**). These results are consistent with those reported by Novella-Rodriguez et al. (2003) who indicated that an increase in cheese proteolysis during ripening could produce an increase in biogenic amines content. Batch 2 made with the *Lb. plantarum* TAUL1588 strain presented the lowest concentration of total biogenic amines, followed by batches 1 and 4.

The information available about the major biogenic amines in sheep cheese is variable, as in the above-case of the TFAAs, because the type and concentration of biogenic amines found in cheese depend on the cheese variety and on the multiple factors involved in the formation and accumulation of these biogenic amines (Renes et al., 2014). The individual biogenic amines concentration in sheep milk cheese batches manufactured with different cultures during ripening are shown in **Table 4**.

Histamine and tryptamine were not detected in the batches examined. Tyramine resulted to be the biogenic amine in the highest concentration throughout the ripening time. At the beginning of ripening, it was not detected in batches 1 and 2. Batch 3 was the one that showed the highest concentration of tyramine throughout the ripening time, followed by batch 4.

Putrescine and phenylethylamine were no detected in batches 1 and 2 during ripening and in batch 4 at the beginning of ripening. Batch 3 presented the highest concentration of these amines at 2, 90, 180 and 240 days of ripening. It was observed an increase in the concentration of these amines of 9 fold for putrescine and 6 fold for phenylethylamine at 240 days of ripening with respect 2 days. As it has been described previously, some biogenic amines may cause undesirable toxicological effects. However, in the case of the phenylethylamine, it has been observed that it has beneficial physiological effects on human health (Irsfeld et al. 2013).

Batches 1 and 2 had the lowest cadaverine concentration without detecting differences ($P > 0.05$) between them. The concentration of cadaverine in these batches was not affected by the ripening time ($P > 0.05$). This same trend was observed in batch 4 until 180 days of ripening at which point the cadaverine concentration increased. Batch 3 showed the highest concentration of cadaverine during cheese ripening. The concentration of this amine increased significantly ($P \leq 0.001$) from 2 days of ripening to 90 days, moment from which it increased slightly until the end of ripening.

CONCLUSIONS

Consumer demand for healthy foods is leading the dairy industry to develop foods in which the nutritional quality has been improved. The culture formed by autochthonous *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* as starters and *Lb. plantarum* as adjunct culture could be a good approach to the development of functional sheep milk cheeses with reduced biogenic amine content.

As cheese ripening is a technological stage that involves the investment of a lot of money, the culture containing the four autochthonous LAB strains of the present study could be an alternative to reduce the ripening time of sheep milk cheeses and it also could

produce high concentrations of bioactive compounds such as GABA and ornithine in cheese.

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DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

En la actualidad la creciente demanda de alimentos con efectos beneficiosos sobre la salud humana incentiva a la industria alimentaria a desarrollar nuevos alimentos que satisfagan las exigencias del consumidor.

En este sentido, el queso, el cual supone un porcentaje importante del comercio agrícola mundial, ha suscitado en los últimos años una connotación negativa por parte del consumidor debido a la relación del contenido en ácidos grasos saturados de este alimento y el desarrollo de algunas enfermedades. Sin embargo, el queso también presenta compuestos que pueden mejorar la salud del consumidor, como es el caso del CLA. Hay que tener en cuenta que la mayor cantidad de este compuesto bioactivo aportada a la dieta proviene de la leche y de los productos lácteos, habiéndose observado que la grasa de la leche de oveja presenta una mayor concentración de CLA que la de vaca o la de cabra.

Ante la situación planteada, la presente Tesis muestra distintas aproximaciones de mejora del perfil nutricional del queso de oveja. Con este propósito, se estudiaron aspectos que van desde la producción animal hasta la tecnología de elaboración del queso.

En este trabajo de investigación, fue identificada leche de oveja de tanque con un perfil en ácidos grasos más saludable a través del estudio del sistema de manejo practicado en granjas de ovino. Igualmente, se ha demostrado la importancia, incluyendo las posibles limitaciones y futuras líneas de investigación, que juega el diseño de cultivos de bacterias lácticas seleccionadas por su capacidad para sintetizar CLA con vistas a su aplicación en la elaboración de quesos de oveja con un perfil nutricional mejorado. Otro factor estudiado ha sido el tiempo de maduración de los quesos, el cual en conjunción con los cultivos empleados ejerce una influencia muy importante en la concentración de compuestos aminoacídicos con efectos beneficiosos sobre la salud humana, como el GABA y la ornitina, y en la concentración de aminas biógenas presentes en el producto final. A lo largo de la elaboración de los quesos también se controló la evolución de los parámetros físico-químicos y de los principales grupos microbianos para garantizar un control del proceso tecnológico en todos los lotes de queso elaborados. Finalmente, se llevó a cabo un análisis sensorial de los quesos porque tal y como ha sido mencionado anteriormente, no sólo es necesario mejorar la calidad nutricional de un alimento sino que también ha de tener unas adecuadas características organolépticas para su aceptación por parte del consumidor. A continuación, los resultados de esta Tesis son discutidos en base a los objetivos específicos establecidos.

4.1. Estudio del perfil en ácidos grasos de leche de tanque procedente de granjas de ovino comerciales

En el capítulo 1 de la presente Tesis se recogen los resultados que a continuación son discutidos.

El efecto de diversos factores asociados al sistema de manejo de los rumiantes sobre el perfil de ácidos grasos de la leche ha sido estudiado ampliamente en rebaños experimentales (Cabiddu et al., 2005; Hervás et al., 2008; Kalač y Samková, 2010; Stanton et al., 2003; Tsiplakou et al., 2008, 2006).

Sin embargo, hay poca información disponible sobre el efecto de la alimentación, de la etapa de lactación, del mes de muestreo y del rebaño en el perfil de ácidos grasos de la leche procedente de granjas de ovino comerciales. En consecuencia, los resultados obtenidos en este estudio proporcionan a la industria láctea nuevos conocimientos sobre las prácticas de manejo habituales en granjas de ovino que podrían conducir a una mejora en la calidad nutricional de la leche de oveja.

En esta Tesis, con el fin de identificar leche de oveja de tanque con alto contenido en CLA y con un perfil en ácidos grasos más saludable, se analizó leche de tanque procedente de 30 granjas comerciales durante los meses de abril, mayo, junio y julio. Un aspecto importante a resaltar es que las granjas de ovino objeto de este estudio fueron seleccionadas y agrupadas en función del régimen de alimentación practicado: en el primer grupo (G1) la alimentación consistió en 50% de pastoreo más ensilado de alfalfa y granos de cebada, en el segundo grupo (G2) la alimentación se basaba en 25% de pastoreo más ensilado de alfalfa y heno y en el tercer grupo (G3) consistió en 0% de pastoreo con alimentación a base de ensilado de alfalfa, heno y un concentrado comercial.

En relación con los ácidos grasos con efectos beneficiosos en la salud humana o sus precursores, cabe destacar que la leche de tanque procedente del G1 presentó la menor proporción de ácido linoleico; sin embargo, mostró la mayor proporción de CLA con respecto a las proporciones de estos mismos ácidos grasos observadas en la leche de tanque procedente de los otros dos grupos estudiados. Este hecho pudo ser parcialmente explicado por la variación observada en la actividad de la Δ-9 desaturasa (en la glándula mamaria) entre los diferentes grupos a través de la relación ácido miristoleico/ácido mirístico. En este contexto, es importante explicar que la enzima Δ-9 desaturasa añade un doble enlace en la posición 9 y la configuración *cis* a ácidos grasos insaturados con una longitud de cadena entre 10 y 18 átomos de carbono y se ha observado que la relación ácido miristoleico/ácido mirístico es la forma más fiable para determinar la actividad de esta enzima. Esto se debe principalmente a que el ácido miristoleico presente en la leche se genera casi exclusivamente (más del 95%) mediante síntesis endógena a partir del ácido mirístico, a diferencia de los otros ácidos grasos monoinsaturados, los cuales también pueden proceder de la dieta (Griinari et al., 2000; Renna et al., 2012). En el presente estudio, el G1 presentó un valor mayor para esta relación que el G2 y el G3, lo que significa que la actividad de la Δ-9 desaturasa fue mayor en el G1 y de ahí que previamente en este grupo se observase una menor proporción de ácido linoleico pero una mayor proporción de CLA en relación con los otros dos grupos estudiados. Estos resultados fueron similares a los descritos por Lock y Garnsworthy (2003), quienes señalaron que las vacas alimentadas bajo sistema de pastoreo mostraron un aumento en la actividad de la Δ-9 desaturasa en la glándula mamaria. Así mismo, esta misma tendencia en la actividad de la Δ-9 desaturasa fue descrita por Renna et al. (2012) en su estudio del perfil de ácidos grasos en leche de cabra. En lo que respecta a la proporción de CLA observada por estos autores y por Couvreur et al. (2006) en leche de vaca, ésta fue mayor que la observada en nuestro estudio, pudiendo ser debido a las diferencias existentes en la composición botánica de los pastos frescos, así como también porque los rebaños estudiados en el presente trabajo fueron comerciales y no experimentales.

Adicionalmente, la leche procedente del G1 presentó una menor relación *omega*-6/*omega*-3 que la leche procedente de los otros dos grupos, lo cual podría ser explicado

por una mayor ingesta de ácido α-linolénico a partir del pasto fresco. No obstante, los cambios descritos en el perfil de ácidos grasos de la leche no sólo se deben al suministro de pasto en la dieta sino que también pueden estar asociados a los forrajes y concentrados incluidos en la dieta de las ovejas. En este sentido, el G3 presentó las proporciones más bajas de CLA y ácido α-linolénico con respecto a los otros dos grupos estudiados. Este hecho pudo ser debido a que fue el único grupo constituido por granjas en las que la alimentación de las ovejas consistió exclusivamente en forraje y concentrado. Según lo descrito por Chillard et al. (2007), este tipo de dieta no contribuye a incrementar la proporción de CLA en la leche, debido a que esta no aporta cantidades suficientes de precursores. En el presente estudio, el G1 estuvo constituido por granjas en las que las ovejas fueron alimentadas con una proporción de forraje menor que las ovejas pertenecientes al G2; sin embargo, las ovejas del G1 pastaron más que las del G2, lo que se tradujo en una mayor proporción de CLA en la leche procedente del G1. Es preciso indicar que cuanto más pastan las ovejas, la microbiota del rumen puede verse afectada, favoreciendo que la biohidrogenación sea incompleta. Este hecho conduce a una mayor síntesis de ácido vacénico, el cual puede ser desaturado en la glándula mamaria por acción de la enzima Δ-9 desaturasa dando lugar una mayor proporción de CLA en la leche (Tsiplakou et al., 2006). De todo esto se deduce que, los factores que pueden afectar a la proporción de este ácido graso beneficioso en la leche de oveja podrían ser: la ingesta de precursores en la dieta y el grado de biohidrogenación que tiene lugar en el rumen.

Las etapas de lactación consideradas en el presente estudio fueron: 20-60 días posparto (inicial), 60-110 días posparto (media) y 110-160 días postparto (final). Este factor explicó menos del 4,18% de la varianza para los ácidos grasos estudiados y no afectó a la proporción de CLA presente en la leche de oveja. Por esta razón, el efecto de la etapa de lactación sobre la proporción de ácidos grasos presentes en la leche de oveja fue menos importante que el del resto de los factores estudiados.

Con respecto al mes de muestreo, la mayor proporción de CLA se observó en el mes de abril seguido de mayo, junio y julio. Estos resultados fueron consistentes con los con los descritos por Tsiplakou et al. (2006), quienes observaron que los valores más altos de CLA en leche de oveja se detectan en abril y mayo, mientras que la proporción de este ácido graso es más baja en enero, marzo y junio. Como en nuestro estudio, estos autores detectaron niveles más altos de CLA en primavera, cuando el forraje es rico en ácido α-linolénico (Tsiplakou et al., 2008). Se ha descrito que la proporción de este ácido graso disminuye a medida que el forraje madura y presenta una mayor proporción de fibra (Dewhurst et al., 2001). En consecuencia, la ingesta de forraje deficiente en ácido α-linolénico conduce a una disminución en la concentración de ácido vacénico, el cual es un intermediario generado durante la biohidrogenación en el rumen. Este hecho a su vez da lugar a una reducción en el CLA sintetizado a partir de ácido vacénico en la glándula mamaria (Nudda et al., 2005), lo que contribuirá a que la concentración de este ácido graso bioactivo presente en la leche sea menor.

Hay que señalar que la interacción entre el mes de muestro y el régimen de alimentación fue significativa ($P \leq 0,01$) para el CLA y para la relación CLA/ácido linoleico. En abril, la leche procedente del G1 y del G2 mostró mayores valores de CLA y de la relación CLA/ácido linoleico que la leche recogida de los mismos grupos en julio. Sin embargo, no se observaron diferencias significativas ($P > 0,05$) entre los valores del CLA y

en la relación CLA/ácido linoleico de la leche del G3 recogida entre abril y julio. Es decir, el mes de muestreo solo influirá significativamente en la proporción de CLA en la leche cuando la alimentación esté basada en el pastoreo.

Por último, en el análisis estadístico mediante agrupación jerárquica utilizando el método Ward de la proporción de ácidos grasos presentes en la leche procedente de los rebaños estudiados se formaron dos *clusters*, reflejando las diferencias existentes entre los distintos rebaños. El primer *cluster* estuvo formado por los rebaños pertenecientes al G3 y por algún rebaño del G2, mientras que el segundo *cluster* estuvo constituido por el resto de los rebaños del G2 y los rebaños del G1. Este último *cluster* mostró una mayor proporción media de CLA que el *cluster* 1. Así mismo, el *cluster* 2 presentó los valores medios más bajos para la relación *omega*-6/*omega*-3 e índice de aterogenicidad en comparación con el *cluster* 1. En consecuencia, estos resultados fueron similares a los observados previamente cuando se analizó el efecto del régimen de alimentación.

4.2. Estudio de la capacidad de cepas de bacterias ácido lácticas para producir CLA

La calidad nutricional de los productos lácteos, en particular del queso de oveja, no sólo puede ser incrementada mediante el empleo de leche con un perfil en ácidos grasos más saludable a través del control del sistema de manejo de los rumiantes sino que también puede ser mejorada mediante el uso de bacterias ácido lácticas con capacidad para convertir el ácido linoleico presente en la leche en CLA por acción de la enzima linoleato isomerasa. Por este motivo, en la presente Tesis se seleccionaron cepas de bacterias ácido lácticas, procedentes de la colección del Departamento de Higiene y Tecnología de los Alimentos de la Universidad de León, con capacidad para sintetizar CLA a partir del ácido linoleico como sustrato. Ante la necesidad de la industria quesera para mantener la singularidad de cada variedad de queso así como para desarrollar quesos con características sensoriales atractivas para el consumidor, las cepas estudiadas en el presente trabajo fueron aisladas de quesos artesanales y seleccionadas en base a la buena aptitud tecnológica que presentaron en estudios previos (González et al., 2015, 2010, 2007; Herreros et al., 2003) con el fin de utilizarlas en el diseño de cultivos iniciadores y adjuntos.

En los capítulos 2 y 3 de la presente Tesis se recogen los principales resultados relacionados con el estudio de la capacidad de producción de CLA por parte de las bacterias ácido lácticas y cuya discusión detallada se presenta a continuación.

En primer lugar, se comprobó la tolerancia al ácido linoleico de las cepas seleccionadas debido a que en varios estudios se ha demostrado que este ácido graso poliinsaturado puede retardar o inhibir el crecimiento de algunas bacterias (Gorissen et al., 2010; Jiang et al., 1998; Nieuwenhove et al., 2007a). La concentración de ácido linoleico utilizada en este estudio se basó en la concentración media (0,5 mg/mL) presente en leche de oveja (De La Fuente et al., 2009), lo que significa que las cepas que fuesen identificadas como productoras de CLA serían capaces de crecer en la leche de oveja empleada como materia prima en la elaboración de los lotes de queso.

De las 85 cepas estudiadas, solo 6 cepas pertenecientes a las especies *Leuconostoc pseudomesenteroides, Leuconostoc mesenteroides* y *Enterococcus faecalis* mostraron

inhibición en su crecimiento y 1 cepa de *Lactococcus lactis* subsp. *lactis* no creció tras 48 h de incubación. Estos resultados confirmaron que la inhibición del crecimiento de las bacterias ácido lácticas por acción del ácido linoleico es dependiente de la cepa y no de la especie, tal y como fue observado también por Gorissen et al. (2010) en cepas de *Bifidobacterium*.

Generalmente, la identificación de cepas productoras de CLA se lleva a cabo mediante análisis cromatográfico. Ahora bien, El-Salam et al. (2010) han señalado que aunque este método proporciona buenos resultados, también implica una inversión económica y de tiempo elevada. Por este motivo, se decidió utilizar el método basado en la determinación espectrofotométrica de los dobles enlaces conjugados presentes en los ácidos grasos. Este método constituye una buena alternativa al análisis cromatográfico, ya que proporciona ventajas a la hora de analizar un amplio número de muestras, como el que se presenta en este trabajo, al implicar un menor tiempo de trabajo y el equipamiento necesario para llevarlo a cabo es más económico (Barrett et al., 2007).

Las cepas analizadas en esta Tesis (85 cepas) pertenecieron a diferentes géneros: *Lactococcus*, *Leuconostoc*, *Lactobacillus* y *Enterococcus*. Únicamente fueron capaces de sintetizar CLA en medio de cultivo suplementado con ácido linoleico, 2 cepas de *Lactobacillus casei* subsp. *casei* (SS 1614 y SS 1644) y 4 cepas de *Lactobacillus plantarum* (TAUL 1539, TAUL 1588, TAUL 1795 y TAUL 1522). Los *Lactobacillus* son bacterias ácido lácticas, *non-starters*, los cuales juegan un papel relevante en la maduración de los quesos, por lo que su estudio como posibles cultivos adjuntos es de especial interés.

Diversos autores han señalado que la concentración de CLA producida por las bacterias ácido lácticas puede verse afectada por diversos factores como es el tiempo de incubación y el pH (El-Salam et al., 2010; Rodríguez-Alcalá et al., 2011). En este sentido y en relación con el tiempo de incubación, todas las cepas estudiadas produjeron un incremento estadísticamente significativo ($P \leq 0{,}001$) de la concentración de CLA entre las 24 h y 48 h de incubación. En consecuencia, el tiempo de incubación es un factor importante a tener en cuenta en la producción de CLA por parte de las cepas de *Lactobacillus*. De igual manera, se observó que *Lactobacillus plantarum* TAUL 1588 resultó ser la cepa que produjo la mayor concentración de CLA tras 24 y 48 horas de incubación alcanzando valores similares a los observados por Rodríguez-Alcalá et al. (2011) para la cepa *Lactobacillus plantarum*-2, empleando el mismo método y por Terán et al. (2015) para *Lactobacillus plantarum* CRL1935 mediante cromatografía de gases. Debido a su capacidad para sintetizar altas concentraciones de CLA, *Lactobacillus plantarum* TAUL 1588 es una cepa de gran interés para investigar su papel como cultivo adjunto en el desarrollo de productos lácteos funcionales.

En lo que respecta a las cepas autóctonas de *Lactobacillus casei* estudiadas; *Lactobacillus casei* subsp. *casei* SS 1644 fue la cepa que produjo la menor concentración de CLA a las 24 h de incubación, mientras que *Lactobacillus casei* subsp. *casei* SS 1614 produjo la menor concentración de CLA a las 48 h. En general, las cepas de *Lactobacillus casei* subsp. *casei* fueron menos eficientes en la síntesis de CLA que las cepas de *Lactobacillus plantarum*. Simultáneamente se observó que las cepas que produjeron un mayor descenso en el pH del medio tras 24 y 48 horas de incubación, *Lactobacillus plantarum* TAUL 1539 y TAUL 1588, fueron también las más productoras de CLA durante estos tiempos de incubación.

Hay que tener en cuenta que la linoleato isomerasa es la enzima responsable de la producción de CLA a partir del ácido linoleico como sustrato en las bacterias ácido lácticas (Chen, 2012; Gorissen et al., 2011). En este estudio, se identificó la presencia del gen que codifica la linoleato isomerasa en las seis cepas detectadas como productoras de CLA. En el caso de las cepas de *Lactobacillus plantarum*, el tamaño aproximado de la banda detectada fue de 500 pares de bases, mientras que para las dos cepas de *Lactobacillus casei* subp. *casei* fue de 600 pares de bases. Este gen también fue detectado en alguna de las cepas que no produjeron CLA. Este hecho ha sido descrito previamente por Gorissen et al. (2011), los cuales observaron que la enzima linoleato isomerasa puede estar presente en las cepas pero, la expresión de la misma podría verse afectada por diversos factores tales como el pH o la temperatura, y estos efectos a su vez son dependientes de la cepa.

Otro aspecto muy importante que hay que resaltar es que aunque los métodos espectrofotométrico y genético empleados son útiles a la hora de identificar cepas de bacterias ácido lácticas productoras de CLA, dichos métodos no permiten detectar los distintos isómeros del CLA producidos (Barrett et al., 2007). La identificación y cuantificación de los isómeros CLA sintetizados por parte de las bacterias ácido lácticas puede ser de gran interés debido a que las propiedades beneficiosas para la salud humana están relacionadas principalmente con dos de ellos: *cis*-9,*trans*-11 $C_{18:2}$ y *trans*-10,*cis*-12 $C_{18:2}$. No obstante, desde hace varios años, el isómero *trans*-9,*trans*-11 $C_{18:2}$ también se encuentra bajo investigación debido a sus potenciales efectos anticarcinogénicos observados en líneas celulares de cáncer de colon humano (Coakley et al., 2006) y por su capacidad para inducir la expresión de genes implicados en el metabolismo lipídico (Ecker et al., 2009). Por esta razón, se realizó un estudio más específico, utilizando cromatografía de gases, que evaluara la capacidad para sintetizar CLA por estas seis cepas autóctonas de *Lactobacillus* en medio de cultivo y en leche desnatada suplementados con ácido linoleico bajo diferentes tiempos de incubación (24, 48 y 72 h).

Los resultados observados en este estudio mostraron que el tiempo óptimo de incubación para la producción de CLA por parte de las cepas estudiadas en medio de cultivo o en leche desnatada fue 48 h. En este sentido, Nieuwenhove et al. (2007b) han descrito la existencia de una relación entre la fase de crecimiento de las bacterias y la concentración de CLA sintetizada en el medio de cultivo. En consecuencia, la mayor concentración de CLA observada a las 48 h de incubación pudo estar relacionada con el incremento en el recuento de células viables tras 48 h en comparación con los recuentos observados a las 24 h de incubación.

Adicionalmente se observó que los niveles de CLA total en medio de cultivo disminuyeron significativamente ($P < 0,05$) a partir de 48 h de incubación. Este hecho pudo ser debido al desarrollo de reacciones de oxidación, así como al metabolismo oxidativo de las cepas durante el tiempo de incubación, lo que favorecería el catabolismo de este compuesto (Ogawa et al., 2001; Rodríguez-Alcalá et al., 2011; Wang et al., 2007).

El tipo de medio de crecimiento (medio de cultivo o leche desnatada) influyó en la producción de CLA por parte de las bacterias ácido lácticas estudiadas. En este contexto, la capacidad de las cepas de *Lactobacillus plantarum* para sintetizar CLA se redujo significativamente cuando se incubaron en leche desnatada. Contrariamente, en el caso de las cepas de *Lactobacillus casei* subsp. *casei*, la producción de CLA fue más eficiente en leche desnatada, alcanzando valores de CLA total a las 48 horas de incubación similares a

los producidos por las cepas de *Lactobacillus plantarum* TAUL 1588 en medio de cultivo durante el mismo periodo de tiempo. Cabe resaltar que tras 24 horas de incubación en leche desnatada, *Lactobacillus plantarum* TAUL 1588, TAUL 1795 y TAUL 1522 fueron incapaces de sintetizar CLA y *Lactobacillus plantarum* TAUL 1539 sólo sintetizó 1,43 µg/mL de CLA total. Por el contrario, *Lactobacillus casei* subsp. *casei* SS 1614 and SS 1644 produjeron 27,16 y 26,52 µg/mL de CLA total, respectivamente. La explicación de estos resultados aún no está clara ya que los mecanismos de síntesis de CLA por parte de las bacterias ácido lácticas son inciertos. Ahora bien, este hecho quizás pudo ser debido a que como Gorissen et al. (2011) han indicado, la actividad de la linoleato isomerasa puede verse afectada por diversos factores ambientales, como el pH.

El análisis mediante cromatografía de gases llevado a cabo en este estudio mostró que bajo las condiciones experimentales, las seis cepas de *Lactobacillus* produjeron tres isómeros CLA: *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ y *trans*-9,*trans*-11 $C_{18:2}$, excepto cuando fueron incubadas en leche desnatada, las cuales sólo fueron capaces de sintetizar dos isómeros CLA: *cis*-9,*trans*-11 $C_{18:2}$ y *trans*-9,*trans*-11 $C_{18:2}$. En definitiva, el tipo de medio empleado afectó a la producción de los diferentes isómeros del CLA sintetizados por las bacterias ácido lácticas.

En relación con este último aspecto, se observó claramente que la mayor concentración de los isómero *cis*-9,*trans*-11 $C_{18:2}$, *trans*-10,*cis*-12 $C_{18:2}$ y *trans*-9,*trans*-11 $C_{18:2}$ en medio de cultivo suplementado con ácido linoleico tras 48 h fue producida por la cepa de *Lactobacillus plantarum* TAUL 1588. En cambio, tras ese mismo periodo de incubación pero utilizando leche desnatada suplementada con ácido linoleico, la cepa que produjo la mayor concentración de los isómeros *cis-9,trans-11* $C_{18:2}$ y *trans-9,trans-11* $C_{18:2}$ fue *Lactobacillus casei* subsp. *casei* SS 1644. Comparativamente, aunque las 6 cepas estudiadas fueron capaces de sintetizar CLA a partir del ácido linoleico como sustrato, se encontraron diferencias significativas ($P \leq 0,05$) en la concentración de los diferentes isómeros CLA sintetizados por cepas de la misma especie, lo que significó que la capacidad para producir este compuesto fue dependiente de cepa y no de la especie.

En cuanto al tiempo de incubación, este afectó a la proporción de isómeros CLA sintetizados por las cepas de *Lactobacillus* estudiadas, siguiendo un patrón común. En medio de cultivo, tras 24 y 48 h de incubación, el isómero mayoritario detectado fue el *cis*-9,*trans*-11 $C_{18:2}$, mientras que tras 72 h de incubación fue el *trans*-9,*trans*-11 $C_{18:2}$. Estos resultados fueron similares a los descritos por otros autores (Ando et al., 2004), quienes señalaron que conforme transcurre el tiempo de incubación, la concentración del isómero *trans*-9,*trans*-11 $C_{18:2}$ tiende a incrementarse con respecto a la concentración del isómero *cis*-9,*trans*-11 $C_{18:2}$. Sin embargo, cuando el medio de crecimiento fue leche desnatada, el *trans*-9,*trans*-11 $C_{18:2}$ fue el principal isómero detectado a lo largo de los distintos tiempos de incubación.

Llegados a este punto, conviene indicar que actualmente los mecanismos de producción e interconversión entre los isómeros CLA no están claros y requieren más estudios.

Tal y como ha sido mencionado con anterioridad, los *Lactobacillus* son bacterias ácido lácticas consideradas como *non-starters*, las cuales constituyen la microbiota predominante de los quesos durante la maduración de los mismos. Los *Lactobacillus* toleran bien los ambientes hostiles e influyen fuertemente en la bioquímica que tiene lugar

durante la maduración de la cuajada, contribuyendo al desarrollo de las características finales del queso (Settanni and Moschetti, 2010). En relación con las concentraciones de CLA producidas por las cepas *Lactobacillus plantarum* TAUL 1588 y *Lactobacillus casei* subsp. *casei* SS 1644 a las 48 h de incubación en medio de cultivo y en leche desnatada suplementados con 0,5 mg/mL de ácido linoleico, estas fueron comparables con los resultados observados en cepas de las mismas especies (Adeghate and Ponery, 2002; Gorissen et al., 2010; Terán et al., 2015). En consecuencia, el estudio de estas cepas como potenciales cultivos adjuntos es de especial interés, ya que estos hallazgos sugieren que podrían ser empleados para el desarrollo de productos lácteos funcionales fermentados con niveles elevados de CLA.

4.3. Estudio de la capacidad de cepas de bacterias ácido lácticas para producir GABA

En los últimos años, el GABA ha suscitado un gran interés ya que ejerce numerosas funciones fisiológicas: neurotransmisor, regulador de la tensión arterial y secretagogo de insulina (Adeghate y Ponery, 2002; Diana et al., 2014a; Okada et al., 2000). El GABA es sintetizado por la glutamato descarboxilasa, una enzima dependiente del piridoxal 5'-fosfato, la cual cataliza la α-descarboxilación del L-glutamato o sus sales a GABA (Narayan and Nair, 1990). Es importante resaltar que esta enzima ha sido detectada en bacterias ácido lácticas (Cotter y Hill, 2003; Komatsuzi et al., 2008) y en consecuencia, el estudio de la capacidad productora de GABA por cepas de bacterias ácido lácticas tiene interés con vistas a su utilización como cultivos iniciadores o adjuntos en la elaboración de productos lácteos. En este sentido, tal y como Siragusa et al. (2007) han señalado, el queso es un buen vehículo de GABA porque las caseínas de la leche presentan una alta proporción de L-glutamato, el cual es liberado durante la maduración de los quesos y puede ser metabolizado a GABA por acción de las bacterias ácido lácticas (Hejtmánková et al., 2012).

Por estas razones, las 85 cepas de bacterias ácido lácticas mencionadas en el apartado anterior también fueron objeto de estudio con el fin de detectar aquéllas con capacidad para sintetizar GABA a partir del glutamato monosódico como sustrato en medio de cultivo (Elliker o MRS). Los resultados obtenidos en este estudio y que son discutidos a continuación, se recogen en el capítulo 2 de la presente Tesis.

Diez de las 85 cepas estudiadas fueron identificadas como productoras de GABA: *Lactococcus lactis* subp. *lactis* (GE 61, GE 118, GE 102, GE 103) y *Lactobacillus brevis* (TAUL 141, TAUL 174, TAUL 69, TAUL 70, TAUL 179, TAUL 195). Estos resultados mostraron la variabilidad existente entre cepas de la misma especie y permitieron confirmar que la capacidad para sintetizar GABA es dependiente de la cepa, tal y como fue descrito por Siragusa et al. (2007).

Así mismo, se estudió el efecto del tiempo de incubación sobre la producción de GABA por parte de las 10 cepas identificadas como productoras ya que como han señalado Dahakal et al. (2012), diferentes factores implicados en el proceso de fermentación, entre ellos el tiempo de incubación, pueden afectar a la síntesis de GABA por parte de los microorganismos. En relación con la evolución de la producción de GABA por parte de las cepas de bacterias ácido lácticas estudiadas a lo largo de los distintos tiempos de incubación en el medio de cultivo suplementado con glutamato monosódico, se observó

que todas las cepas mostraron un incremento estadísticamente significativo ($P \leq 0,05$) en la síntesis de GABA de 24 a 72 horas de incubación. Estos resultados fueron similares a los descritos por Wu y Sha (2015), quienes detectaron un incremento en la producción de GABA por parte de 9 bacterias ácido lácticas tras 72 horas de incubación en medio de cultivo MRS suplementado con 50 mg/mL de glutamato monosódico como sustrato.

Uno de los resultados más importantes de nuestro estudio fue que la cepa que produjo la mayor concentración de GABA (2524,05 µg/mL) fue *Lactobacillus brevis* TAUL 195 tras 72 horas de incubación, lo que la convierte en una cepa autóctona con gran interés para ser utilizada como cultivo adjunto en la elaboración de productos lácteos.

Los resultados observados en el presente estudio mostraron que las cepas de *Lactobacillus brevis* fueron más eficientes que las cepas de *Lactococcus lactis* en la producción de GABA, coincidiendo con lo descrito por otros autores (Barrett et al., 2012; Wu y Shah, 2017; Zhang et al., 2012).

Cabe señalar que Diana et al. (2014a) han indicado que el empleo de cultivos compuestos por varias cepas productoras de GABA puede constituir una estrategia nueva y eficiente para incrementar la concentración de GABA en los productos lácteos. Así mismo, Settani y Moschetti (2010) han descrito que la combinación de cepas de *Lactococcus lactis* y de cepas de *Lactobacillus* puede mejorar las características sensoriales del queso. En base a estas consideraciones, sería interesante llevar a cabo más estudios para diseñar cultivos con las cepas detectadas como productoras de GABA en este estudio, pertenecientes a las especies *Lactococcus lactis* y *Lactobacillus brevis,* ya que no sólo nos permitirían obtener quesos con buenas características sensoriales sino que también con un elevado contenido en GABA, el cual se ha observado que tiene efectos beneficiosos sobre la salud humana.

Es importante indicar que el sistema glutamato descarboxilasa, el cual está formado por la enzima glutamato descarboxilasa y por el *antiporter* glutamato-GABA, es el responsable de la producción de GABA por parte de las bacterias ácido lácticas. Por este motivo, en este estudio, se comprobó la presencia de los genes que codifican la enzima glutamato descarboxilasa y el *antiporter* glutamato-GABA en las cepas detectadas como productoras de GABA y en algunas de las no productoras.

En este contexto, Nomura et al. (1999) han observado que las cepas de *Lactococcus lactis* sólo presentan un gen que codifica la enzima glutamato descarboxilasa mientras que Li et al. (2013) han identificado dos genes que codifican esta enzima en cepas de *Lactobacillus brevis*. Ambos hechos fueron observados también en el presente estudio.

En el caso de las cepas pertenecientes a la especie *Lactobacillus brevis*, se detectó la presencia de los genes que codifican la enzima glutamato decarboxilasa y el *antiporter* glutamato-GABA en las cepas identificadas como productoras de GABA y también en alguna de las cepas no productoras. Tal y como Siragusa et al. (2007) han indicado, este hecho pudo ser debido a que la expresión de estos genes puede verse afectada por diversos factores como por ejemplo el pH del medio.

No obstante, cuando las cepas estudiadas pertenecieron a la especie *Lactococcus lactis* sólo se detectó la presencia de estos genes en las cuatro cepas identificadas previamente como productoras de GABA. Estos resultados abren la posibilidad de utilizar esta técnica como método para identificar cepas de *Lactococcus lactis* productoras de

GABA en base a la presencia de estos genes. Sin embargo, esto debería ser estudiado en un mayor número de cepas.

4.4. Diseño de co-cultivos constituidos por cepas de bacterias ácido lácticas con capacidad para sintetizar CLA

Otro de los aspectos estudiados en esta Tesis fue la posibilidad de diseñar cultivos que pudieran incrementar el contenido en CLA en el queso de oveja, debido a la buena capacidad para sintetizar este compuesto que presentaron las 6 cepas de *Lactobacillus*. Los resultados que son discutidos a continuación aparecen recogidos en el capítulo 3 de la presente Tesis.

En base a que los cultivos iniciadores comerciales incluyen cepas de *Lactococcus lactis* como microorganismos acidificantes, en este estudio se diseñaron 4 cultivos constituidos por cepas de esta especie combinadas con dos de las cepas de *Lactobacillus* (TAUL 1588 y SS 1644) identificadas como productoras de CLA (**Figura 4**). Dos de estos cultivos (1 y 3) incluyeron cepas de *Lactococcus lactis* subsp. *lactis* y *Lactococcus lactis* subsp. *cremoris* de origen comercial (CHOOZIT™ LYO MA 011) y los otros dos (2 y 4) estuvieron constituidos por las cepas autóctonas *Lactococcus lactis* subsp. *lactis* TAUL 238 y *Lactococcus lactis* subsp. *cremoris* TAUL 1239. Es necesario indicar que ninguna cepa de *Lactococcus lactis* incluida en este estudio fue capaz de sintetizar CLA. Estas cepas de *Lactococcus* se seleccionaron para el diseño de los cultivos porque en estudios previos mostraron una buena aptitud tecnológica (Herreros et al., 2003) y en el test de compatibilidad, realizado siguiendo el método descrito por Schillinger y Lücke (1989), fueron las cepas que presentaron el mejor comportamiento entre ellas y con las cepas de *Lactobacillus* productoras de CLA.

El objetivo de este planteamiento fue establecer si el empleo de cepas de *Lactococcus lactis* comerciales o autóctonas podría afectar a la síntesis de CLA por parte de las cepas de *Lactobacillus* productoras de este compuesto. El criterio seguido para la selección de las cepas de *Lactobacillus* se basó en que la cepa de *Lactobacillus plantarum* TAUL 1588 fue la cepa que produjo la mayor concentración de CLA en medio de cultivo y que la cepa de *Lactobacillus casei* subsp. *casei* SS 1644 fue la que sintetizó mayor concentración de este ácido graso biactivo en leche desnatada. A su vez, estas cepas presentaron una buena compatibilidad entre ellas. A continuación, en el caso de los cultivos 3 y 4 se pretendía estudiar si la combinación de las dos cepas productoras de CLA podría aumentar la producción de este compuesto más que ellas mismas por separado (cultivos 1 y 2) (**Figura 4**).

En base a los planteamientos descritos, se estudió la capacidad para sintetizar CLA por parte de estos cuatro cultivos en medio de cultivo y en leche desnatada suplementados con ácido linoleico durante tres tiempos de incubación (24, 48 y 72 h).

En relación con los isómeros CLA sintetizados por los diferentes cultivos a lo largo de la incubación, cuando se utilizó el medio de cultivo para el crecimiento, fue observado que el contenido en el isómero *cis*-9,*trans*-11 $C_{18:2}$ presentó diferencias estadísticamente significativas ($P \leq 0,05$) entre los cultivos a las 24, 48 y 72 h de incubación. En todos los casos, la mayor concentración de este isómero se produjo a las 48 h y los cultivos que

mostraron una mayor producción de este isómero fueron el 1 y el 2, no encontrándose diferencias estadísticamente significativas ($P > 0,05$) entre ellos.

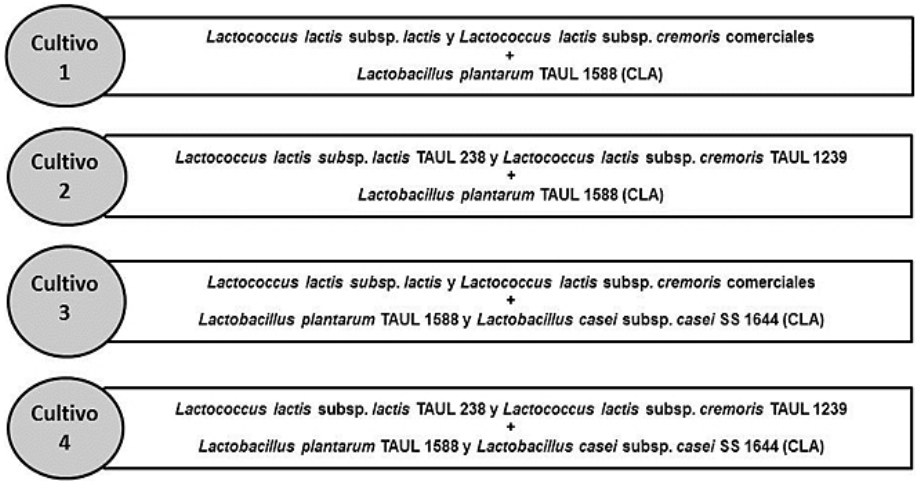

Figura 4. Descripción de cada cultivo diseñado con cepas de *Lactococcus lactis* (comerciales y autóctonas) y las cepas de *Lactobacillus* productoras de CLA.

En el caso de del isómero *trans*-10,*cis*-12 $C_{18:2}$, no se observaron diferencias estadísticamente significativas ($P > 0,05$) entre los distintos cultivos a lo largo de la incubación. Sin embargo, el contenido en el isómero *trans*-9,*trans*-11 $C_{18:2}$ presentó diferencias estadísticamente significativas ($P \leq 0,01$) entre los cuatro cultivos a las 72 horas de incubación, observándose la mayor concentración del mismo en el caso del cultivo 2. Con respecto al CLA total producido por los cultivos, y al igual que en el estudio descrito anteriormente con respecto a las seis cepas de *Lactobacillus*, la mayor producción de CLA total se produjo a las 48 horas de incubación, momento a partir del cual la concentración de dicho compuesto comenzó a disminuir significativamente ($P \leq 0,05$). Cabe resaltar que las mayores concentraciones de CLA a las 48 h fueron producidas por el cultivo 2 y por el cultivo 1. En el estudio llevado a cabo en medio de cultivo como medio de crecimiento, estos cultivos produjeron casi el doble de la concentración de CLA que produjo individualmente la cepa de *Lactobacillus plantarum* TAUL 1588 en el mismo medio. En este contexto, Andrade et al. (2012) han indicado que existen diversos factores ambientales que pueden afectar a la síntesis de CLA por parte de las bacterias ácido lácticas, explicando el motivo por el cual la presencia de cepas de *Lactococcus lactis* en este estudio hicieron más propicias las condiciones para la producción de CLA por parte de la cepa *Lactobacillus plantarum* TAUL 1588. En relación con que las cepas de *Lactococcus lactis* fuesen autóctonas o comerciales, este hecho no afectó significativamente ($P > 0,05$) a la producción de CLA por parte de los cultivos, al menos hasta las 72 h de incubación. En el caso del cultivo 2, constituido por las cepas autóctonas de *Lactococcus lactis*, el descenso en la concentración de CLA tras 72 h de incubación fue menos acusada que en el resto de los cultivos estudiados. De igual manera, en lo que respecta a la combinación de cepas productoras de CLA de diferentes especies, este hecho no incrementó la

concentración de CLA en medio de cultivo para ninguno de los tiempos de incubación analizados.

Por el contrario, el contenido en CLA total y de cada uno de los isómeros producidos por los cuatro cultivos diseñados en los distintos tiempos de incubación fue diferente en leche desnatada con respecto al observado en el medio de cultivo. En base a las consideraciones indicadas previamente en la presente Tesis en relación con la importancia que tienen los distintos isómeros CLA sobre la salud humana, cabe señalar que el contenido en el isómero *cis*-9,*trans*-11 $C_{18:2}$ presentó diferencias estadísticamente significativas ($P \leq 0,05$) entre los cultivos a las 48 y a las 72 h de incubación. Durante este último tiempo de incubación, los cultivos 1, 2 y 3 produjeron una mayor concentración de este isómero, mientras que en el caso del cultivo 4 se observó un descenso de 9,40 µg/mL con respecto a la concentración observada a las 48 h. La mayor concentración del isómero *trans*-9,*trans*-11 $C_{18:2}$ producida a las 72 h de incubación por el cultivo 4 podría estar relacionada tanto con su síntesis a partir del ácido linoleico como con una mayor interconversión del isómero *cis*-9,*trans*-11 $C_{18:2}$ hacia éste, coincidiendo con lo descrito por Ogawa et al. (2005). Este hecho a su vez explicaría el descenso anteriormente observado en la concentración del isómero *cis*-9,*trans*-11 $C_{18:2}$ producida por el cultivo 4 tras 72 h de incubación en comparación con los otros cultivos.

De igual manera, tal y como había sido observado cuando se estudió la producción de CLA por parte de las cepas de *Lactobacillus* en leche desnatada, ninguno de los cultivos diseñados tuvo la capacidad de sintetizar el isómero *trans*-10,*cis*-12 $C_{18:2}$.

Por último, es importante señalar que la mayor concentración de CLA total se observó a las 72 h de incubación, la cual fue producida por el cultivo 4, constituido por cepas autóctonas de *Lactococcus lactis* y las cepas productoras de CLA *Lactobacillus plantarum* TAUL 1588 y *Lactobacillus casei* subsp. *casei* SS 1644. Sin embargo, el menor contenido de CLA fue descrito para los cultivos 1 y 2, no incluyendo ninguno de ellos la cepa *Lactobacillus casei* subsp. *casei*, la cual resultó ser en el estudio de las cepas, la especie más eficiente sintetizando CLA en leche desnatada en comparación con las cepas de *Lactobacillus plantarum*. En consecuencia, la presencia de *Lactobacillus casei* subsp. *casei* SS 1644 en leche desnatada conllevó a un incremento en la producción de CLA en este medio. Así mismo, la presencia de cepas autóctonas de *Lactococcus lactis* mejoró significativamente ($P \leq 0,05$) la producción de CLA en la leche desnatada en el caso del cultivo 4 con respecto al cultivo 3 y del cultivo 2 en relación con el cultivo 1.

4.5. Evolución de los parámetros físico-químicos y de los principales grupos microbianos de los quesos de oveja elaborados con diferentes cultivos productores de CLA a lo largo de la maduración

En base a los resultados descritos anteriormente, los quesos de oveja fueron elaborados empleando como materia prima leche de oveja procedente de una de las granjas que fueron estudiadas en el capítulo 1 de la presente Tesis y cuya recogida fue programada para el mes de abril, periodo en cual las ovejas fueron alimentadas principalmente bajo régimen de pastoreo. Así mismo, teniendo en cuenta la capacidad de los co-cultivos diseñados para producir CLA *in vitro* utilizando leche desnatada como medio de crecimiento, fueron elaborados 4 lotes de queso, los cuales se describen a

continuación. El lote 1 o control se elaboró con el cultivo iniciador que incluyó las cepas autóctonas de *Lactococcus lactis* subsp. *lactis* TAUL 238 y *Lactococcus lactis* subsp. *cremoris* TAUL 1239, ninguna de ellas productora de CLA. Los lotes 2 y 3 se elaboraron con los cultivos constituidos por estas mismas cepas de *Lactococcus lactis* y una de las cepas de *Lactobacillus* productoras de CLA, *Lactobacillus plantarum* TAUL 1588 y *Lactobacillus casei* subsp. *casei* SS 1644, respectivamente. Finalmente, el lote 4 fue elaborado empleando el cultivo formado por las cepas de *Lactococcus lactis* más las dos cepas de *Lactobacillus* productoras de CLA (**Figura 5**).

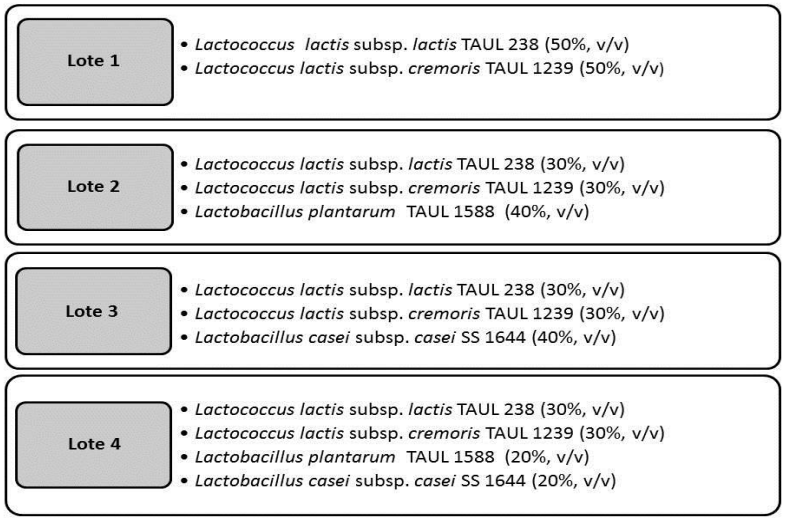

Figura 5. Cultivos autóctonos empleados en la elaboración de los lotes de queso de oveja. Proporción individual de cada cepa con respecto a la proporción total de las cepas que componen cada cultivo.

Los resultados que son discutidos en detalle en este apartado aparecen recogidos en el capítulo 5 de la presente Tesis.

Los parámetros físico-químicos de los quesos juegan un papel fundamental durante la maduración de los mismos al influir no sólo sobre el desarrollo de la microbiota presente sino también sobre la actividad enzimática que cataliza los diferentes procesos bioquímicos que se desarrollan durante la misma y que van a ser los responsables de las características finales que presentan los quesos (Fox et al., 2016).

Los diferentes cultivos empleados en la elaboración de los cuatro lotes de queso no tuvieron un efecto significativo ($P > 0,05$) sobre los diferentes parámetros físico-químicos para el mismo tiempo de maduración. Únicamente en el caso de la acidez titulable, sus valores para los lotes 3 y 4 (los cuales incluyeron en el cultivo la cepa de *Lactobacillus casei* subsp. *casei* SS 1644) fueron superiores a los observados en los otros dos lotes de queso al cabo de 90 y 180 días de maduración. Este hecho pudo venir determinado por la

mayor capacidad acidificante presentada por esta cepa con respecto a la de *Lactobacillus plantarum* TAUL 1588, tal y como fue descrito en estudios previos (González et al., 2015; Herreros et al., 2003).

De igual manera, todos los parámetros físico-químicos analizados en este trabajo mostraron un comportamiento muy similar a lo largo de la maduración en los 4 lotes de queso. A los 90 días el valor de pH fue ligeramente superior al que presentaron los quesos al inicio de la maduración, permaneciendo luego constante o disminuyendo ligeramente hasta el final de la misma. Mejor indicador que el pH de la actividad microbiana en los quesos durante la maduración es la acidez titulable, cuyos valores se incrementaron durante los 6 primeros meses para luego disminuir ligeramente. Además, la acidificación que tuvo lugar durante el proceso de maduración de los lotes de queso contribuyó a la pérdida de humedad de éstos como fue reflejado en la correlación negativa observada entre los valores de la acidez y de la humedad (ρ= -0,84; $P \leq 0,001$). El contenido en humedad descendió a lo largo de la maduración alcanzando valores inferiores al 30% en todos los lotes de queso. Los valores de la actividad del agua siguieron una tendencia muy parecida, presentando valores medios de 0,989 al inicio de la maduración y de 0,915 al final de la misma. Así mismo, se observó que conforme la relación sal/humedad aumentaba, los valores de la actividad del agua disminuían (ρ= -0,87; $P \leq 0,01$). En lo referente a los valores observados para estos parámetros fueron similares a los descritos para otros quesos de oveja madurados (Guinee y Fox, 2004).

Cabe destacar que en ninguno de los lotes de queso se observaron diferencias significativas ($P > 0,05$) en el contenido en grasa y proteína (expresados sobre porcentaje de materia seca) durante la maduración. Este hecho fue debido a la estandarización de la materia prima usada en la fabricación (mismo productor y misma época), ya que ambos parámetros son los que mayores variaciones sufren durante el periodo de lactación. Los valores observados para estos dos parámetros fueron similares a los descritos en queso de oveja (Fernández et al., 2012).

En relación con la evolución de los principales grupos microbianos, se observó que tras la adición de los cultivos a la leche pasterizada, se incrementaron los recuentos de la microbiota aerobia mesófila, bacterias ácido lácticas en general y lactobacilos en particular durante la etapa inicial de la maduración. En este contexto, Diezhandino et al. (2015) han indicado que el aumento en los recuentos microbianos se debe tanto a la retención física de los microorganismos en la cuajada como a la multiplicación microbiana durante la fase de coagulación que tiene lugar en el proceso de elaboración de los quesos.

Los mayores recuentos en microbiota aerobia mesófila se observaron a los 2 días de maduración (10 log UFC/mL), excepto en el caso del lote 3, en el cual los recuentos máximos (11 log UFC/mL) se detectaron a los 90 días. A partir de este momento, estos recuentos descendieron hasta el final de la maduración, alcanzando valores entre 7 (lote 1) y 9 (lote 3) log UFC/mL.

Los recuentos de bacterias ácido lácticas a lo largo de la maduración fueron similares a los descritos previamente para la microbiota aerobia mesófila, aspecto que refleja que las bacterias ácido lácticas fueron los microorganismos predominantes en los cuatro lotes de queso. Al término de la maduración, los recuentos de bacterias ácido lácticas fueron del orden de 1-2 unidades logarítmicas inferiores a los observados a los 2 días de maduración. Este hecho pudo ser debido a que se produjo un importante

incremento en los valores de la relación sal/humedad en los quesos a los 90 días de maduración, lo que pudo tener un efecto inhibitorio en el crecimiento de las bacterias ácido lácticas. De hecho, fue observada una correlación negativa (ρ= -0,57; $P \leq 0,01$) entre los recuentos de bacterias ácido lácticas y la relación sal/humedad de los quesos.

Comparando la evolución de los lactobacilos en los puntos de muestreo realizados, se pudo observar que en los lotes de queso elaborados con los cultivos que incluyeron la cepa de *Lactobacillus plantarum* TAUL 1588 (lotes 2 y 4) los mayores recuentos se alcanzaron a los 90 días, mientras que en el lote 3, fabricado con el cultivo que incorporaba la cepa *Lactobacillus casei* subsp. *casei* SS 1644, los recuentos máximos se observaron tras 2 días de maduración, disminuyendo a partir de ese momento hasta el final de la misma. En el lote 1 no se detectaron recuentos de lactobacilos hasta los 180 días de maduración, siendo los mismos más bajos que los observados en los otros lotes de queso, consecuencia de la no inclusión de cepas de *Lactobacillus* en el cultivo iniciador.

Finalmente, los recuentos de *Enterobacteriaceae* son considerados como un buen indicador de las condiciones higiénicas durante el proceso de elaboración de los quesos (Renes et al., 2014). Las excelentes prácticas higiénicas llevadas a cabo durante la elaboración de los cuatro lotes de queso se vieron reflejadas en estos recuentos, donde únicamente fueron detectados en las muestras de 2 días de maduración y con valores inferiores a 4 log UFC/mL. A estos bajos recuentos también contribuyeron las condiciones de acidez y fuerza iónica instauradas en los quesos, como demuestran las correlaciones negativas observadas entre los recuentos de *Enterobacteriaceae* y los valores de acidez (ρ= -0,73; $P \leq 0,01$) así como entre los recuentos de *Enterobacteriaceae* y la relación sal/humedad (ρ= -0,95; $P \leq 0,01$), coincidiendo con lo descrito por otros autores en queso de oveja (Piras et al., 2013).

4.6. Efecto de cultivos productores de CLA y del tiempo de maduración sobre el perfil de ácidos grasos del queso de oveja

De acuerdo con los planteamientos que se han venido realizando en esta Tesis, se llevó a cabo el estudio del perfil de ácidos grasos de los cuatro lotes de queso elaborados con los diferentes cultivos autóctonos a lo largo de la maduración (2, 90, 180 y 240 días). El objetivo principal de este estudio fue conocer si los cultivos seleccionados por su capacidad para sintetizar CLA *in vitro* también presentaban esta capacidad cuando se utilizaban en la elaboración de los quesos de oveja.

Los resultados que a continuación son discutidos se presentan en el capítulo 4 de la presente Tesis.

El tiempo de maduración de los quesos no tuvo un efecto significativo ($P > 0,05$) sobre el contenido de los 86 ácidos grasos analizados en este estudio. De manera similar a este trabajo, varios autores han confirmado que el tiempo de maduración no afecta al perfil en ácidos grasos de los quesos (Bodas et al., 2010; dos Santos et al., 2012; Luna et al., 2007). En base a esto, es importante resaltar que la matriz del queso puede proporcionar estabilidad a los ácidos grasos biactivos, asegurando que sus concentraciones serán similares tanto en quesos frescos como en quesos madurados.

Sin embargo, el tipo de cultivo utilizado en la elaboración de queso de oveja si influyó significativamente ($P \leq 0,05$) sobre la concentración de los ácidos grasos. En todos

los lotes de queso, los ácidos grasos saturados fueron el grupo mayoritario representando el 73% del contenido total de ácidos grasos. El ácido palmítico ($C_{16:0}$) seguido del ácido mirístico ($C_{14:0}$) fueron los predominantes dentro del grupo de los ácidos grasos saturados. Estos resultados fueron similares a los descritos por Bodas et al. (2010) en su estudio del perfil de ácidos grasos en quesos de oveja elaborados con leche procedente de rebaños alimentados con diferentes dietas. Cabe destacar que el descenso observado en la concentración de ácido palmítico y mirístico del lote 4 en relación con los otros lotes de queso puede ser considerado como positivo desde el punto de vista nutricional. Sin embargo, hay que mencionar que la relación entre el consumo de ácidos grasos saturados y el desarrollo de enfermedades cardiovasculares es controvertida debido a que los datos observacionales procedentes de diversos estudios no han permitido probar la relación causa-efecto (Parodi, 2004).

Uno de los resultados más relevantes de este estudio fue que la combinación de las dos cepas de *Lactobacillus* en el cultivo empleado en la elaboración del lote 4 condujo a una mayor concentración de los ácidos grasos $C_{6:0}$, $C_{8:0}$ y $C_{10:0}$, los cuales han sido asociados con efectos beneficiosos en la salud humana (Nagao y Yanagita, 2010; Parodi, 2004). Además, estos ácidos grasos de cadena corta juegan un papel interesante en las características sensoriales de los quesos debido a que presentan umbrales de percepción más bajos que los ácidos grasos de cadena larga (Laskaridis et al., 2013).

Cabe destacar que el lote de queso elaborado con la combinación de ambas cepas de *Lactobacillus* mostró la menor concentración de $C_{17:0}$ así como de ácidos grasos de cadena ramificada en comparación con los lotes elaborados con las cepas de *Lactobacillus* individualmente. Este hecho mostró que el metabolismo de los cultivos utilizados para elaborar queso juega un papel importante en el contenido de estos ácidos grasos minoritarios, confirmando la relación entre la producción de ácidos grasos de cadena impar y ramificada y el tipo de microorganismo involucrado en el proceso de fermentación.

La proporción total de ácidos grasos monoinsaturados fue significativamente ($P \leq 0,05$) diferente entre los distintos lotes de queso estudiados. De nuevo el lote 4 presentó una mayor proporción (24,64%) de estos ácidos grasos en comparación con el lote 1 (22,68%). Dentro de este grupo de ácidos grasos, el ácido oleico ($C_{18:1}$ *cis*-9) fue el mayoritario, constituyendo entre el 78-80% del total de los ácidos grasos monoinsaturados en los cuatro lotes de queso. En el presente estudio se observó que el uso de cepas autóctonas productoras de CLA generó un incremento en la proporción de los isómeros *cis*-$C_{18:1}$, principalmente del isómero *cis*-9 en comparación con el lote control. Estos resultados difieren de los descritos por Mohan et al. (2013), los cuales observaron que el queso Cheddar elaborado empleando una cepa productora de CLA presentaba una menor proporción de ácido oleico en comparación con el lote control. De igual manera, en el presente estudio se comprobó que el empleo de cepas productoras de CLA en los cultivos utilizados en la elaboración de los quesos incrementaron la proporción de los ácidos grasos *trans*-$C_{18:1}$, siendo el ácido vacénico el isómero mayoritario, representando aproximadamente el 44% del total de los ácidos grasos *trans*- $C_{18:1}$. Estos resultados fueron similares a los descritos por dos Santos et al. (2012) y Taboada et al. (2015) para queso de cabra elaborado con cepas productoras de CLA.

La presencia de una concentración elevada de ácido vacénico en queso es deseable debido a que este ácido graso puede ser utilizado para la síntesis endógena (mediante la

enzima estearoil-CoA) del ácido ruménico en humanos, proporcionando los efectos beneficiosos para la salud humana que son atribuidos a este isómero CLA (Turpeinen et al., 2002).

De igual manera, el tipo de cultivo empleado influyó en la proporción de ácidos grasos poliinsaturados de los quesos de oveja estudiados. Dentro de este grupo de ácidos grasos, destacan el CLA y los ácidos grasos *omega*-3, los cuales han sido relacionados con efectos beneficiosos sobre la salud humana, siendo su ingesta a través de los alimentos esencial para alcanzar tales efectos positivos (Swanson et al., 2012; Yang et al., 2015). En este sentido, el lote 4 elaborado con una combinación de las cepas *Lactobacillus plantarum* TAUL 1588 y *Lactobacillus casei* subsp. *casei* SS 1644 presentó una mayor concentración de CLA total que el resto de los lotes estudiados. Este hecho podría ser debido a que la combinación de las dos cepas generaría condiciones más favorables para que tuviese lugar la actividad de la linoleato isomerasa de ambas cepas. Tal y como ha sido mencionado en apartados anteriores de esta Tesis, Gorissen et al. (2011) señalaron que la actividad de la linoleato isomerasa en las bacterias ácido lácticas es dependiente de factores ambientales tales como el pH o la temperatura, así como del tipo de cepa. Desafortunadamente, los mecanismos y factores que pueden afectar a la síntesis de CLA por parte de las bacterias ácido lácticas son inciertos y es necesario llevar a cabo más estudios.

El método cromatográfico empleado en este estudio permitió detectar diversos isómeros CLA en los cuatro lotes de queso de oveja. El ácido ruménico fue el isómero mayoritario detectado en todos los lotes, representando más del 80% del CLA total. Este hecho mostró concordancia con lo descrito por otros autores en queso de oveja (Mele et al., 2011; Zlatanos et al., 2002). Adicionalmente, la proporción de CLA total observada en el lote 4 fue similar a la descrita por El-Salam y El-Shibiny (2014) en queso.

En consecuencia destaca el gran interés que supone la combinación de estas cepas productoras de CLA en la elaboración de queso, al generar una mayor proporción de este ácido graso biactivo en comparación con el lote control.

Cabe señalar que el ácido α-linolénico fue el ácido graso *omega*-3 mayoritario en todos los lotes de queso, siendo los lotes de queso elaborados con los cultivos que incluyeron la cepa de *Lactobacillus casei* subsp. *casei* SS 1644 los que presentaron mayor proporción de este ácido graso. Por contra, en el lote 2 elaborado con el cultivo incluyendo la cepa de *Lactobacillus plantarum* TAUL 1588, la proporción de ácido α-linolénico fue similar a la observada en el lote control. Por tanto, y de acuerdo con estos resultados, la cepa de *Lactobacillus casei* subsp. *casei* SS 1644 podría ser la principal responsable del mayor contenido en ácido α-linolénico observado en estos quesos.

En general, los cambios descritos previamente en el perfil de ácidos grasos de los quesos dieron lugar a diferencias significativas ($P \leq 0,05$) en los índices de ácidos grasos relacionados con la salud humana y que han sido objeto del presente estudio. Altos valores en la relación *omega*-6/*omega*-3 y en el índice de aterogenicidad en los alimentos, si bien no conllevan que su consumo tenga que ser evitado, implicarían que este ha de ser moderado (Taboada et al., 2015). Cabe señalar que en el lote control, los valores observados para la relación *omega*-6/*omega*-3 y en el índice de aterogenicidad fueron significativamente ($P \leq 0,05$) superiores a los detectados en los lotes elaborados con cepas autóctonas con capacidad para sintetizar CLA. Estos resultados mostraron que el uso de estas cepas autóctonas, previamente seleccionadas como productoras de CLA *in vitro*, en la

elaboración de queso da lugar a un perfil en ácidos grasos beneficioso para la salud humana (más aún cuando se emplea una combinación de ellas) en comparación con la proporción de ácidos grasos observada en el queso de oveja elaborado con el cultivo que no incluyó cepas con mencionada capacidad.

Adicionalmente, se llevó a cabo un análisis de componentes principales para visualizar de una manera más gráfica el perfil de ácidos grasos que presentaron los cuatro lotes de queso de oveja a lo largo de la maduración. Se identificaron 3 grupos a lo largo de los dos primeros componentes, los cuales explicaron el 91% del total de la variación observada. Los quesos elaborados con la combinación de las dos cepas fueron situados en el lado derecho del gráfico y estuvieron correlacionados con la proporción de ácidos grasos monoinsaturados *cis* y *trans*, CLA total y ácidos grasos poliinsaturados. Por contra, los quesos control fueron localizados en el lado izquierdo del gráfico y estuvieron asociados con el contenido en ácidos grasos saturados, la relación *omega*-6/*omega*-3 y el índice de aterogenicidad. Los quesos pertenecientes a los lotes 2 y 3 formaron un único grupo con valores intermedios entre el lote 1 y el lote 4.

Estos resultados permitieron confirmar que el tipo de cultivo empleado en la elaboración de los quesos fue el factor que más afectaba al perfil de ácidos grasos de los quesos de oveja estudiados y que las cepas previamente detectadas como productoras de CLA *in vitro* también presentan esta capacidad cuando se usan combinadas en un cultivo. Estos estudios se consideran de gran interés para la industria quesera al contribuir al desarrollo de productos lácteos con un mejor perfil nutricional a partir de leche de oveja.

4.7. Efecto de los diferentes cultivos diseñados sobre el perfil de aminoácidos libres, la microestructura y el contenido en aminas biógenas de los quesos de oveja a lo largo de la maduración

Las bacterias ácido lácticas que son empleadas habitualmente en la elaboración de queso, en especial las utilizadas como cultivos adjuntos, juegan un papel muy importante en la proteólisis que tiene lugar durante la maduración del queso debido a que estas bacterias contienen proteinasas y peptidasas que pueden dar lugar a la producción de aminoácidos libres (Fox et al., 2016). Algunos de estos aminoácidos a su vez pueden servir como sustrato en reacciones secundarias catabólicas, llevadas a cabo por estas bacterias ácido lácticas, que pueden dar lugar a la síntesis de compuestos como el GABA y la ornitina con propiedades beneficiosas en la salud humana (Adeghate y Ponery, 2002; Diana et al., 2014b; Sugino et al., 2008). No obstante, los aminoácidos libres también pueden sufrir reacciones de descarboxilación por acción de las bacterias ácido lácticas que conducen a la síntesis de aminas biógenas, cuyo consumo en elevadas concentraciones puede tener efectos negativos en la salud humana (Linares et al., 2011; Manca et al., 2015).

En base a lo expresado anteriormente, se llevó a cabo un estudio que permitió evaluar el efecto de los diferentes cultivos empleados en la elaboración de los cuatro lotes de queso de oveja, descritos en el apartado anterior, sobre el perfil de aminoácidos libres, con especial énfasis en el GABA y la ornitina, la microestructura y el contenido en aminas biógenas de los quesos de oveja a lo largo de la maduración (2, 90, 180 y 240 días).

Los resultados que son discutidos a continuación, en relación con el estudio descrito en este apartado, aparecen recogidos en el capítulo 5 de la presente Tesis.

El tiempo de maduración de los quesos conllevó a un incremento significativo ($P \leq 0{,}001$) en la concentración de los aminoácidos libres totales de los cuatro lotes de queso. Este aumento fue debido principalmente a la actividad proteolítica de las cepas que formaron parte de los cultivos (González et al., 2015; Herreros et al., 2003), dando lugar a que la concentración de aminoácidos libres totales fuera superior a la observada por Poveda et al. (2004) en queso de oveja elaborado con diferentes cultivos iniciadores y con un tiempo de maduración similar.

La proteólisis que tuvo lugar durante la maduración de los cuatro lotes de queso queda reflejada en las imágenes obtenidas cuando se analizó la microestructura de los cuatro lotes de queso utilizando el método basado en la microscopía confocal de barrido láser. La microestructura observada en cada una de las imágenes obtenidas para los cuatro lotes estudiados y mismo tiempo de maduración fue muy similar. Al inicio de la maduración (2 días), se observó una matriz proteica continua y fibrosa conteniendo glóbulos grasos que presentaban una forma irregular. La fase proteica y grasa mostraron una ligera orientación lineal, al igual que también fue observado por Auty et al. (2001) en queso Cheddar, atribuyendo este hecho a la etapa de prensado que tiene lugar durante el proceso de elaboración de estos quesos. Por un lado, conforme transcurrió el tiempo de maduración, la matriz proteica fue presentando gradualmente una estructura amorfa, como consecuencia de la proteólisis. Por otro lado, la coalescencia de los glóbulos grasos a lo largo de la maduración dio lugar a la formación de grandes partículas de grasa y la actividad lipolítica que tuvo lugar contribuyó a la salida de parte de la grasa de los glóbulos. Estos resultados fueron similares a los observados en el análisis de la microestructura de otros quesos (Everett y Auty, 2008; O'Reilly et al., 2003).

En lo que respecta a los distintos cultivos empleados en la elaboración de queso, éstos tuvieron un efecto significativo ($P \leq 0{,}001$) en la concentración de aminoácidos libres totales. El lote 3, elaborado con el cultivo que incluyó la cepa de *Lactobacillus casei* subsp. *casei* SS 1644, fue el que presentó la mayor concentración de aminoácidos libres totales a lo largo de todo el periodo de maduración de los quesos, alcanzando unos valores al cabo de 90 días de maduración muy similares a los detectados en el resto de lotes al final de la maduración (240 días). En este sentido, Arzania et al. (2006) han señalado que la maduración se trata de un proceso que implica costes relativamente altos a la industria quesera. En consecuencia, el empleo de este cultivo podría reducir el tiempo de maduración del queso de oveja no solo proporcionando beneficios tecnológicos sino también económicos para las industrias.

Los lotes 1, 2 y 4 no presentaron diferencias significativas ($P > 0{,}05$) en la concentración de aminoácidos libres totales entre ellos hasta el final de la maduración. En este momento, el lote 2, elaborado con el cultivo formado por las cepas autóctonas de *Lactococcus lactis* y el *Lactobacillus plantarum* TAUL 1588, presentó una concentración de aminoácidos libres totales similar a la descrita en el lote 1 que fue elaborado con el cultivo que únicamente incluía las cepas de *Lactococcus lactis*. Sin embargo, el lote 4, elaborado con las cuatro cepas autóctonas presentó una concentración mayor de aminoácidos libres totales que los lotes 1 y 2 aunque inferior a la del lote 3.

En general, de los 21 aminoácidos analizados en los cuatro lotes de queso a lo largo de la maduración, los mayoritarios fueron leucina, ácido glutámico, fenilalanina, prolina, alanina y valina, los cuales representaron aproximadamente el 60% del total de aminoácidos libres. Por el contrario, tirosina, histidina, glicina, triptófano y arginina fueron los aminoácidos libres minoritarios, representando menos del 5% del total de aminoácidos libres analizados. La información disponible en la literatura en relación con los aminoácidos libres mayoritarios y su concentración en queso de oveja varía ampliamente (Madrau et al., 2006; Mangia et al., 2008; Poveda et al., 2015). En este hecho podrían haber intervenido las diversas bacterias ácido lácticas empleadas en la elaboración de los quesos debido a que éstas presentan un complejo sistema proteolítico y dependiendo de la especie implicada, este sistema puede estar constituido por diferentes proteinasas y peptidasas (Fox et al., 2016). Adicionalmente, Poveda et al. (2004) han indicado que la composición de las caseínas es diferente dependiendo del tipo del que se trate; así, las caseínas α_{S1} presentan un contenido alto en leucina, fenilalanina y valina, mientras que las caseínas β tienen un elevado contenido en prolina. Por consiguiente, dependiendo del sustrato específico de las enzimas presentes en el sistema proteolítico de las bacterias ácido lácticas empleadas como cultivos, el tipo y la concentración de aminoácidos libres en el queso serán diferentes.

La escasa información disponible sobre el efecto de cultivos autóctonos en la concentración de GABA y ornitina a lo largo de la maduración del queso de oveja hacen que los resultados obtenidos en este estudio sean muy relevantes. El tiempo de maduración fue un factor importante en la concentración final de GABA en los cuatro lotes de queso, ya que se incrementó más de 80 veces a lo largo de la misma. El lote 1 fue el que presentó la mayor concentración de GABA tras 240 días de maduración seguido de los otros tres lotes de queso, entre los cuales no se observaron diferencias significativas ($P > 0,05$). La importancia de este estudio vino determinada por la alta concentración de GABA detectada en todos los lotes al final de la maduración. En relación con la dosis de GABA que conlleve efectos beneficiosos para la salud de los consumidores, se ha descrito que es necesaria una ingesta oral diaria de 26,4 mg para ejercer un efecto positivo en el tratamiento de ciertos desórdenes neurológicos (Okada et al., 2000). Por consiguiente, sería necesario consumir 15 g de queso al día del lote 1 y 23 g de los otros tres lotes para alcanzar esta dosis recomendada de GABA. Este hecho implica que pequeñas porciones de estos quesos proporcionarían la concentración necesaria de GABA para alcanzar los efectos fisiológicos indicados anteriormente. De igual manera, en otro estudio llevado a cabo por Pouliot-Mathieu et al. (2013) observaron que una ingesta de diaria de 50 g de un queso experimental (conteniendo 16 mg de GABA) producía un descenso de la presión sanguínea en humanos. En base a los resultados obtenidos en nuestro estudio, el consumo de 50 g de queso al día de los diferentes lotes elaborados en este trabajo proporcionaría entre 60 mg y 90 mg de GABA. No obstante, serían necesarios otros estudios, fuera del ámbito de esta Tesis, para profundizar en estos aspectos.

En lo que respecta a la ornitina, la presencia de este compuesto en los alimentos está ganando interés debido a las funciones bioactivas en la salud humana que han sido descritas en varios estudios (Kurata et al., 2012; Sugino et al., 2008). Actualmente, no hay disponible información sobre la dosis efectiva de la ornitina para alcanzar los beneficios fisiológicos que se le han atribuido. Lo que si se conoce es que la ornitina puede ser

sintetizada por la actividad enzimática de las bacterias ácido lácticas sobre la arginina y la citrulina durante la maduración de los quesos (Diana et al., 2014b). En este sentido, en los cuatro lotes de queso se produjo un incremento ($P \leq 0,001$) en la concentración de ornitina a lo largo de la maduración. Así mismo, no se observaron diferencias significativas ($P > 0,05$) en la concentración de ornitina entre los cuatro lotes de queso analizados hasta los 240 días de maduración. Como fue descrito previamente para el GABA, el lote 1 fue el que presentó la mayor concentración de ornitina seguido de los lotes 2, 3 y 4, entre los cuales no se observaron diferencias ($P > 0,05$). Las concentraciones de ornitina observadas al final de la maduración en los cuatro lotes fueron superiores a las señaladas por Diana et al. (2014b) en queso de oveja. Los resultados descritos hasta el momento, abren la posibilidad de llevar a cabo más estudios con estos cultivos para el posible desarrollo de quesos funcionales.

Finalmente, se analizó el efecto del cultivo empleado y del tiempo de maduración sobre el contenido en aminas biógenas de los cuatro lotes de queso. Durante el transcurso del tiempo de maduración, el contenido total de aminas biógenas aumentó significativamente ($P \leq 0,001$), siendo estos valores muy similares a los descritos por otros autores en queso de oveja (Renes et al., 2014; Schirone et al., 2013). Es importante indicar que la concentración máxima permitida de aminas biógenas en productos lácteos no ha sido establecida todavía desde un punto de vista legislativo y que la mayoría de los trabajos de investigación se han centrado casi exclusivamente en el estudio de la histamina y de la tiramina, debido a que éstas son las aminas biógenas que con más frecuencia se asocian con intoxicaciones alimentarias, así como las más abundantes en queso (Linares et al., 2016). En el presente estudio, no se detectó la presencia de histamina en ninguno de los lotes de queso analizados, mientras que las concentraciones de tiramina oscilaron entre 308,65 mg/kg de queso y 585,47 mg/kg queso al final de la maduración, encontrándose estos valores dentro los límites máximos tolerables (100-800 mg/kg de queso) indicados por ten Brink et al. (1990). No obstante, es necesario resaltar que la cadaverina y la putrescina, las cuales no han sido asociados con intoxicaciones alimentarias, pueden aumentar la toxicidad de la histamina y la tiramina. En base a esto, el contenido total de aminas biógenas no debería exceder la cantidad de 900 mg/kg de queso establecida por Valsamaki et al. (2000), y en este sentido, ninguno de los lotes de quesos estudiados excede este límite, por lo que no supondrían un riesgo para la salud del consumidor.

En relación con el efecto del cultivo empleado en la elaboración de queso, el lote 3, elaborado con el cultivo que incluyó la cepa de *Lactobacillus casei* subsp. *casei*, fue el que presentó la mayor concentración de aminas biógenas totales a lo largo de la maduración. Simultáneamente, y como ha sido mencionado previamente, este lote fue el que mostró la mayor concentración de aminoácidos libres totales. Estos resultados fueron coincidentes con los descritos por Novella-Rodríguez (2003), quienes indicaron que el aumento de la proteólisis durante la maduración de los quesos, puede conducir a un incremento del contenido en aminas biógenas. Otro resultado destacable en el presente estudio fue que el lote 2 elaborado con el cultivo que incluía la cepa de *Lactobacillus plantarum* TAUL 1588 presentó la menor concentración de aminas biógenas totales, seguido de los lotes 1 y 4. El hecho de que el lote 4 presente una menor concentración de aminas biógenas totales respecto al lote 3, podría estar relacionado con la capacidad de la cepa *Lactobacillus*

plantarum TAUL 1588 para degradar las aminas biógenas producidas por las otras cepas de bacterias ácido lácticas empleadas en los cultivos. Guarcello et al. (2016) han indicado que la existencia de cepas de *Lactobacillus* con capacidad para degradar aminas biógenas abre nuevas líneas de investigación para aumentar la disponibilidad de cultivos adjuntos para elaborar quesos. Aun así, sería necesario llevar a cabo más estudios con la cepa de *Lactobacillus plantarum* TAUL 1588 para demostrar si realmente presenta la capacidad para degradar aminas biógenas.

La información disponible sobre las aminas biógenas mayoritarias en queso de oveja es variable al igual que en el caso de los aminoácidos libres totales. Esta variabilidad puede ser debida a que el tipo y concentración de aminas biógenas detectadas en los quesos dependen del tipo de queso que se trate, así como de múltiples factores involucrados en la formación y acumulación de éstas (Renes et al., 2014). Los lotes 1 y 2 presentaron un patrón común con respecto a la concentración de cada una de las aminas biógenas identificadas a lo largo de la maduración. En ambos lotes no se detectó la putrescina ni la feniletilamina en ninguno de los tiempos de maduración considerados, ni de tiramina al inicio de la maduración, siendo el lote 2 el que presentó menor concentración de esta amina biógena. Además, estos lotes fueron los que presentaron la menor concentración de cadaverina sin observarse diferencias ($P > 0,05$) entre ellos. Cabe destacar que el tiempo de maduración no afectó a la concentración de esta amina biógena en estos lotes.

En lo referente al lote 3, elaborado con el cultivo que incluyó la cepa de *Lactobacillus casei* subsp. *casei*, éste fue el que presentó las mayores concentraciones de todas estas aminas biógenas a lo largo de la maduración. Por contra, el lote 4 que fue elaborado con el cultivo que incluyó esta cepa conjuntamente con la cepa de *Lactobacillus plantarum* TAUL 1588, presentó de manera general, unas concentraciones de putrescina, feniletilamina, tiramina y cadaverina intermedias entre las observadas para el lote 3 y los lotes 1 y 2.

4.8. Estudio de las características sensoriales del queso de oveja elaborado con distintos cultivos

En los apartados anteriores se ha descrito la posible mejora del perfil nutricional del queso de oveja. Sin embargo, también se consideró necesario el estudio de las características sensoriales de los cuatro lotes de queso por ser éste un aspecto determinante en la aceptación del queso por parte de los consumidores.

En base a estas consideraciones, se llevó a cabo un análisis del perfil de textura y del color empleando métodos instrumentales y también se analizaron las características sensoriales de los quesos mediante un panel de catadores. Los resultados obtenidos en este estudio y que a continuación son discutidos, aparecen recogidos en el capítulo 4 de la presente Tesis.

El perfil de textura y el color de los quesos se analizaron a lo largo del tiempo de maduración, mientras que el análisis sensorial con el panel de catadores sólo fue llevado a cabo en los quesos con 180 y 240 días de maduración al ser éstos los tiempos en que se comercializa habitualmente este tipo de quesos.

El análisis del perfil de textura permitió observar cambios significativos ($P \leq 0,05$) en los parámetros de dureza, cohesividad, elasticidad y masticabilidad a lo largo de la maduración en los cuatro lotes de queso estudiados. De manera general, los valores de dureza aumentaron hasta los 240 días de maduración, siendo los lotes 1 y 2 los que presentaron mayor dureza. Por contra, los valores de elasticidad disminuyeron hasta la mitad al final de la misma. En el caso de la cohesividad, de manera general, los valores observados para estos parámetros disminuyeron tras 2 días de maduración, momento a partir del cual permanecieron constantes. Al final de la maduración, el efecto del cultivo empleado en la elaboración de los quesos no tuvo un efecto significativo ($P > 0,05$) en los valores observados para la elasticidad y cohesividad. Los valores de masticabilidad después del descenso producido durante los tres primeros meses, siguieron un comportamiento diferente entre lotes. Así, en los lotes 1 y 2 sus valores se incrementaron hasta los 180 días, mientras que en los lotes 3 y 4 dichos valores permanecieron sin variaciones durante el resto de la maduración, alcanzando valores similares en los cuatro lotes al final de la misma.

Con respecto al análisis del color, el tipo de cultivo empleado en la elaboración de los quesos no tuvo un efecto significativo ($P > 0,05$) en los parámetros L*, a* y b* analizados. Por contra, el tiempo de maduración sí influyó significativamente ($P \leq 0,05$) sobre estos parámetros. Se observó un descenso más acusado en la luminosidad (L*) de los quesos de los 2 días a los 90 días de maduración, momento a partir del cual este parámetro permaneció constante o disminuyó ligeramente. De manera inversa, los valores de los parámetros a* y b* aumentaron hasta los 90 días, momento a partir del cual permanecieron constantes o disminuyeron ligeramente hasta el final de la maduración. Estos resultados reflejaron un incremento tanto en la coloración rojiza (a*) como en la coloración amarilla (b*) de los quesos a lo largo de la maduración. Una tendencia muy similar ha sido descrita por otros autores (Lurueña-Martínez et al., 2010; Pinho et al., 2004) en otros estudios sobre quesos de oveja madurados.

Finalmente y por lo que respecta al análisis sensorial, en los quesos con 180 días de maduración no se observaron cambios significativos en la mayoría de los atributos evaluados por los catadores en función del tipo de cultivo iniciador utilizado en su elaboración. El lote control (lote 1) fue el que presentó mayores valores para la intensidad de color y olor. En lo referente al atributo color, no se encontró una correlación con los resultados observados en el análisis instrumental ya que para este tiempo de maduración los valores L*, a* y b* fueron similares en los cuatro lotes. Así mismo, ninguno de los lotes presentó olores que estuvieran asociados a defectos en los quesos.

De igual manera, a los 240 días de maduración, los catadores no detectaron diferencias entre los lotes de queso con respecto a los atributos evaluados, excepto para la intensidad de olor y flavor, siendo los lotes 2 y 3 los que presentaron la menor puntuación para ambos atributos.

De cualquier modo, las diferencias observadas entre los lotes de queso fueron muy pequeñas, siendo en todos los casos inferiores a 1 punto en la escala de percepción empleada. Esta homogeneidad en los diferentes atributos sensoriales fue corroborada durante la evaluación global de los quesos, obteniéndose tanto a los 180 como a los 240 días de maduración, una puntuación media de 8,25 sobre 10, conllevando una buena aceptación por parte de los catadores.

En definitiva, los datos obtenidos en el análisis de la textura, color y a través del panel de catadores mostraron que el empleo de diferentes cultivos adjuntos en la elaboración de los quesos no implicó cambios importantes en los parámetros sensoriales analizados.

4.9. Referencias

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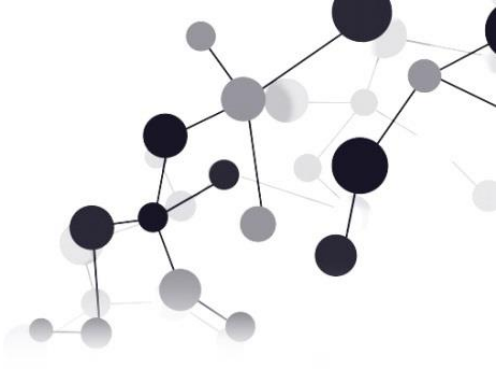

CONCLUSIONES/CONCLUSIONS

5. CONCLUSIONES

PRIMERA. Actualmente la producción de leche de oveja se ha intensificado dando menor importancia al valor nutricional de la misma. Sin embargo, prácticas de manejo menos intensivas basadas en un mayor pastoreo de las ovejas en detrimento de las dietas basadas en forraje y concentrado, no supondrían una inversión económica elevada para los ganaderos, mejorarían la calidad de la leche y en consecuencia, el valor nutricional del queso. Por consiguiente, este aspecto sería de gran interés para la industria láctea.

SEGUNDA. Considerando los efectos beneficiosos sobre la salud humana del CLA y del GABA, el desarrollo de cultivos lácticos constituidos por cepas autóctonas con capacidad demostrada para sintetizar estos compuestos bioactivos, puede hallar aplicación en la elaboración de productos lácteos funcionales.

TERCERA. Los cultivos compuestos por cepas de *Lactobacillus* productoras de CLA en combinación con cepas de *Lactococcus lactis* autóctonas, fueron más efectivos en la producción de CLA que los cultivos que incluyeron cepas comerciales de *Lactococcus lactis* y los *Lactobacillus* productores de CLA.

CUARTA. El cultivo autóctono compuesto por cepas de *Lactococcus lactis* y la combinación de las cepas productoras de CLA, *Lactobacillus plantarum* TAUL 1588 y *Lactobacillus casei* subsp. *casei* SS1644, mejoró la calidad nutricional de la grasa del queso de oveja en relación con los ácidos grasos con efectos beneficiosos sobre la salud humana. Este hecho es de gran interés ya que permitiría satisfacer la demanda de los consumidores en lo que respecta a alimentos más saludables y con unas buenas características sensoriales.

QUINTA. Los quesos de oveja elaborados con los cultivos autóctonos que incluyeron la cepa de *Lactobacillus plantarum* mostraron un elevado contenido en GABA y una baja concentración en aminas biógenas, constituyendo una buena aproximación para el desarrollo de quesos de oveja funcionales.

SEXTA. Este trabajo demuestra la gran importancia que tienen las prácticas de manejo del ganado ovino en el perfil de la grasa láctea así como el interés de diseñar cultivos lácticos con capacidad para sintetizar compuestos bioactivos con efectos beneficiosos sobre la salud humana en el desarrollo de productos lácteos con calidad nutricional mejorada.

5. CONCLUSIONS

FIRST. Currently, sheep milk production is being intensified giving less importance to the nutritional value of the milk. However, less intensive sheep management practices based on higher sheep grazing instead of forage- and concentrate-based diets, would not mean high capital investment for farmers, it would lead to an improvement in sheep milk quality and consequently, in the nutritional value of cheese. Therefore, this aspect would be of great interest to the dairy industry.

SECOND. Considering the health benefits of CLA and GABA, the development of lactic cultures constituted by autochthonous strains with demonstrated ability to produce these bioactive compounds, could contribute to the production of functional dairy products.

THIRD. Cultures including *Lactobacillus* CLA-producing strains in combination with autochthonous *Lactococcus lactis* strains, were more effective on CLA production than the cultures including commercial *Lactococcus lactis* strains and the CLA-producers *Lactobacillus*.

FOURTH. The autochthonous culture including the *Lactococcus lactis* strains and the combination of both CLA-producers strains, *Lactobacillus plantarum* TAUL 1588 and *Lactobacillus casei* subsp. *casei* SS1644, improved the nutritional quality of sheep milk cheese fat in relation to the fatty acids with beneficial effects on human health. This fact is of great interest in order to meet consumer demand for healthier foods with good sensory characteristics.

FIFTH. Sheep milk cheeses produced with the autochthonous cultures including the *Lactobacillus plantarum* TAUL 1588 strain showed high GABA content and low biogenic amines concentration, being a good approach to develop functional sheep milk cheeses.

SIXTH. This work shows the great importance of sheep management practices on the milk fat profile as well as the relevance of designing lactic cultures with ability to synthesize bioactive compounds with beneficial effects on human health in order to develop dairy products with improved nutritional quality.