Programa de Doctorado en Biotecnología Alimentaria

Departamento de Ingeniería Química y Tecnología del Medio Ambiente

Caracterización microbiológica de productos lácteos tradicionales para el diseño de cultivos iniciadores

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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Español: **Caracterización de productos lácteos tradicionales para el diseño de cultivos iniciadores.**	Inglés: **Microbial characterization of traditional dairy products for the design of starter cultures.**

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RESUMEN (en español)

El principal objetivo de este trabajo ha consistido en estudiar la diversidad microbiana presente en los procesos de elaboración y maduración de productos lácteos tradicionales, identificar y caracterizar los microorganismos con interés tecnológico que se han encontrado y utilizar las cepas seleccionadas para el diseño fermentos (mezclas de microorganismos responsables de la acidificación y la maduración de los quesos).

En primer lugar estudiamos la diversidad microbiana del queso tradicional asturiano Casín mediante técnicas clásicas de cultivo y la técnica independiente de cultivo DGGE. Sorprendentemente se determinó que el proceso de acidificación estaba dominado por cepas de *Lactococcus garvieae* y se detectó la presencia de *Streptococcus thermophilus*.

El segundo producto analizado fue el queso tradicional polaco Oscypek, que se estudió mediante las técnicas mencionadas junto con la pirosecuenciación de amplicones del gen ARNr 16S, siendo este un trabajo pionero en la aplicación de esta última técnica al estudio de un producto lácteo tradicional. Mediante esta técnica se detectaron poblaciones subdominantes no habituales en quesos, pertenecientes a las familias *Bifidobacteriaceae* y *Moraxellaceae*.

Las técnicas clásicas de cultivo y la DGGE se emplearon en el estudio de una leche fermentada natural. Ambas técnicas revelaron la presencia de *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* y *Lactobacillus plantarum*. Mediante técnicas de tipificación determinamos que la diversidad microbiana se reduce a una sola cepa de cada una de las especies y subespecies, que parecen formar una asociación microbiana estable que se mantiene a lo largo del tiempo.

El análisis individualizado de los microorganismos con potencial tecnológico procedentes de productos tradicionales comenzó por el estudio de las características fenotípicas y genotípicas de 20 cepas de *Lc. lactis*. Con el objetivo de determinar sus relaciones filogenéticas y su potencial como cultivos inciadores, las cepas se sometieron a una serie de pruebas fenotípicas y genotípicas. Los resultados confirman la idea de que,

independientemente de su fenotipo, los genotipos *lactis* y *cremoris* constituyen verdaderas subespecies. Además varias cepas de ambas subespecies presentan propiedades deseables para su inclusión en cultivos iniciadores.

También se examinaron 305 aislados de *Lc. lactis* con la intención de encontrar cepas productoras de bacteriocinas (agentes antimicrobianos naturales). Se encontraron 11 que producían nisina, la única bacteriocina autorizada como aditivo alimentario en determinados productos lácteos. Además, aparecieron otras cinco cepas productoras de lactococina 972 y una de lactococina G/Q. Las cepas productoras de bacteriocinas podrían utilizarse como cultivos protectores, inhibiendo o eliminando bacterias patógenas o alterantes.

El último grupo de microorganismos caracterizados estaba constituido por una serie de 42 aislados identificados por métodos moleculares y asignados a las especies *Leuconostoc citreum*, *Leuconostoc mesenteroides* y *Leuconostoc lactis*. Tras una selección preliminar de 14 cepas, se examinaron detalladamente sus características bioquímicas, tecnológicas y de seguridad y se seleccionaron las más indicadas para ser utilizadas como cultivos adjuntos.

En la última parte del trabajo, distintas cepas microbianas se combinaron para formar nueve mezclas acidificadoras. En estas mezclas se estudiaron las propiedades tecnológicas más relevantes para su uso como fermentos lácticos. Las cuatro mezclas con mejores aptitudes se ensayaron en sendas elaboraciones experimentales de queso. En estas elaboraciones se analizaron las características físico-químicas básicas y la evolución de poblaciones microbianas durante la elaboración y maduración. Por último, los quesos maduros fueron sometidos a una evaluación sensorial por un panel de cata que confirmó la idoneidad de algunas de las mezclas para su uso como fermentos lácteos.

RESUMEN (en Inglés)

The principal aim of this work consisted in studying the composition and diversity of the microbiota involved in the manufacturing and ripening of traditional dairy products, identification and characterization of microorganisms with a technological interest, and use of selected strains for the designing of specific starter and adjunct cultures (mixtures of microorganisms responsible for acidification and ripening, respectively) for dairy.

In the first place, the microbiota of the Spanish traditional Casín cheese was studied trough classical culturing and DGGE techniques. Surprisingly, the species *Lactococcus garvieae* dominated the acidification process; and DGGE detected DNA corresponding to *Streptococcus thermophilus*.

The second product studied was the traditional Polish Oscypek cheese, using the techniques mentioned above and pyrosequencing of 16S rRNA gene amplicons, this

resulting in one of the pioneering works applying this state-of-the-art powerful sequencing technique to a traditional dairy fermentation. This technique detected subdominant uncommon bacteria in cheeses, belonging to the families *Bifidobacteriaceae* and *Moraxellaceae*.

A natural, homemade fermented milk was studied through culturing and DGGE. Both techniques revealed the presence of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lactobacillus plantarum*. Molecular typing techinques identified single strains each of the species and subspecies. These three strains formed a stable microbial consortium (association), which has been maintained in this ecosystem for decades.

Analysis of the microbial components with potential technological interest was initiated by studying the phenotypic and genetic properties of twenty *Lc. lactis* strains. With the aims of determining their phylogenetic relationships and their suitability as starters, all strains were subjected to a series of phenotypic and genetic analyses. The results obtained supported the idea that the *lactis* and *cremoris* genotypes, no matter what phenotype they show, actually represent true subspecies. Further, some strains of the two subspecies appeared to be good starter candidates.

We also examined 305 isolates of *Lc. lactis* in order to find bacteriocin (natural antimicrobial agents) producers. Eleven strains were identified as producing nisin, the only bacteriocin authorized as a food additive in dairy products. In addition, five other strains produced lactococcin 972, and one produced lactococcin G/Q. These bacteriocin producers could be used as protective starters against pathogens and spoiling bacteria.

The last group of microorganisms characterized was a set of 42 isolates identified by molecular methods and assigned to the species *Leuconostoc citreum*, *Leuconostoc mesenteroides* and *Leuconostoc lactis*. After a preliminary selection of 14 strains, their biochemical, technological and safety aspects were deeply examined and strains with desirable properties were identified and appointed as candidate adjunct starters.

Finally, different components of lactic acid bacteria strains were combined to create nine distinct starter mixtures. These were then analyzed for the most important technological parameters while growing in milk, including acidification rate, total growth and the production of organic acids, volatile compounds and free amino acids. The suitability of the four most promising cheese starter mixtures was finally evaluated in experimental trails. Sensory evaluation of the cheeses qualified some of the mixtures as appropriate starter cultures.

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Resumen

Resumen

La elaboración de queso y otros productos lácteos fermentados es una de las formas de conservación de alimentos más antiguas que existen. Desde hace más de 7000 años, los procesos de fermentación de la leche se han extendido por todos el mundo de manera pareja a la domesticación de animales. Aunque en el presente la producción mundial de leche y derivados lácteos está dominada por industrias con producción a gran escala, los pequeños productores como las queserías tradicionales siguen siendo importantes todavía en algunas regiones del mundo. Para asegurar la supervivencia de los productos tradicionales, estos deben de mantener sus propiedades físico-químicas y organolépticas típicas, así como unas buenas condiciones higiénicas y de conservación. Una de las maneras de alcanzar estos objetivos es la identificación y caracterización de los microorganismos presentes en los productos tradicionales con el propósito de utilizarlos como fermentos. Los fermentos o cultivos iniciadores se definen como los microorganismos que se inoculan en una materia prima para iniciar y controlar su fermentación. Su diseño es un aspecto clave, puesto que ayudarán a reducir la variabilidad entre lotes y a mantener las cualidades sensoriales típicas de los productos tradicionales.

El principal objetivo de este trabajo es estudiar la diversidad microbiana presente en los procesos de elaboración y maduración de productos lácteos tradicionales, identificar y caracterizar los microorganismos con interés tecnológico y utilizar las cepas seleccionadas para el diseño cultivos iniciadores.

En primer lugar, y para familiarizarnos con las técnicas cultivo-independientes que se utilizaron más adelante, estudiamos la composición y evolución de las poblaciones microbianas durante la elaboración y maduración de un queso azul estándar elaborado con pasteurizada y fermentos comerciales. La caracterización de las poblaciones microbianas se llevó a cabo mediante técnicas clásicas de cultivo y la técnica molecular, cultivo-independiete de la DGGE. Lo más sobresaliente de los resultados fue la detección mediante esta última técnica de una población abundante de *Streptococcus termophilus*. Esta población procedía de la leche de fabricación ya que los fermentos no contenían cepas de esta especie. Los resultados mostraron que la caracterización de las poblaciones microbianas que intervienen en la producción del queso mejora considerablemente cuando se combinan los dos tipos de técnicas.

A continuación estudiamos la diversidad microbiana y la dinámica de la microbiota a lo largo de la elaboración y maduración del queso tradicional asturiano Casín mediante técnicas convencionales de cultivo y la DGGE. Los cultivos mostraron que las poblaciones mayoritarias estaban compuestas por especies de bacterias del ácido láctico (BAL). De forma sorprendente, el proceso de acidificación estaba dominado por cepas de la especie *Lactococcus garvieae*. Esta especie era reemplazada durante la maduración por cepas de *Lactococcus lactis* subsp. *lactis*. Los perfiles de DGGE, por su parte, revelaron de nuevo la presencia de ADN perteneciente a la especie *Streptococcus thermophilus* a lo largo de todo el proceso de producción del queso. Esta especie no suele detectarse en los quesos tradicionales españoles mediante las técnicas de cultivo.

La microbiota del queso tradicional polaco Oscypek se estudió también mediante técnicas clásicas de cultivo y las técnicas cultivo-independientes DGGE y pirosecuenciación de amplicones del gen ARNr 16S. Este ha sido uno de los trabajos pioneros en la aplicación de esta última técnica al estudio de un producto lácteo fermentado. Además de los géneros bacterianos más abundantes en los productos lácteos, como *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus*, y *Enterococcus,* que fueron detectados por los tres métodos, otras bacterias subdominantes pertenecientes a las familias *Bifidobacteriaceae* y *Moraxellaceae*, fueron

identificados únicamente por pirosecuenciación. La detección de ADN de bifidobacterias en queso sugiere que quizá estas bacterias puedan sobrevivir en la leche durante algún tiempo antes de abandonar la ubre. Además de bacterias, la DGGE mostró una gran diversidad de especies de levaduras en el queso Oscypek. Los métodos de cultivo por su parte permitieron identificar más de 300 aislados de diversas cepas bacterianas. Estos aislados se contemplan como un reservorio de biodiversidad fenotípica y genética con una potencial aplicación para el desarrollo de frementos específicos o generales de queso.

Las técnicas clásicas de cultivo y la DGGE fueron empleadas de nuevo en el estudio de una leche fermentada natural, elaborada por el método de "backslopping". Los cultivos y la DGGE mostraron que *Lc. lactis* subsp. *lactis* y *Lc. lactis* subsp. *cremoris* son dominantes en este producto, mientras que *Lactobacillus plantarum* está presente a un nivel subdominante. Los aislados de estas especies se tipificaron mediante la técnica deamplificación por PCR de repeticiones extragénicas palindrómicas (rep-PCR). Mediante esta técnica de tipificación determinamos que la diversidad microbiana se reduce a una sola cepa de cada una de las especies y subespecies presentes en el producto. Estas tres cepas de *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* y *Lb. plantarum* parecen formar una asociación microbiana que se mantiene estable a lo largo del tiempo.

El análisis individualizado de los microorganismos con potencial tecnológico comenzó por el estudio de las características fenotípicas y genotípicas de 20 cepas de *Lc. lactis* aisladas de cinco quesos tradicionales elaborados con leche cruda sin la adición de fermentos. Todas las cepas presentaban un fenotipo de *Lc. lactis* subsp. *lactis* pero sus genotipos correspondían a *Lc. lactis* subsp. *lactis* y *Lc. lactis* subsp. *cremoris* (10 cepas a cada uno). Con el objetivo de determinar sus relaciones filogenéticas y su potencial como cultivos inciadores, las cepas se sometieron a una batería de pruebas fenotípicas (fermentación de carbohidratos, perfiles de actividad enzimática, producción de compuestos voláties) y genotípicas (electroforesis de campo pulsante y análisis de secuencias multilocus). Los resultados obtenidos confirman la idea de que, los genotipos *lactis* y *cremoris* constituyen verdaderas subespecies y pueden distinguirse por pruebas fenotípicas y genotípicas, aunque no

por las pruebas clásicas. Además varias cepas de ambas subespecies presentaban propiedades deseables para su inclusión como componentes de cultivos iniciadores.

También se examinaron 305 aislados de *Lc. lactis* obtenidos de quesos asturianos elaborados con leche cruda con la intención de identificar cepas con potencial antimicrobiano contra bacterias indeseables. Los aislados se tipificaron, encontrando 60 cepas distintas. Once de estas cepas se identificaron como *Lc. Lactis* subsp. *lactis* productoras de nisina, una de las bacteriocinas más potentes producida por las bacterias lácticas y la única autorizada hasta el momento como aditivo alimentario en determinados productos lácteos. Además, otras cinco cepas de *Lc. Lactis* subsp. *lactis* producían lactococina 972 y, finalmente, una cepa de *Lc. Lactis* subsp. *cremoris* era productora de lactococina G/Q. Las bacteriocinas son agentes antimicrobianos que pueden ser empleados como conservantes naturales. Por si solas o combinadas con otras cepas de *Lc. lactis*, las cepas productoras de bacteriocinas podrían utilizarse como cultivos protectores, inhibiendo o eliminando microorganismos patógenos y alterantes.

El último grupo de microorganismos caracterizados estaba constituido por una serie de 42 aislados de leuconostocs. Los aislados se identificaron por métodos moleculares y se asignaron a las especies *Leuconostoc citreum* (24), *Leuconostoc mesenteroides* (13) y *Leuconostoc lactis* (5). Tras una selección preliminar basada en su crecimiento en leche, 14 cepas fueron estudiadas con más detalle. Sus características bioquímicas, tecnológicas y de seguridad fueron examinadas cuidadosamente y se seleccionaron las más indicadas para ser utilizadas como cultivos adjuntos en la elaboración de productos lácteos que lo requieran.

En la última parte del trabajo, distintas cepas de BAL se combinaron para formar nueve mezclas acidificadoras. En estas mezclas se estudiaron *in vitro* las propiedades tecnológicas más relevantes, incluyendo el crecimiento en leche, el potencial acidificador y la capacidad de coagulación, así como la producción de ácidos orgánicos, compuestos volátiles y aminoácidos libres. Las cuatro mezclas con mejores aptitudes como fermentos se ensayaron en sendas elaboraciones experimentales de queso. En estas elaboraciones experimentales se analizaron las

características físico-químicas básicas y la evolución de poblaciones microbianas totales e indicadoras a lo largo de los procesos de elaboración y maduración. Por último, los quesos maduros fueron sometidos a una evaluación sensorial por un panel de cata entrenado que confirmó la utilidad de algunas de las mezclas bajo condiciones reales de elaboración de productos lácteos.

Summary

Summary

Cheese and other fermented dairy commodities are amongst the most ancient foods. Since more than 7000 years ago the fermentation process spread all around the world wherever milk-producing animals existed. Although in present times the dairy world production is mostly dominated by large-scale industries, traditional cheese makers are still important in some regions of the world. For the survival of traditional products, these must keep stable physical-chemical and organoleptic properties during their production and ripening, as well as good hygienic (safety) and conservation conditions. Thus, the identification and characterization of the microorganisms present in these products are essential in order to reproduce the fermentation process in a controlled way. In this respect, the design of starters and adjunct cultures (mixtures of microorganisms responsible for acidification and ripening, respectively) is a key point, because they would allow a controlled production, while reducing the variability among batches and maintaining the original sensory profiles of the traditional products.

The principal aim of this work consisted in studying the composition and diversity of the microbiota involved in the manufacturing and ripening of traditional dairy products, identification and characterization of microorganisms with a technological interest, and use of selected strains for the design of specific starter and adjunct cultures for dairy.

As a preliminary study, in order to get familiar with conventional and state-of-the-art, culture-independent, microbial techniques -which were used on subsequent works-, the composition and development of the microbial populations during the manufacture and ripening of a blue-veined cheese produced from pasteurized milk with the addition of commercial starters, was followed by culturing and the culture independent PCR-DGGE technique. It was noteworthy the detection by DGGE of an abundant population of *Streptococcus thermophilus*, which was not present in the commercial starter's formula. The results showed that the characterization of the microbial populations interacting and evolving during the cheese-making process is certainly improved by combining culturing and molecular methods.

The first traditional dairy product analyzed in this study was the Spanish Casín cheese. Classical culturing and DGGE techniques were both used for studying the microbial diversity and dynamics of the cheese microbiota during manufacturing and ripening. The culture technique showed that lactic acid bacteria (LAB) species constituted the majority of the microbial populations. Surprisingly, the species *Lactococcus garvieae* dominated the acidification process; although this species was replaced during repining by strains of *Lactococcus. lactis* subsp. *lactis* strains. Additionally, the DGGE profiles revealed the presence of DNA corresponding to *S. thermophilus* throughout the whole manufacturing process. This species has rarely been reported previously from traditional Spanish cheeses.

The microbiota of the traditional, Polish Oscypek cheese was studied using both culturing and the culture-independent methods of DGGE and pyrosequencing of 16S rRNA gene amplicons. This has been one of the pioneering works applying this last powerful sequencing technique to a traditional dairy fermentation. Besides members of the most abundant bacterial genera in dairy products, e.g., *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus*, and *Enterococcus*, which were identified by all three methods, subdominant microorganisms belonging to the families *Bifidobacteriaceae* and *Moraxellaceae*, were only identified by pyrosequencing. This is one of the first reports detecting the presence of DNA sequences belonging to bifidobacteria in a cheese system. In addition to bacteria, a great diversity of yeast

produced lactococcin 972, and one strain belonging to *Lc. Lactis* subsp. *cremoris* encoded lactococcin G/Q. Bacteriocins are natural antimicrobial agents that can be used as natural preservatives. Alone or in combination with other *Lc. lactis* strains, bacteriocin producers could be used as starters with a superior protection activity against pathogens and spoiling microorganisms.

The last group of microorganisms characterized was a set of 42 *Leuconostoc* isolates. They were identified by molecular methods and assigned to the species *Leuconostoc citreum* (24), *Leuconostoc mesenteroides* (13) and *Leuconostoc lactis* (5). After a preliminary selection based on their growth in milk, 14 strains were studied in more detail. Biochemical, technological and safety aspects of these strains were deeply examined in order to select appropriate strains to be used as adjunct cultures in dairy. Strains with desirable properties were identified and appointed as adjunct starter candidates.

Finally, different lactic acid bacteria strains were combined to create nine distinct starter mixtures. These were then analyzed for the most important technological parameters while growing in milk, including acidification rate, total growth and the production of organic acids, volatile compounds and free amino acids. The suitability of the four most promising cheese starter mixtures was finally evaluated in experimental trails. The basic microbial and chemical parameters were then evaluated during manufacturing and ripening. Sensory evaluation of the experimental cheeses qualified some of the mixtures as appropriate starter cultures.

species was demonstrated in Oscypek by the DGGE method. Culturing methods enabled the identification of more than 300 microorganisms from different microbial groups. Isolates are envisioned as a reservoir of phenotypic and genetic biodiversity for potential applications as general and specific cheese starter cultures.

A natural homemade fermented milk with good sensorial properties produced by backslopping inoculation in Spain was studied by culturing and DGGE. Both techniques showed that *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were dominant in this product, while *Lactobacillus plantarum* appeared at a lower subdominant level. Repetitive extragenic palindromic (rep)-PCR typing of the isolates identified single strains each of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lb. plantarum* as the microbial types dwelling in this niche. These three strains formed a stable microbial consortium (association), which has been maintained in this ecosystem for decades.

Analysis of the microbial components with potential technological interest was initiated by studying the phenotypic and genetic properties of twenty *Lc. lactis* strains isolated from five traditional cheeses made of raw milk with no added starters. All of them presented a *Lc. lactis* subsp. *lactis* phenotype, but their genotypes belonged to *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (10 strains each). With the aims of determining their phylogenetic relationships and their suitability as starters, all strains were subjected to a series of phenotypic (fermentation of carbohydrates, enzyme activity profiles, production of volatile compounds) and genetic (pulsed field gel electrophoresis, multilocus sequence typing) analyses. The results obtained supported the idea that the *lactis* and *cremoris* genotypes, which can be distinguished by phenotypic and genetic tests, actually represent true subspecies. Further, some strains of the two subspecies appeared to be good starter candidates.

We also examined 305 isolates of *Lc. lactis* obtained from raw milk Asturian cheeses, in order to check their antimicrobial properties. Among these isolates, 11 nisin-producing strains were identified as *Lc. Lactis* subsp. *lactis*. Nisin is one of the strongest bacteriocins from lactic acid bacteria and the only one authorized as a food additive in dairy products. In addition, five other *Lc. Lactis* subsp. *lactis* trains

Introducción

Introducción

1. La leche

La leche es el fluido secretado por las hembras de los mamíferos como alimento para sus crías durante los primeros días de vida, por lo que contiene los nutrientes necesarios para cubrir los requerimientos nutricionales de la descendencia. La leche presenta una gran variabilidad de composición entre las distintas epecies de mamíferos (Jensen y col., 1995). Dentro de cada especie la composición de la leche varía también en función de factores como la alimentación del animal, la edad, la salud y el periodo de lactancia (Fox y col., 2000).

La leche es un producto con pocos sistemas antimicrobianos y poco potentes (inmunoglobulinas, lactoperoxidasa, lactoferrina) por lo que es un medio de cultivo excelente para el desarrollo de un gran número de bacterias Gram positivas (lactococos, lactobacilos, leuconostoc, enterococos, estafilococos, corinebacterias, etc.) y Gram negativas (coliformes, pseudomonas, aeromonas, campilobacterias, etc.), así como muchos hongos y levaduras. Los microbios proceden del ambiente, la piel y las mucosas de los animales, los utensilios y el personal que participa en su obtención y procesado, etc. En general, crecen bien aquellos microorganismos capaces de aprovechar las principales fuentes de carbono (lactosa) y nitrógeno (caseínas) de la leche.

1.1. Composición química de la leche

1.1.1. Materia Grasa

La leche es una emulsión tipo aceite en agua en la que la materia grasa forma glóbulos cuyo diámetro varía entre 0,1 y 20 μm, siendo su valor medio de 3-4 μm. De todos los constituyentes de la leche, la materia grasa es el que presenta mayor variación entre las distintas especies de mamíferos, oscilando entre el 2% (caballo) y el 50% (foca). Además, la materia grasa de la leche se ve muy influenciada también por la alimentación de los animales. Los lípidos de la leche son en su mayoría triglicéridos (98%), mientras que el 2% restante está compuesto por diglicéridos, monoglicéridos, ácidos grasos libres, fosfolípidos, esteroles (principalmente colesterol) y trazas de vitaminas liposolubles (A, D, E y K).

1.1.2. Proteínas

Desde el punto de vista tecnológico, las proteínas son los constituyentes más importantes de la leche puesto que su capacidad de coagular posibilita la obtención de numerosos productos lácteos de textura firme. La diferencia en porcentaje proteico entre especies varía del 1,3-1,5% en humanos hasta el 20% en pequeños mamíferos como ratones o ratas. Las proteínas de la leche pueden separarse en tres grandes grupos: las caseínas, las proteínas del suero y las proteínas de la membrana del glóbulo graso. El grupo proteico mayoritario es el de las caseínas. Estas son fosfoproteínas que a su vez puede dividirse en cuatro grandes tipos: α_{s1}, α_{s2}, β y κ. A temperatura ambiente, las caseínas precipitan a pH 4,6, cuando alcanzan su punto isoeléctrico. En las especies productoras de leche más importantes para la industria láctea (vaca, oveja, cabra y búfala), la caseína comprende el 80% del nitrógeno de la leche.

El segundo grupo de proteínas más numeroso lo forman las proteínas del suero. Éstas permanecen en solución en sus puntos isoeléctricos a menos que se desnaturalicen (por ejemplo por calor). Dentro de las proteínas del suero destacan la α-lactoalbúmina, presente en la leche de todos los mamíferos, la β-lactoglobulina que aparece únicamente en la leche de los mamíferos ungulados (donde es

mayoritaria) y en menor concentración proteínas como las inmunoglobulinas, la lactoferrina y la lactoperoxidasa. Las proteínas de la membrana del glóbulo graso forman una membrana estabilizadora y son mayoritariamente lipoproteínas.

1.1.3. Carbohidratos

La lactosa es el principal carbohidrato de la leche de todos los mamíferos, aunque posee también trazas de otros azúcares como glucosa, frutosa, glucosamina, galactosamina, ácido neuramínico y algunos oligosacáridos. La lactosa es un disacárido de galactosa y glucosa. En la naturaleza se encuentra únicamente en la leche donde se sintetiza a partir de la glucosa absorbida de la sangre. La concentración de lactosa varía entre especies y disminuye siempre dentro de una misma especie a lo largo de la lactancia, siguiendo una tendencia opuesta a la de los lípidos y las proteínas. Desde el punto de vista tecnológico la lactosa tiene una importancia capital pues es la principal fuente de carbono para los microorganismos involucrados en la obtención de los productos lácteos fermentados.

1.1.4. Sales minerales

El contenido en sales minerales de la leche se sitúa alrededor del 1%. Las sales se encuentran en disolución o formando complejos con las caseínas. Por orden de abundancia, los principales iones en la leche son Na, Ca, K y Mg. Estos se encuentran en forma de fosfatos, cloruros, citratos y caseinatos. La cantidad de sales, y en particular los cloruros aumentan a lo largo de la lactación. Entre los iones el calcio es sin duda el más importante: ayuda a mantener la estructura de las micelas de caseína, y tiene una doble función nutritiva y tecnológica.

1.2. El sector lácteo

En este apartado se recogen los datos más significativos sobre la producción, consumo y comercio de leche y productos derivados, tanto a nivel mundial como nacional y regional.

1.2.1. El mercado global

La producción mundial de leche ha aumentado durante la última década pasando de 580 millones de toneladas en el año 2000 a 749 millones de toneladas en el 2011. A lo largo de este periodo, el consumo por habitante se ha incrementado también, hasta alcanzar la cifra de 107,3 kg por habitante y año. En términos absolutos Europa está a la cabeza del consumo, superando los 280 kg por habitante y año. Sin embargo, el consumo en Europa se ha reducido en un 3% en los últimos seis años, mientras que en este mismo periodo la demanda ha crecido mucho en América del Sur (26%), África (22%) y Asia (13%) (FIL-IDF, 2012).

El queso es el derivado lácteo más importante en términos de producción y consumo global. Se estima que la producción mundial ronda los 20 millones de toneladas anuales. Los quesos industriales de leche de vaca representan aproximadamente el 80% de la producción mundial de queso, incluyendo el 20% restante los quesos producidos a pequeña escala y de forma artesanal y los producidos con leche de otras especise (oveja, cabra y búfala, principalmente). La producción mundial de queso aumentó de manera sostenida hasta el año 2007, produciéndose a partir de esta fecha un estancamiento tanto en la UE como en USA.

En términos globales, las cuatro principales potencias productoras y/o exportadoras de leche y derivados son la Unión Europea, USA, Australia y Nueva Zelanda. El precio de la leche líquida y sus productos derivados experimentó un descenso general entre 2009 y 2010, hasta los 2000 USD/tonelada. En el año 2011 se ha producido un repunte, alcanzando los niveles anteriores a 2009 de 4000 USD/tonelada (FIL-IDF, 2012).

1.2.2. El sector lácteo en España

La producción de leche de vaca en España se ha mantenido constante en la última década, produciéndose en torno a los 5,8 millones de toneladas al año. De estas, en el año 2011, 3,67 millones se destinaron al consumo de leche líquida, el resto se destinó a la transformación, obteniendo 798.000 toneladas de leches

acidificadas (principalmente yogur) y 128.000 toneladas de queso. La producción restante se destinó a la fabricación de otros productos (nata, mantequilla o leche en polvo). En estas transformaciones se generaron 1,52 millones de toneladas de lactosuero. La leche procedente de otros animales, 369.000 toneladas de leche de oveja y 315.000 toneladas de leche de cabra, se dedicó prácticamente en su totalidad a la elaboración de quesos. La producción total de queso en España en el año 2011, incluyendo el elaborado con leche de las tres especies y sus mezclas, alcanzó las 307.000 toneladas (http://www.magrama.gob.es).

En cuanto al consumo, el de leche líquida en España en el año 2009 fue de 88 kg por habitante, sensiblemente superior al consumo medio europeo (62 kg por habitante). Sin embargo, el consumo de queso (8,2 kg por habitante y año) representó menos de la mitad de la media europea (17 kg por habitante) (FIL-IDF, 2010). Como se puede inferir de estas cifras, el consumo de leche y productos lácteos en España es mucho mayor que la producción, razón por la que nuestro país es un importador neto de leche y derivados, procedentes en su mayoría de la Unión Europea.

1.2.3. El sector lácteo en Asturias

La actividad ganadera y la industria láctea asociada cuentan con un gran arraigo Asturias. Hasta 1999 se produjo un aumento gradual de la cabaña bovina y a partir de ese año, con algunos altibajos, se inició una tendencia descendente que se materializó en una considerable disminución de la cabaña de animales de producción de leche. El censo de ganaderías lecheras en nuestra región ha pasado de 7809 explotaciones en el año 2000 a menos de 2500 explotaciones en el año 2011. Esta reducción, más acusada en los años recientes, se debe al cierre masivo de explotaciones ganaderas de medio y pequeño tamaño, acompañado, sin embargo, de un ligero aumento de las explotaciones de más de 50 cabezas de ganado (Instituto Asturiano de Estadística, 2011).

La producción de leche en Asturias se determina por cuota lechera global asignada a Asturias por la Unión Europea. En el año 2011 la cuota para Asturias fue de 580.000 toneladas.

1.3. La fermentación de la leche

La fermentación es una de las formas de conservación de alimentos más antiguas que se conocen. Por su simpleza, es fácil suponer que la leche fuera uno de los primeros alimentos sometidos a fermentación. Los estudios más recientes, gracias a técnicas de datación mediante isótopos de carbono, han desvelado que ya se practicaba el ordeño hace 9000 años en la región de Anatolia (Evershed y col., 2008) y se han encontrado evidencias arqueológicas de elaboración de queso en regiones centroeuropeas hace 7400 años (Salque y col., 2012) (**Figura 1**) y de yogur hace 7000 años en el noroeste del Sahara (Dunne y col., 2012).

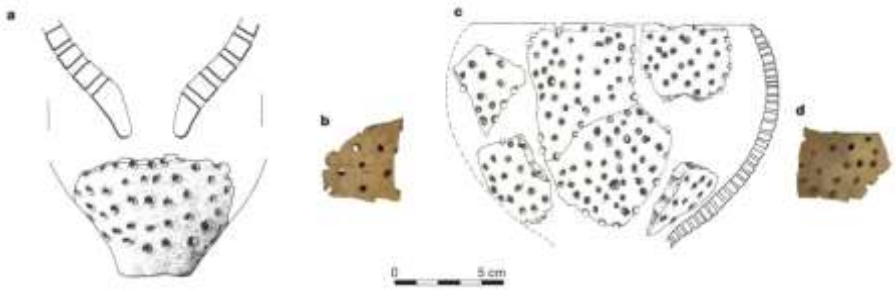

Figura 1. Dibujos de las vasijas utilizadas para elaborar queso, reconstruida a partir de las piezas halladas en Smolsk, en la actual Polonia. Las piezas datan de 7.400 años de antigüedad. (Salque y col. 2012).

La fermentación espontánea de la leche se debe al desarrollo de diversas especies de bacterias del ácido láctico (BAL). El crecimiento de estas bacterias provoca un rápido descenso del pH (acidificación) lo que, al mismo tiempo, resulta en una inhibición de muchos microorganismos patógenos y alterantes. El desarrollo de las BAL juega también un papel importante en la predigestión de los nutrientes de la leche, aumentando su biodisponibilidad. Se producen también nuevos metabolitos que mejoran las propiedades organolépticas de los alimentos derivados respecto a las de la materia prima.

1.3.1. Leches fermentadas

La leche puede consumirse en su forma fluida original o puede ser transformada mediante fermentación en una gran variedad de productos derivados (**Figura 2**). Con seguridad, las primeras leches fermentadas se produjeron de

manera accidental, mediante una acidificación espontánea causada por componentes de la microbiota típica de la leche y otros microorganismos del ambiente. Con el tiempo, las distintas formas de obtener derivados fermentados de la leche se han diversificado y extendido tanto que han dado lugar a la amplia variedad de productos que encontramos en la actualidad alrededor del mundo, incluyendo un incontable número de leches fermentadas naturales, como el Filmjölk, Langfil o Vilii, típicos del norte de Europa, la mazada fermentada o Buttermilk, muy popular en Holanda y Estados Unidos, el kéfir, producido por un consorcio de bacterias lácticas, bacterias del ácido acético y levaduras envueltas en una matriz inerte de polisacáridos y proteínas, y el yogur, de origen caucásico y probablemente la leche fermentada actual más popular en todo el mundo (Tamang y Kailasapathy, 2010).

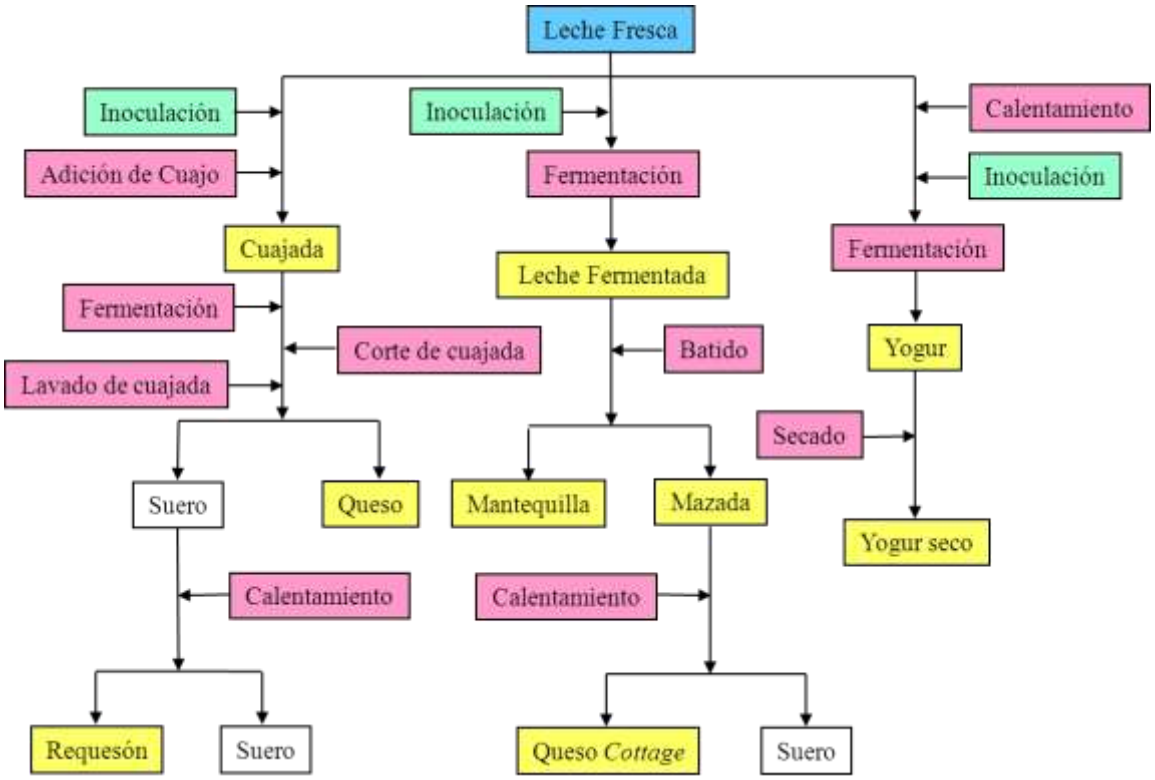

Figura 2. Esquema simplificado de la elaboración de distintos productos lácteos fermentados. Código de colores: En azul la leche, el material de partida; en rosa los procesos tecnológicos, en verde la inoculación con microorganismos, en amarillo los productos finales y en blanco el principal producto residual, el suero.

1.3.2. El queso

El queso es el producto que se obtiene mediante la coagulación de la leche y el escurrimiento parcial del suero. Bajo esta simple definición se agrupa una numerosa variedad de productos que resultan de la utilización y combinación de leches de distinto origen y de la multitud de variantes de los métodos de elaboración (**Tabla 1**). Según diversos autores se estima que se producen entre 500 y 1000 tipos de queso distintos en todo el mundo (Kosikowski, 1977; Burkhaler, 1981; Sandine y Elliker, 1970). En la composición microbiana del queso influyen múltiples factores como la materia prima, el proceso de producción, las condiciones de maduración, etc. Los componentes microbianos esenciales en la fabricación de queso son las BAL cuya función principal es convertir la lactosa de la leche en ácido láctico. Las BAL responsables de la fermentación se desarrollaban en el queso de manera espontánea. Sin embargo, hoy en día, las BAL que se utilizan en la industria quesera son cuidadosamente seleccionadas y añadidas a la leche de manera deliberada en forma de fermentos lácticos (Parente y Cogan, 2004). En la **Tabla 1** se recogen también los principales mircroorganimos involucrados en la elaboración y maduración de distintos tipos de queso y en la **Figura 3** pueden observarse los quesos tradicionales que fueron objeto de estudio en esta tesis.

Figura 3. Dos quesos tradicionales estudiados en el presente trabajo: el queso Casín (izquierda) y el queso Oscypek (derecha), ambos elaborados con leche cruda y sin adición de fermentos.

Tabla 1.- Principales categorías de quesos, fermentos y microorganismos implicados en la maduración.

Tipos de queso	Queso	Tipo de Fermento[a]	Funcion del Fermento[b]	Microbiota del queso[c]								
				Lc	Lc c	Ln	Ec	Sc	Lb	Ll	Lh	Otros microorganismos
• Queso de suero	Ricotta Requesón	FM	LA, D	++	++	+						
• Cuajada ácida	Cottage Quark Afuega'l Pitu	FM, FD	LA, D	++	++	+						
• Cuajada enzimática												
○ Maduración bacteriana												
- Pasta Filata	Mozarella	FN, FM	LA, AR				+	++	+	+	+	
- Alto contenido en sal	Feta	FM	LA	++	+		++					Lb HOMO
- Quesos con ojos	Edam, Gouda	FM	LA, C, P	++	++	+	+					
	Emmental	FM	LA, C, P	++	++	+	+	++	+	+	+	*Propionibacterium freudenreichii/P. shermanii*
- Semi Duro	Caerphilly Queso Tetilla **Casín***	FM, *SF	LA, D	++			+					Lb HOMO/Lb HETERO
- Duro	Cheddar Manchego **Oscypek***	FD, FM, *SF	LA,P, D, AR	++	+	+	++		-			Lb HOMO/Lb HETERO
- Extra duro	Grana Padano Parmesano	FN	LA, P, AR				++	++	++	++	++	Lb HOMO/Lb HETERO
○ Maduración con mohos												
- Interno (Quesos azules)	Roquefort Gorgonzola Stilton Cabrales	FM, FD	LA, C, P, L	++		+	++					*Penicillium roqueforti*/Lb HOMO/Lb HETERO
- Externo (Quesos de moho blanco)	Brie Camembert	FM, FD	LA, P, L	++	++	+	+					*Penicillium camemberti/Geotrichum candidum/* Lb HOMO
○ Maduración con lavado de corteza	Brick Limburger Munster	FM	LA, AR	++			++					*Brevibacterium linens/Kokuria* spp.*/ Micrococcus* spp.

Modificado de Parente y Cogan (2004). Resaltados en negrita los quesos estudiados en este trabajo.

[a]FM fermento mixto indefinido, FD fermento definido, FN fermento natural, *SF elaborado sin fermento en los casos de Casín y Oscypek.

[b]AL, ácido láctico; D, diacetilo; AR, aromas distintos al diacetilo; C, CO2; P, proteólisis; L, lipolisis; PG ácido propiónico y gas.

[c]Lc: *Lactococcus lactis* subsp *lactis,* Lc c: *Lactococcus lactis* subsp. *cremoris*, Ln: *Leuconostoc mesenteroides*, Ec: *Enterococcus faecium* y/o *E. feaecalis*, Sc: *Streptococcus thermophilus,* Lb: *Lactobacillus delbrueckii* subsp. *bulgaricus*, Ll: *Lactobacillus delbrueckii* subsp. *lactis,* Lh: *Lactobacilus helveticus,* Lb HOMO (*Lactobacillus plantarum, Lactobacillus casei*), Lb HETERO (*Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus fermentum*). ++ Especide dominante, + Especie presente en bajas densidades poblacionales u ocasionalmente

2. Microbiología de la leche y los productos lácteos

El pH de la leche (alrededor de 6,6), su temperatura a la salida de la ubre (37ºC) y su alto valor nutricional son ideales para el crecimiento de los microorganismos. Salvo en caso de infección, la leche se considera estéril dentro de la ubre. Las infecciones más corrientes son las mastitis, que están causadas principalmente por *Staphylococcus aureus*, seguido en menor número por *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* y *Corynebacterium* spp. Sin provocar infección, muchas otras bacterias colonizan el extremo interior y exterior de la tetilla y están presentes en los equipos de ordeño y por supuesto en el ambiente, incluyendo en éste a los operarios, la piel de los animales, el aire, etc. Para que la leche no se altere debido a la acción microbiana son necesarias unas correctas condiciones higiénicas de producción y conservación.

En las mejores condiciones higiénicas, es factible conseguir tras el ordeño leche con menos de 5.000 unidades formadoras de colonia (ufc) por mililitro (mL). Sin embargo hace 30 años era difícil producir leche con menos de 100.000 ufc/mL (Fox y col., 2000). Esta mejora general de la higiene se debe principalmente a mejoras en las condiciones higiénicas del ordeño, a un mejor diseño de los equipos utilizados (más fáciles de limpiar), al enfriamiento rápido de la leche a menos de 5ºC tras el ordeño y al mantenimiento de la cadena de frío desde la producción hasta su entrega a la industria.

2.1. Bacterias del Ácido Láctico (BAL)

2.1.1. Características generales de las BAL

Las BAL son microorganismos Gram positivos generalmente inmóviles, no esporulados, no pigmentados y que no reducen los nitratos. Tampoco licuan la gelatina y no producen indol ni sulfuro de hidrógeno. Aunque algunas especies son anaerobias estrictas, la mayoría son microorganismos anaerobios aerotolerantes o microaerófilos. En general carecen de catalasa, pero poseen peroxidasas y superóxido dismutasas que destruyen el H_2O_2 y los radicales O_2^- que se forman en condiciones de aerobiosis (Carr y col., 2002).

Las bacterias de este grupo colonizan hábitats muy variados desde el punto de vista físico-químico y biológico. Algunas especies aparecen asociadas a material vegetal y otras forman parte de la microbiota normal del cuerpo de los animales y del hombre, encontrándose en los tractos respiratorio, intestinal y genitourinario (Eckburg y col., 2005; Marchesi y Shanahan, 2007). En estos espacios las BAL, parecen ejercer un papel beneficioso, debido a que antagonizan la colonización por patógenos, actúan como coadyuvantes inmunológicos y mejoran la digestibilidad de ciertos alimentos (Kleerebezem y Vaughan, 2009).

Podemos destacar que tanto las BAL como sus productos metabólicos se han venido consumiendo (en diversos alimentos fermentados) desde tiempo inmemorial y estas bacterias no se suelen asociar con procesos patológicos (Chamba y Jamet, 2008). Por este motivo se les ha otorgado el *status* de bacterias GRAS (Generally Recognized As Safe) o QPS (Qualified Presumption of Safety) por la FDA y la EFSA (EFSA, 2007), respectivamente; es decir, se las considera bacterias seguras desde el punto de vista higiénico-sanitario.

Debido a su limitada capacidad biosintética, las BAL son bacterias nutricionalmente muy exigentes. Además de una fuente de carbono fermentable, requieren factores de crecimiento complejos como vitaminas, aminoácidos, ácidos grasos y ácidos nucleicos o sus precursores (Dellaglio y col., 1994). No sorprende, por tanto, que sus hábitats, aunque variados, son todos nutricionalmente ricos.

Uno de los rasgos fisiológicos más característicos de las BAL es la tolerancia al ácido, consecuencia obligada de su metabolismo. Esta cualidad ofrece a las bacterias lácticas una ventaja selectiva en los hábitats en que se desarrollan. Así, muchas BAL son capaces de acidificar el medio y sobrevivir a la acidez (por debajo de pH 4.0 en ocasiones). Esta propiedad les confiere una gran importancia en la preservación de alimentos, porque al acidificarlos favorecen su propio crecimiento e inhiben el desarrollo de los microorganismos indeseables sensibles a la acidez. La producción de ácido puede realizarse por medio de un catabolismo homofermentativo de los azúcares, siendo el ácido láctico el producto mayoritario de la fermentación, o de un proceso heterofermentativo en el que además de ácido láctico se produce CO_2, ácido acético y etanol (Mayo y col., 2010).

La gran importancia aplicada de las bacterias de este grupo, ha propiciado el estudio de sus características bioquímicas y genéticas más importantes desde los años 80 (Davies y Gasson 1984; McKay y Balwin, 1990; Salminen y von Wright, 1993). En el presente, con las nuevas técnicas genómicas y proteómicas, se están realizando grandes avances en el estudio de la base genética y fisiológica de las BAL, profundizando en el conocimiento de sus características diferenciales con el fin de hacer uso de todo su potencial (Kok y col., 2005; Makarova y col., 2006; Bachman y col., 2009; Kelly y col., 2010).

2.1.2. Género *Lactococcus*

Los lactococos son BAL mesófilas homofermentadoras con morfología cocoide que producen ácido láctico de tipo L(+). De manera tradicional, el género *Lactococcus* incluye las especies *Lc. lactis* (con tres subespecies: *lactis, cremoris y hordniae*), *Lc. garvieae, Lc. raffinolactis, Lc. plantarum y Lc piscium.* Un tipo especial de *Lc. lactis* subsp. *lactis* lo constituye la biovariedad *diacetylactis*, que posee la capacidad de metabolizar el citrato de la leche produciendo diacetilo. El diacetilo es un compuesto responsable del olor de la mantequilla y clave también en el aroma de muchos quesos. Ninguna de las especies de lactococos es patógena, a excepción de *Lc. garvieae* que causa la lactococcis en peces y mastitis en ganado (Casalta y Montel, 2008). De forma reciente se han añadido al género nuevas especies como *Lc chungangensis* (Cho y col., 2008) y *Lc fujiensis* (Cai y col., 2011), aisladas de sedimentos activos en plantas depuradoras y de col china, respectivamente. Además, se han identificado variantes de *Lc. lactis* en la mucosa intestinal de peces para las que se ha propuesto una nueva subespecie: *Lc. lactis* subsp. *tructae* (Pérez y col., 2010).

En la industria quesera la única especie empleada es *Lc. lactis* y en particular las cepas de la subespecie *cremoris* (Mills y col., 2010). Tradicionalmente, la subespecie *lactis* se distinguía de la *cremoris* de acuerdo a criterios fenotípicos como su habilidad para crecer a 40º, en 4% de NaCl y a pH 9,2, así como a la capacidad de las cepas para fermentar la maltosa y desaminar la arginina (Holt y col., 1994). Sin embargo, la distinción entre las subespecies es muy difícil, dado que se basa en unos pocos caracteres fenotípicos que presentan gran variabilidad entre cepas. Además, desde hace mucho tiempo, se sabe que algunas cepas con fenotipo de *Lc. lactis*

subsp. *lactis* presentan un genotipo de *Lc. lactis* subsp. *cremoris* (Jarvis y Jarvis, 1981). Más recientemente, también se han descrito genotipos de *Lc. lactis* subsp. *lactis* con fenotipo de *L. lactis* subps. *cremoris* (Kelly y col., 2010; Tanigawa y col., 2010). De esta manera la especie *Lc. lactis* tiene una estructura poco usual con dos fenotipos (*lactis* y *cremoris*) y dos genotipos distintos (*lactis* y *cremoris*) entre los que no hay una clara correspondencia (Urbach y col., 1997; Nomura y col., 2002; Rademaker y col., 2007). El nicho natural de estas dos subespecies no está muy bien definido y existe cierta controversia sobre si ambas son capaces de sobrevivir fuera de los ambientes lácteos donde habitualmente se encuentran (Salama y col., 1995, Klijn y col. 1995, Urbach y col., 1997).

2.1.3. Género *Lactobacillus*

Los lactobacilos son bacterias Gram positivas, no esporuladas, capaces de colonizar hábitats tan diversos como la materia vegetal, los productos lácteos y el tracto gastrointestinal del hombre y los animales (Klander y Weiss, 1986). En el género *Lactobacillus* se incluyen más de 100 especies con relaciones fiologenéticas diversas y propiedades muy heterogéneas (http://www.bacterio.cict.fr/l/lacto-bacillus.html). Se clasifican de forma práctica en tres grupos en función de sus características fermentativas, siguiendo las divisiones clásicas que propuso Jensen en 1919 (Kandler y Weiss, 1986). De esta forma, se distingue entre homofermentadores estrictos, heterofermentadores facultativos y heterofermenta-dores obligados. En cuanto a su temperatura óptima de crecimiento, se dividen en mesófilos y termófilos.

El grupo de los lactobacilos heterofermentadores obligados incluye entre otras las especies *Lb. acidophilus, Lb. helveticus* y *Lb. delbrueckii* [con tres subespecies clásicas, *delbrueckii, bulgaricus* y *lactis*, a las que se han añadido dos más de forma reciente, *indicus* (Dellaglio y col., 2005) y *sunkii* (Kudo y col., 2012)]. De éstos, los lactobacilos más habituales en los productos lácteos son *Lb. delbrueckii* subsp. *bulgaricus* (denominado comúnmente como *Lb. bulgaricus*) y *Lb. helveticus*. Cepas de estas dos especies se utilizan como componentes de los fermentos de yogur y en los quesos suizos e italianos de pasta cocida. Además, algunas cepas de *Lb. acidophilus* se utilizan desde hace mucho tiempo como probióticos (Rettger y col., 1935).

Aunque no se incluyen como componentes de los fermentos, diversas especies heterofermentadoras facultativas (como *Lb. plantarum* y *Lb. casei*) y otras heterofermentadoras estrictas (como *Lb. brevis y Lb. fermentum*) forman parte de la microbiota secundaria de una gran mayoría de quesos.

2.1.4. Género *Leuconostoc*

Los leuconostocs son bacterias lácticas heterofermentativas que producen ácido láctico de tipo D(-) y cantidades equimoleculares de CO_2, etanol y ácido acético (Mayo y col., 2010). El género *Leuconostoc* comprende actualmente 13 especies: *Leuc. carnosum, Leuc. citreum, Leuc. fallax, Leuc. gasicomitatum, Leuc. gelidum, Leuc. holzapfelii, Leuc. inhae, Leuc. kimchi, Leuc. lactis, Leuc. mesenteroides* (con cuatro subespecies, *cremoris, dextranicum, mesenteroides* y *suionicum*), *Leuc. miyukkimchii, Leuc. palmae,* y *Leuc. pseudomesenteroides* (Ogier y col., 2008). De todas estas especies, solo *Leuc. mesenteroides* subsp. *cremoris* y *Leuc. lactis* se incluyen habitualmente como componentes de fermentos mesófilos comerciales (Hemme y Foucaud-Scheunemann, 2004).

El interés tecnológico de los leuconostocs para la industria láctea viene dado por su capacidad para metabolizar el citrato con la consiguiente producción de diacetilo y otros compuestos aromatizantes. Como consecuencia de su metabolismo heterofermentativo, pueden participar también en la apertura de la matriz del queso, favoreciendo el crecimiento de microorganismos aerobios.

2.1.5. Género *Streptococcus*

Las bacterias del género *Streptococcus* son cocos Gram positivos que se asocian en parejas o cadenas. En la actualidad hay más de 80 especies descritas dentro de este género, que incluye muchos representantes patógenos. De todas ellas, sin embargo, solo una especie, *S. termophilus,* se utiliza como cultivo iniciador en la industria láctea. Esta especie está bien caracterizada desde el punto de vista fenotípico y genético (Hols y col., 2005). Se trata de una bacteria homofermentadora y anaerobia facultativa, filogenéticamente emparentada con *S. salivarius,* una especie patógena cuyo hábitat natural es la boca. Sin embargo, la adaptación de *S.*

thermophilus a la leche parece haberse producido mediante una reducción genómica en la que desaparecen los genes de patogenicidad y virulencia y se adquieren varias habilidades clave para el desarrollo en este medio. *S. thermophilus* es una bacteria termófila y crece bien a 45ºC. Se utiliza junto con *Lb. delbrueckii* subsp. *bulgaricus* en la producción de yogur y es un cultivo iniciador habitual junto a diversas especies de lactobacilos en los quesos italianos y suizos. En los últimos años ha crecido también su utilización junto a *Lc. lactis* en otros muchos tipos de quesos como el Cheddar y el Manchego (Parente y Cogan, 2004).

2.2. Otros tipos bacterianos

2.2.1. Género *Propionibacterium*

Las propionibacterias son bacterias Gram positivas que pueden presentarse en forma de bacilos, cocos, bífidos o incluso de células ramificadas. A pesar de ser catalasa positivos son microorganismos esencialmente anaerobios o microaerófilos. Como las brevibacterias y las bifidobacterias, las propionibacterias pertenecen al filo *Actinobacteria* y a la clase *Actinobacteria,* que comprende bacterias Gram positivas con alto contenido G+C. Estos tipos bacterianos no están emparentados filogenéticamente con las verdaderas BAL (Stackebrandt y col., 1997) como queda reflejado en el árbol filogenético de la **Figura 4**. El género *Propionibacterium* incluye 12 especies que se dividen en dos grandes grupos: las propionibacterias "clásicas" o "lácteas", que se encuentran fundamentalmente en productos lácteos (también en encurtidos y ensilados), y las propionibacterias "cutáneas", de las que la especie más representativa es *Propionibacterium acnes* (Tierry y col., 2011). Las propionibacterias poseen requerimientos nutricionales relativamente simples, aunque necesitan ácido pantoténico, biotina o tiamina para crecer, y muchas de ellas pueden utilizar NO_3^- como única fuente de nitrógeno. Las propionibacterias clásicas son seguras desde el punto de vista de la salud (Meile y col., 2008). Este grupo comprende cuatro especies típicas: *P. freudenreichii*, la especie más común, y las especies *P. jensenii*, *P. thoenii* y *P. acidipropionici*. *P. freudenreichii* subsp. *shermanii* juega un papel importante en la maduración tardía de algunos quesos, al transformar el ácido láctico del queso en ácido propiónico y CO_2, que lentamente formará burbujas de gas en el interior de la pasta, dando lugar a los "ojos" típicos de quesos como el Emmental y el Gruyère

(Tierry y col., 2011). Junto a otros compuestos volátiles, el ácido propiónico confiere a los quesos un aroma típico a avellana.

2.2.2. Género *Brevibacterium*

Las brevibacterias son bacilos Gram positivos, catalasa positivos y aerobios estrictos de la clase *Actinobacteria*. Pocas son las brevibacterias de importancia en quesería. La especie más reconocida en este ámbito es *Brevibacterium linens* que se aisla de la superficie de algunos quesos, principalmente de los de pasta lavada (smear ripened) en los que se utiliza también como cultivo de maduración (Onraedt y col., 2005). Otras especies que se aíslan de leche y productos lácteos son *B. casei, B. iodinum* y *B. epidermidis*; especies que parecen ser patógenas oportunistas para el hombre. Las brevibacterias apenas pueden crecer a 5ºC y su temperatura óptima se sitúa entre los 20 y 25ºC. Son capaces de crecer en presencia de altas concentraciones de sal, hasta un 8-10% en el caso de *B. linens* y *B. iodinum* y hasta un 15% en el caso de *B. casei* y *B. epidermidis*. Su metabolismo es respiratorio y no producen ácido a partir de glucosa, siendo las brevibacterias sensibles al ácido e incapaces de crecer a valores de pH por debajo de 6.0. Poseen complejos sistemas proteolíticos y lipolíticos que contribuyen al aroma del queso. En particular, son capaces de transformar la metionina en metanotiol y otros compuestos sulfurosos, responsables del particular olor de los quesos de pasta lavada como el Limburger.

2.2.3. Género *Bifidobacterium*:

Las bifidobacerias son bacterias Gram positivas, no esporuladas y generalmente ramificadas (bífidas) que producen ácido láctico y acético como resultado de sus procesos fermentativos. Como las otras actinobacterias, se incluyen dentro de las BAL porque aparecen juntas en la leche y en el tracto gastrointestinal del hombre y de los animales, aunque no están muy relacionadas filogenéticamente (**Figura 4**). Además se utilizan también como probióticos, fundamentalmente a través de los productos lácteos (Leahy y col., 2005). Entre los del hombre y de los animales. Además se utilizan también como probióticos, fundamentalmente a través de los productos lácteos (Leahy y col., 2005). Entre los mencionar su contribución al equilibrio microbiano gastrointestinal, su participación en la maduración y activación

del sistema inmune, el mantenimiento de la integridad intestinal y la resistencia a patógenos (Leahy y col., 2005; Kleerebezem y Vaughan, 2009).

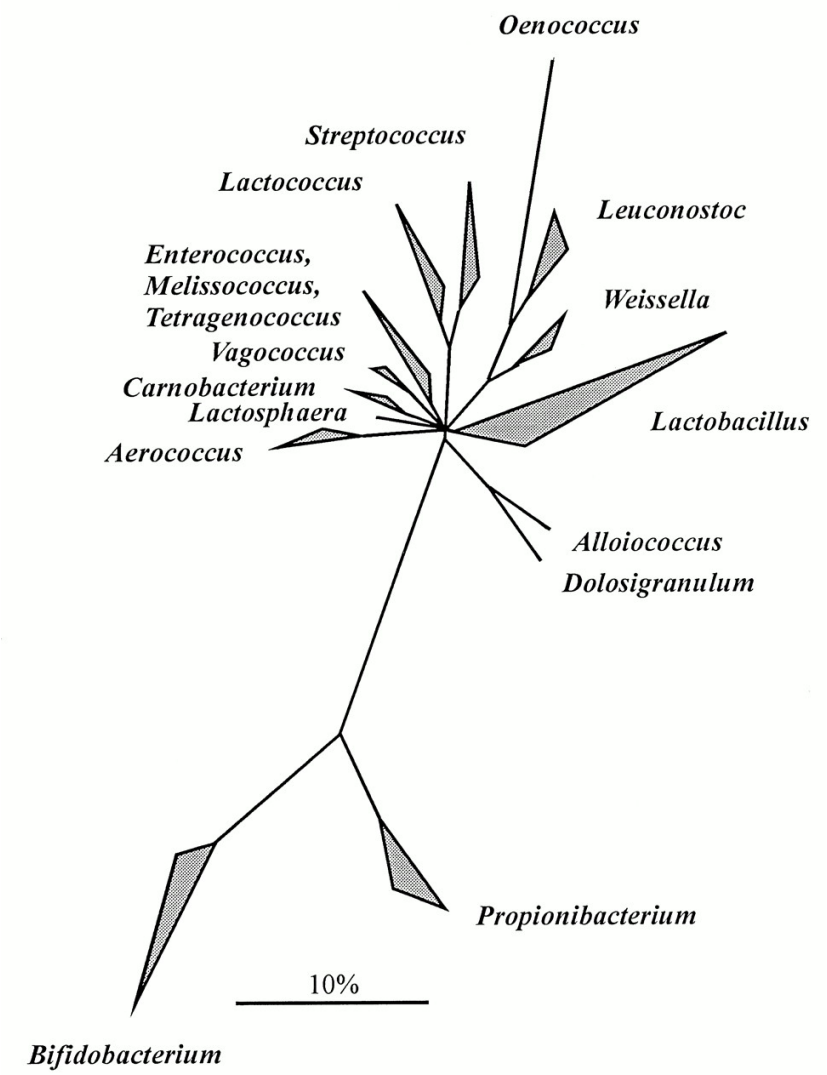

Figura 4. Árbol consenso basado en las secuencias del ARNr 16S, que incluye los principales grupos filogenéticos de BAL y los géneros gram-positivos no relacionados *Bifidobacterium* y *Propionibacterium*. (Holzapfel, 2001)

2.3. Mohos y levaduras

Entre los microorganismos que acceden a la leche procedentes del ambiente se incluyen diversas especies de mohos y levaduras. Algunas de ellas encuentran en los productos lácteos fermentados un nicho ecológico favorable para su desarrollo. La capacidad para asimilar o fermentar la lactosa y otros ácidos orgánicos de la leche (láctico, cítrico, succínico), así como sus actividades proteolíticas o lipolíticas,

posibilitan el aprovechamiento de los nutrientes de este medio. Además mohos y levaduras toleran niveles reducidos de pH y a_w y son capaces de crecer a bajas temperaturas y en elevadas concentraciones de sal (Pitt y Hocking, 1997; van den Tempel y Nielsen, 2000), condiciones corrientes durante los procesos de maduración de muchos productos lácteos. Los mohos y levaduras más extendidos en la leche y sus derivados pertenecen a los géneros *Penicillum, Debaryomyces, Kluyveromyces, Candida, Yarrowia, Zygosaccharomyces y Saccharomyces* (Beresford y col. 2001; Wouters y col. 2002).

En ocasiones, la presencia de estos microbios se relaciona con diversas alteraciones de los alimentos. Los defectos más típicos que causan las levaduras en los productos lácteos son una excesiva producción de gas, cambios de color por la síntesis de diferentes pigmentos, cambios en la textura provocados por una intensa proteolisis, producción de aromas indeseados, producción de toxinas, etc. (Pitt y Hocking, 1997). En otros casos confieren al producto unas características organolépticas originales y deseables, por lo que su presencia es beneficiosa. Así, diversas levaduras son esenciales en la elaboración de productos como el kéfir, el Koumis, el Viili y otras leches fermentadas del norte de Europa y del continente africano. En los quesos participan en la degradación del ácido láctico, desacidificando la pasta y posibilitando el asentamiento de poblaciones secundarias.

Las especies de levaduras predominantes en los productos lácteos son *Debaryomyces hansenii, Kluyveromyces marxianus, Kluyveromyces lactis, Yarrowia lipolytica, Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Pichia fermentans, Pichia membranaefaciens* y *Rhodotorula mucilaginosa* y contribuyen también a los procesos de maduración por medio de sus variadas actividades enzimáticas (Beresford y col., 2001; Wouters y col., 2002).

En cuanto a los mohos, diversas especies de *Penicillium* colonizan y se desarrollan sobre muchos tipos de alimentos, incluyendo productos lácteos. Las especies más utilizadas en la tecnología quesera son, sin duda, *Penicillium camemberti* y *Penicillium roqueforti*. Estos mohos son esenciales en la maduración de los quesos tipo Brie y Camembert (*P. camemberti*) y los quesos azules (*P. roqueforti*) (Gripon, 1999). Los mohos son responsables, en primera instancia del aspecto externo y al corte de

los quesos, lo que los hace inconfundibles. Además, la maduración se produce por la intensa lipolisis y proteolisis debida a las potentes actividades enzimáticas de los mohos. Estos procesos generan un gran número de compuestos que determinan sus características organolépticas y reológicas diferenciales. Entre estos compuestos destacamos varias metilcetonas, alcoholes secundarios, ésteres, lactonas y aldehídos derivados de los ácidos grasos, así como diversos compuestos sulfurados que proceden de la degradación de la fracción proteica. Además de los penicilios, con los quesos se relaciona un variado grupo de mohos entre los que se pueden citar especies de *Mucor, Epicoccum, Sporotrichum*, etc.

A caballo entre mohos y levaduras se encuentra *Geotrichum candidum*, uno de los mohos lácticos más típicos, aunque en la actualidad se considera una verdadera levadura (Wouters y col., 2002). *G. candidum* consume lactato, lo que favorece el crecimiento de poblaciones secundarias incapaces de multiplicarse a pH ácido (como *B. linens*). Al mismo tiempo, algunas cepas inhiben el desarrollo de microorganismos indeseables (bacterias y hongos).

3. Fermentos lácticos

Tal como se ha mencionado anteriormente, la fabricación de productos lácteos fermentados constituye uno de los procedimientos de conservación más antiguos y es también una fuente inagotable de nuevos productos. Tradicionalmente, los procesos fermentativos se han llevado a cabo por procedimientos empíricos que a lo largo de las últimas décadas se han ido sustituyendo por procedimientos muy controlados, basados en el conocimiento científico sobre las propiedades de los productos lácteos y las de los microorganismos que se utilizan en su elaboración.

La acidificación espontánea de la leche conduce en muchas ocasiones a productos finales con características poco uniformes y con defectos y alteraciones no deseadas. Además, la implicación de la leche y los productos lácteos en la transmisión de enfermedades generalizó la utilización de la pasterización en el siglo pasado (Mills y col., 2010). Junto a los microorganismos patógenos, la pasteurización elimina también los microorganismos deseables que dirigen la

fermentación. Por este motivo, tras la pasteurización, los microorganismos necesarios han de ser añadidos de forma exógena en forma de fermentos (Mills y col., 2010).

Los fermentos, cultivos iniciadores o "starters" se definen como una o más cepas de una o más especies microbianas que se inoculan en una materia prima para iniciar y controlar su fermentación. De forma más general y aplicada, el término fermento engloba a todos los microorganismos que se añaden a la leche para promover su transformación, incluyendo diversos cultivos adjuntos y de maduración. Los constituyentes de los fermentos participan en los cambios más importantes que tienen lugar durante la elaboración y la maduración, modificando la textura, el aroma y el sabor de la materia prima para generar las características organolépticas típicas del producto final (Smit y col., 2005).

3.1. Funciones de los fermentos lácticos

En la elaboración de los productos lácteos fermentados, los cultivos iniciadores tienen varias funciones esenciales:

- Producción de ácido, principalmente láctico, a partir de la lactosa de la leche. El ácido láctico contribuye al aroma y sabor de los productos fermentados. En el queso, la acidez incrementa la actividad del cuajo y facilita el desuerado de la cuajada, siendo un factor tecnológico de gran importancia.

- Producción de proteasas y lipasas que degradan las proteínas y las grasas de la leche, generando componentes de sabor y aroma o precursores que se transfomarán en éstos a través de diversas reacciones catabólicas posteriores (Smit y col., 2005).

- Producción de compuestos aromáticos volátiles como el diacetilo, el acetaldehído y los ácidos grasos de cadena corta (Hemme and Foucaud-Scheunemann, 2004; Smit y col., 2005; Settanni and Moschetti, 2010).

- Inhibición de bacterias alterantes y/o patógenas por diversos procesos de antagonismo microbiano, entre los que cabe destacar la producción de ácidos

orgánicos (láctico, acético), la competencia por espacio y nutrientes, la producción de bacteriocinas y de H_2O_2. Todos estos compuestos contribuyen a la conservación del producto (Topisirovic y col., 2006). Las bacteriocinas, por ejemplo, no solo mejoran la calidad general (higiénico-sanitaria y organoléptica) de los productos lácteos mediante la inhibición de alterantes y patógenos (Gálvez y col., 2008), sino que además, pueden provocar la lisis del cultivo iniciador o de los cultivos adjuntos, con lo que se acelera la maduración (Leroy y de Vuyst , 2004).

- En algunos productos, las especies heterofermentativas pueden formar CO_2 a partir de la lactosa y el citrato, lo que contribuye al desarrollo de las típicas aberturas (ojos) en la pasta de algunos quesos o a la sensación de frescor de algunas leches fermentadas. En los quesos azules la formación de ojos puede favorecer también el desarrollo de *P. roqueforti*.

- Mantenimiento del equilibrio bacteriano intestinal y la salud. En los últimos tiempos, a los productos lácteos se incorporan diversos microorganismos con el fin de mantener o recuperar el equilibrio intestinal o para reforzar las defensas; lactobacilos y bifidobacterias son los tipos más empleados en este sentido (Ouwenhand y col., 2002; Leahy col., 2005; Kleerebezen y Vaugahn, 2009).

3.2. Clasificación de los fermentos

Los fermentos pueden clasificarse atendiendo a la temperatura óptima de crecimiento de sus componentes (fermentos mesófilos y termófilos), a su función principal (acidificadores, aromatizantes, de maduración), a su composición (fermentos de cepa única y fermentos mixtos; éstos a su vez pueden ser de cepas de una sola especie bacteriana, de mezclas definidas de especies, o de mezclas no definidas), al modo de presentación (liofilizados, congelados), etc. En todo caso, los tipos bacterianos utilizados son solo unos pocos. En la tabla 2 se recogen las especies más importantes y sus características bioquímicas principales.

Una categoría especial de fermentos son los fermentos específicos (**Figura 5**). Estos son los que se desarrollan para un producto en particular a partir de microorganismos aislados de dicho producto, respetando los tipos microbianos

presentes en la fermentación y sus proporciones relativas. El objetivo de estos fermentos es reproducir de forma fiel las cualidades típicas y diferenciadoras de cada producto al mismo tiempo que contribuyen a aumentar la calidad higiénico-sanitaria y a reducir los accidentes tecnológicos. Al estar constituidos por microorganismos "autóctonos", los componentes de los fermentos específicos estarán en equilibrio con los numerosos virus bacterianos (bacteriófagos) endémicos de la zona de producción (Madera y col., 2004), limitando así los efectos devastadores del ataque fágico (Parente y Cogan, 2004).

Figura 5. Fermentos específicos para el queso de Cabrales diseñados por el grupo de Cultivos Funcionales del IPLA, comercializados en forma liofilizada por la empresa Bioges Starters.

3.2.1. Cultivos iniciadores

Tal y como ya se mencionó anteriormente, los cultivos iniciadores más utilizados en la industria quesera **(Tabla 2)** están constituidos por cepas del género *Lactococcus* y su principal cometido es provocar una rápida acidificación de la leche. En otros productos lácteos (yogur, quesos suizos e italianos), las especies de BAL utilizadas como fermentos son *Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus* y/o *S. thermophilus.*

3.2.2. Microbiotas secundarias y cultivos adjuntos

Al lado de los cultivos iniciadores, en los productos lácteos se desarrollan otros tipos microbianos denominados de forma genérica microbiotas secundarias. Estas microbiotas secundarias son muy variadas desde el punto de vista taxonómico y funcional. En ellas podemos encontrar a BAL que no participan directamente en la acidificación, microorganismos que se denominan de forma corriente como BAL no

Tabla 2.- Bacterias lácticas habituales en los fermentos comerciales y sus características fisiológicas más relevantes.

Especie	Forma	Tipo[a]	Ácido láctico producido en leche (%)[b]	Isómero del ácido láctico	Metabolismo del citrato	Formación de NH_3 a partir de Arginina	Crecimiento a (ºC)			
							10	15	40	45
Lactococcus lactis subsp. *lactis*	Coco	M	0,8	L	+++/-	+	+	+	+	-
Lactococcus lactis subsp. *cremoris*	Coco	M	0,8	L	-	-	+	+	-	-
Lactobacillus delbrueckii subsp. *bulgaricus*	Bacilo	T	1,8	D	+	+/-	-	-	+	+
Lactobacillus delbrueckii subsp. *lactis*	Bacilo	T	1,8	D	+	+/-	-	-	+	+
Lactobacillus helveticus	Bacilo	T	2,0	DL	+	-	-	-	+	+
Leuconostoc lactis	Coco	M	<0,5	D	+++	-	+	+	-	-
Leuconostoc mesenteroides subsp. *cremoris*	Coco	M	0,2	D	+++	-	+	+	-	-
Streptococcus thermophilus	Coco	T	0,6	L	-	-	-	-	+	+

Modificado de Fox y col. (2000). [a]M: mesófilos, T: termófilos. [b]Valores aproximados; +/- resultado variable entre cepas.

iniciadoras (Non-Starter Lactic Acid Bacteria; NSLAB), y a otros tipos como propionibacterias, corinebacterias, estafilococos, mohos y levaduras, etc. Los microorganismos NSLAB pertenecen mayoritariamente a los géneros *Lactobacillus* y *Leuconostoc* y alcanzan densidades elevadas durante la maduración, superando en ocasiones a las de los fermentos (Beresford y col., 2001; Wouters y col., 2002).

En algunos productos el desarrollo de estas poblaciones es indispensable para generar las características típicas de los mismos. En otras ocasiones, sin embargo, las microbiotas secundarias dan lugar a defectos de textura, aroma y/o sabor, o representan un riesgo sanitario (Crow y col., 2001). Así, en muchos quesos artesanales, especialmente los producidos en el arco mediterráneo, es corriente la presencia de poblaciones elevadas de *Enterococcus faecalis, Enterococcus faecium* y otras especies de *Streptococcus* (Estepar y col., 1999; Flórez y col., 2006). La presencia de estas bacterias es controvertida, puesto que, aunque pueden contribuir a la maduración, muchas especies son patógenas oportunistas y presentan numerosos determinantes de virulencia y de resistencia a antibióticos (Giraffa, 2003; Foulquié-Moreno y col., 2006).

Los componentes beneficiosos de estas microbiotas se pueden añadir en forma de cultivos, denominándose cultivos adjuntos. El hecho de que a pesar de la utilización de fermentos los quesos elaborados con leche pasteurizada presenten menos sabor que los de leche cruda se ha relacionado con la eliminación de

microorganismos tecnológicamente relevantes (McSweeney y col., 1993). La adición de cultivos adjuntos con los tipos microbianos que la pasteurización elimina podría restaurar los perfiles organolépticos originales (Parente y Cogan, 2004).

3.3. Propiedades fisiológicas y tecnológicas de los fermentos

Las propiedades tecnológicas que se exigen a los fermentos tienen que ver con las funciones que llevan a cabo en las distintas fermentaciones, tal y como se han enumerado en párrafos precedentes. Para realizar una acidificación rápida, necesitan desarrollarse de manera acelerada en leche y alcanzar densidades celulares elevadas (Parente y Cogan, 2004). La bajada rápida del pH inhibe el desarrollo de microorganismos patógenos y alterantes y crea las condiciones químicas necesarias para que tengan lugar las transformaciones de la maduración. El crecimiento en leche exige un equipamiento enzimático para utilizar la lactosa y otro para el aprovechamiento del nitrógeno de las caseínas de la leche, ya que la leche presenta un reducido nivel de aminoácidos libre y péptidos pequeños de asimilación directa (Fernández y Zúñiga, 2006).

Las LAB homofermentadoras (*Lactococcus, Streptococcus, Pediococcus, Enterococcus* y algunas especies de *Lactobacillus*) utilizan la lactosa por medio de la ruta Embden-Meyerhoff-Parnas (EMP) dando lugar a piruvato, el cual se convierte en lactato mediante la lactato deshidrogenasa (Mayo y col., 2010). Por su parte, las BAL heterofermentadoras (*Leuconostoc, Oenococcus* y algunas especies de *Lactobacillus*) fermentan la lactosa por medio de la ruta de la fosfocetolasa o de Warburg-Dickens (WD), de manera que como producto final generan lactato, CO_2 y etanol. Las BAL poseen dos mecanismos distintos de transporte y degradación de lactosa: (I) un sistema fosfotransferasa dependiente de fosfoenulpirúvico (PEP-PTS) que fosoforila la lactosa durante su incorporación y (II) una permeasa que internaliza la lactosa sin modificar. Los enzimas claves en la degradación son una β-fosfo-galactosidasa en el primer caso y una β-galactosidasa en el segundo (de Vos y Vaughan, 1994). El primer sistema lo presentan las cepas *starter* de *Lc. lactis* y algunas cepas de *Lactobacillus casei*; todas las demás BAL utilizan el segundo mecanismo.

La utilización de las caseínas necesita de un complejo sistema proteolítico con varios componentes. En primer lugar, es necesaria una degradación parcial de las caseínas en el exterior celular por medio de una proteinasa específica. Luego, los péptidos y aminoácidos generados se transportan al interior de la célula utilizando transportadores de amino ácidos y péptidos específicos (Fernández y Zúñiga, 2006). Finalmente los péptidos son hidrolizados por la acción de las peptidasas citoplasmáticas (Christensen, y col., 1999; Savijoki y col., 2006).

Además de contribuir al desarrollo de las BAL, la proteólisis de la leche durante la maduración genera péptidos y amino ácidos que son precursores de muchos compuestos aromáticos típicos de los productos lácteos (Liu y col., 2008).

Otra de las características tecnológicas interesantes de las BAL es su capacidad para producir compuestos antimicrobianos que antagonizan el desarrollo de los microorganismos. De estos compuestos, los más importantes son los ácidos orgánicos, el H_2O_2 y las bacteriocinas. Estas últimas son sustancias proteicas capaces de inhibir o eliminar especies de otros grupos bacterianos. Desde el punto de vista bioquímico, en las BAL se han identificado dos tipos de bacteriocinas: (i) las que no contienen aminoácidos modificados y (ii) los lantibióticos, caracterizados por la presencia de aminoácidos modificados (deshidratados y con enlaces tioéter) (de Vuyst y Leroy, 2007). Ambos tipos se sintetizan en los ribosomas, aunque los segundos se modifican enzimáticamente con posterioridad. Las bacteriocinas se contemplan como conservantes de grado alimentario por su capacidad para inhibir patógenos y alterantes y su facilidad de degradación en el intestino (Gálvez y col., 2008).

A escala industrial, los fermentos compuestos por una sola cepa son inviables debido a la amenaza de los bacteriófagos: el principal problema de las fermentaciones lácteas (Madera y col., 2004). Un escaso desarrollo de los fermentos puede provocar defectos en el sabor, la textura y afectar a la seguridad higiénica de los productos fermentados (Garneau y Moineau, 2011). Para reducir el impacto económico causado por las infecciones de bacteriófagos se pueden adoptar medidas como el uso de cubas cerradas, la propagación de los fermentos en medios sin calcio o la selección de cepas resistentes a fagos (Everson, 1991). Sin embargo, en la

práctica, la rotación de mezclas de cepas resistentes a distintos tipos fágicos continúa siendo el método más simple, barato y efectivo (Madera y col., 2004; Garneau y Moineau, 2011).

3.4. Propiedades probióticas de los fermentos

Los probióticos se definen como "microorganismos vivos que ingeridos en cantidades adecuadas, ejercen un efecto beneficioso sobre la salud del consumidor" (FAO/WHO, 2006). La mayoría de los probióticos usados en la actualidad pertenecen a unas pocas especies de bacterias intestinales de los géneros *Lactobacillus* y *Bifidobacterium* (Leahyy col., 2005; Kleerebezem y Vaughan, 2009), aunque también se emplean cepas bacterianas de otros géneros (*Streptococcus, Propionibacterium, Bacillus,* etc.) e incluso cepas de levaduras (*Saccharomyces cerevisieae boulardii*) (Ouwehand y col., 2002).

En los últimos tiempos, se estudian también las propiedades probióticas de las BAL procedentes de los productos lácteos tradicionales (Collado y col., 2007; Foligné y col., 2010; Settanni y Moschetti, 2010; Ayeni y col., 2011). Estas bacterias tendrán mejor capacidad de crecimiento y supervivencia en los productos lácteos fermentados que a las cepas de origen intestinal.

3.5. Diseño de los fermentos

Las industrias dedicadas a la producción de fermentos lácteos reconocen la necesidad de encontrar nuevas cepas de BAL con características adecuadas para reemplazar a las que están actualmente en uso (van Hylckama Vlieg y col., 2006; Mills y col., 2010). Por esta razón, desarrollan una búsqueda constante de nuevas cepas o de cepas con nuevas propiedades beneficiosas, de cara a mejorar las fermentaciones tradicionales o a desarrollar nuevas aplicaciones (Rademaker y col., 2007; Brandsma y col., 2008). En el marco de esta búsqueda, los productos lácteos tradicionales elaborados con leche cruda y sin adición de fermentos comerciales son un importante reservorio de diversidad fenotípica y genética y resultan una fuente excelente de nuevas cepas de BAL (Kelly y Ward, 2002; Rademaker y col., 2007).

Para su utilización como nuevos fermentos iniciadores o como cultivos adjuntos, las cepas han de ser identificadas de forma precisa y caracterizadas en profundidad

3.6. Ensayo experimental de fermentos

En los fermentos lácticos mixtos con presencia de cepas o especies distintas son corrientes las interacciones entre los microorganismos. Estas interacciones pueden tener efectos negativos o positivos. Un ejemplo clásico es la simbiosis que ocurre en el yogur entre las cepas de *S. thermophilus* y las de *Lb. delbrueckii* subsp. *bulgaricus* (Herve-Jimenez y col., 2009). Los lactobacilos producen bases púricas que los estreptococos necesitan y los estreptococos proveen a los lactobacilos de nutrientes esenciales para su crecimiento como son los ácidos fórmico y fólico (Derzelle y col., 2005; Crittenden y col., 2003). Interacciones positivas similares se han descrito también entre los microorganismos del kéfir (Simova y col., 2006). Otro efecto positivo interesante es la complementación de las rutas de formación de compuestos de aroma y sabor que se produce, por ejemplo, entre cepas distintas de *Lc. lactis* (Ayad y col., 2001, Kimoto-Nira y col., 2012). Además, como ya vimos antes, algunas cepas producen compuestos antimicrobianos capaces de inhibir el desarrollo de otras lo que resulta en una interacción negativa (de Vuyst y Leroy, 2007; Gálvez y col., 2008). Todas estas razones hacen que, más allá de la caracterización tecnológica individualizada, sea necesario también analizar el comportamiento de las mezclas de cepas mediante la realización de fermentaciones controladas.

4. Caracterización microbiana de productos lácteos

Las propiedades sensoriales de los productos lácteos tradicionales dependen de una larga serie de factores entre los que podemos destacar la alimentación de los animales, las prácticas y tecnologías de elaboración y la composición cualitativa y cuantitativa de los microorganismos responsables de la acidificación y maduración (Cogan, 1997; Corroler y col., 1998). La composición y actividad de los tipos microbianos presentes en los productos lácteos juegan un papel crucial en el desarrollo de sus cualidades higiénicas y de conservación (Guinane y col., 2005). De esta forma, resulta esencial la identificación y caracterización de los

microorganismos que participan en la elaboración de los productos tradicionales para poder llegar a reproducir las fermentaciones de manera controlada. La estandarización es una importante forma de incrementar la calidad y salubridad de estos productos.

De manera tradicional, la caracterización microbiana de los productos lácteos se ha realizado con técnicas dependientes de cultivo utilizando utilizando medios ricos no selectivos, combinados con recuentos en medios selectivos y diferenciales. De esta forma se lleva a cabo la enumeración y seguimiento de las poblaciones microbianas tecnológicamente relevantes y otras poblaciones indicadoras. En las últimas décadas, en estas caracterizaciones microbionas se ha comenzado a aplicar también una amplia variedad de técnicas moleculares independientes de cultivo (Giraffa y Neviani, 2001; Jany y Barbier, 2008). Estas técnicas han revelado una diversidad microbiana muy superior a la que anticiparon las técnicas de cultivo.

Los métodos moleculares permiten evitar algunos de los inconvenientes asociados al cultivo microbiano como la baja especificidad de los medios selectivos y la imposibilidad de detectar células viables pero no cultivables. La mayor parte de estos nuevos métodos se basan en la amplificación de ADN o ARN ribosómico mediante la reacción en cadena de la polimerasa (PCR) y un posterior análisis de los amplicones obtenidos (Amman y col., 1995). Estas técnicas son menos laboriosas, más fiables, más rápidas y más baratas que las técnicas convencionales de cultivo. Además ofrecen una descripción microbiana más completa de los ecosistemas alimentarios. Sin embargo, como señalan varios autores, no están libres de inconvenientes y limitaciones que es necesario conocer para una mejor interpretación de los resultados (von Wintzingerode y col., 1997; Ercolini, 2004).

4.1. Microbiología convencional de quesos tradicionales españoles

Numerosos quesos tradicionales españoles se han analizado en el pasado por medio de las técnicas de cultivo. Las técnicas microbiológicas clásicas utilizadas en los estudios pioneros sobre el queso Cabrales (Núñez, 1978), el queso Manchego (Ordóñez y col., 1978), el queso de La Serena (Del Pozo y col., 1988) y otros (Fontecha y col., 1990), se han continuado utilizando hasta el presente. Las técnicas

de cultivo además son indispensables para la obtención de cepas que, una vez identificadas y caracterizadas, se puedan utilizar como fermentos. De esta forma, desde los años 90 se han caracterizado microbiológicamente los quesos de La Armada (Tornadijo y col., 1995), Afuega'l Pitu (Cuesta y col., 1996), Arzúa (Centeno y col., 1996), Peñamellera (Estepar y col., 1999), queso de cabra de Tenerife (Zarate y col., 1997), el queso Cabrales (Flórez y col., 2006), el queso Manchego (Ballesteros y col., 2006) y otros muchos. El objetivo de todos estos estudios es conocer la composición y diversidad microbiana de los quesos tradicionales y su evolución a lo largo de la maduración. Este conocimiento podrá emplearse para mejorar la calidad organoléptica e higiénica de estos productos. Los trabajos se dirigen también a preservar la microbiota tecnológicamente relevante con el fin de elaborar fermentos específicos para los distintos quesos, o para utilizar sus componentes en otras aplicaciones biotecnológicas.

4.2. Estudios microbiológicos independientes de cultivo

Con la implantación de las técnicas moleculares independientes de cultivo, en los últimos años, se ha profundizado aún más en el conocimiento de la composición y estructura de las poblaciones que gobiernan las fermentaciones lácteas y en la dinámica de estas a lo largo de la maduración. Estas técnicas se han aplicado ya al estudio del queso de Cabrales (Flórez y Mayo, 2006) y a algunos quesos tradicionales de Andalucía como el de Cueva de la Magahá (Martín-Platero y col., 2008) o el de Sierra de Aracena (Martín-Platero y col., 2009). Entre las técnicas independientes de cultivo que se han empleado en estas caracterizaciones podemos destacar la DGGE (Flórez and Mayo, 2006; Capítulo 3 de esta memoria), la LH-PCR (Martín-Platero y col., 2008; Martín-Platero y col., 2009) y la pirosecuenciación (Capítulo 5 de esta memoria).

4.2.1. Electroforesis en gel con gradiente desnaturalizante (DGGE)

La electroforesis en gel con gradiente químico desnaturalizante (*Denaturing Gradient Gel Electrophoresis* o DGGE) y su técnica gemela, la electroforesis en gel con gradiente temporal de temperatura (TTGE), se desarrollaron con el fin de analizar comunidades microbianas. Ambas se basan en una migración secuencia-específica de amplicones de un gen polimórfico (como por ejemplo el que codifica el ARNr

16S) obtenidos mediante PCR, a través de un gel con un gradiente desnaturalizante (Muyzer y Smalla, 1998). La separación de los amplicones en la electroforesis se basa en una reducción de movilidad de la doble hélice de ADN a medida que se va desnaturalizando a su paso por el gel de poliacrilamida con el gradiente (sea químico o de temperatura). Con el objetivo de prevenir la completa disociación de las dos moléculas de la doble cadena, se añade al extremo 5' de uno de los cebadores una secuencia de citosinas y guaninas (denominada pinza GC) de alrededor de 50 bases. Ambas técnicas (PCR-DGGE y PCR-TTGE) han sido usadas recientemente en estudios microbiológicos de la leche y diferentes tipos de quesos (Coppola y col., 2001; Ogier y col., 2002; Randazzo y col., 2002; Ercolini y col., 2004; Ogier y col., 2004). Con ellas se pueden analizar las poblaciones mayoritarias de una forma rápida y sencilla (Muyzer y col., 1993). Si en vez de ADN se analiza el ARN microbiano total, los perfiles de DGGE revelan las poblaciones mayoritarias y metabólicamente activas.

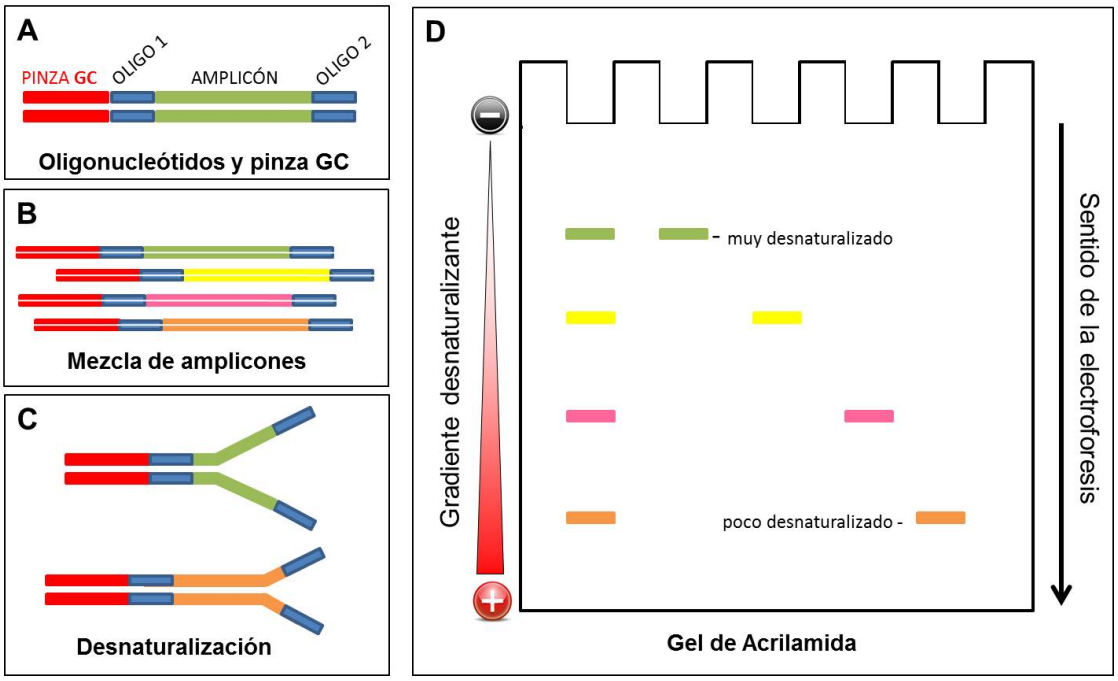

Figura 6. Esquema simplificado del funcionamiento de la DGGE. **6A**: Diseño de la amplificación por PCR. Uno de los dos oligos lleva unida una pinza GC para evitar la completa desnaturalización del amplicón. **6B**: Tras la amplificación se obtiene una mezcla de amplicones con distinta secuencia pero mismo tamaño que dan una única banda en un gel de agarosa convencional. **6C:** Los amplicones se desnaturalizan en función de su secuencia, debido a que las bases GC tienen uniones más fuertes. **6D:** La electroforesis se realiza en presencia de un agente desnaturalizante. La desnaturalización del amplicón hace que este migre menos a través del gel de acrilamida.

4.2.2. LH-PCR

LH-PCR hace referencia a la heterogeneidad de longitud en una amplificación por PCR (*length heterogeneity*-PCR). Uno de los genes con mayor significado en ecología microbiana, es el que codifica el ARNr 16S (Suzuki y col., 1998). Este gen tiene regiones muy conservadas en todos los grupos de procariotas separadas por regiones hipervariables que son distintas entre grupos, géneros y especies. Las regiones hipervariables se pueden amplificar en conjunto o por separado con distintos cebadores. Lo que se registra mediante esta técnica es la diferencia de longitud del amplicón (sea de uno o de varios segmentos del gen o del gen completo) tras una amplificación utilizando cebadores fluorescentes, seguida de una electroforesis capilar. La técnica LH-PCR se ha utilizado para analizar la composición y evolución de los lactobacilos en quesos tradicionales andaluces (Martín-Platero y col., 2008; Martín-Platero y col., 2009).

4.2.3. Nuevas técnicas de secuenciación

Las nuevas técnicas de secuenciación o NGS (Next Generation Sequencing) engloban a un grupo de tecnologías que permiten una secuenciación masiva simultánea de muchos fragmentos de ADN. Las técnicas NGS son de gran utilidad para el estudio de la composición, estructura y evolución de las poblaciones microbianas. Las dos principales tecnologías utilizadas hasta la fecha son el Pirosecuenciador 454 de LifeScience (Roche) y la plataforma de secuenciación Illumina (Illumina Company). Ambos sistemas tienen un rendimiento infinitamente superior a los secuenciadores capilares tradicionales. En general, la pirosecuenciación consigue secuencias más largas (hasta las 600 pares de bases), mientras que la tecnología Illumina presenta unos costes inferiores y supera a la tecnología 454 en número de secuencias obtenidas por reacción (10^9 con el equipo HiSeq2000 de Illumina frente a 10^6 con el equipo 454FLX+). El análisis de una secuencia más larga es más fiable desde el punto de vista taxonómico. Sin embargo algunos autores defienden que 150 pares de bases localizadas en regiones hipervariables son suficientes para identificar la mayor parte de las especies microbianas (Liu y col., 2007).

La pirosecuenciación fue la primera de las tecnologías NGS disponible en el mercado y ha sido la opción mayoritaria para el estudio de poblaciones microbianas en alimentos. En lácteos, esta técnica se ha utilizado para el estudio de las poblaciones microbianas asociadas al kéfir, (Dobson y col., 2011; Leite y col., 2012), a quesos daneses (Masoud y col., 2011), a la Mozzarella (Ercolini y col. 2012) a queso de tipo latino (Lusk y col. 2012) y los microorganismos presentes en quesos irlandeses tradicionales (Quigley y col., 2012). La técnica se ha aplicado también en la caracterización microbiológica del queso Oscypek que se relaciona en esta memoria (Primera parte, Artículo III).

5. Genómica de microorganismos de interés tecnológico en productos lácteos

Otra herramienta clave para el estudio de la genética, fisiología y metabolismo de las BAL consiste en la secuenciación completa de sus genomas. Esta área de conocimiento ha experimentado un rápido crecimiento en las dos últimas décadas. La primera bacteria láctica cuyo genoma se secuenció fue *Lc. lactis* subsp. *lactis* IL1403 en el año 1999 (Bolotin y col.,1999). Hoy en día, la base de datos más actualizada (GOLD genome online database; http://www.genomesonline.org) cuenta con 87 genomas completos de especies y cepas de BAL (9 *Lactococcus*, 39 *Lactobacillus*, 6 *Leuconostoc*, 5 *S. thermophilus*, 2 *Oenococcus*, 2 *Pediococcus*, 1 *Weissella* y 23 *Bifidobacterium*) y más de 300 genomas en proceso de secuenciación.

El genoma típico de una BAL es relativamente pequeño (entre 1,7 Mb en el caso de *S. thermophilus* y 3,9 Mb en el caso de *Lactobacillus pentosus*), con capacidad para codificar, por tanto, un número de genes limitado (desde unos 1.600 hasta unos pocos más de 3.000). Estos genomas tan reducidos contienen, sin embargo, una gran variedad de secuencias que codifican transportadores para la asimilación de las diversas fuentes de carbono y nitrógeno propias de los nichos que las BAL ocupan (Mayo y col., 2008). Al mismo tiempo, presentan una escasez de genes implicados en procesos biosintéticos y catabólicos. La amplia variación en el número de genes que presentan, incluso cepas de una misma especie, sugiere que el genoma de las BAL es plástico y dinámico (Mayo y col., 2008). Se cree que la diversificación genómica de las BAL a partir de sus ancestros se ha producido por la pérdida de genes mediante procesos de mutación y deleción y por la adquisición de otros genes

(mediante procesos de transferencia génica horizontal) que resultan ser clave para la adaptación a los nichos ecológicos que habitan (Price y col., 2011; Siezen y col., 2011).

La disponibilidad de los genomas de diversas cepas de BAL ha mejorado el conocimiento en detalle de muchas rutas metabólicas implicadas en las fermentaciones industriales y otras de gran importancia para la aplicación de las BAL como probióticos. En la industria alimentaria este conocimiento permitirá una mejor explotación de la capacidad fermentativa de las BAL (Liu y col., 2008; Branco dos Santos y col., 2013) y posibilitará la selección de los candidatos más adecuados para cada aplicación. El conocimiento detallado de los genomas facilitará también su manipulación genética, lo que resulta de mucho interés para la utilización de las BAL como factorías celulares. Por su carácter GRAS las BAL se contemplan como sistemas muy adecuados en aplicaciones biotecnológicas como la expresión de proteínas heterólogas, la síntesis de aditivos de grado alimentario y de compuestos beneficiosos para la salud (Hugenholtz y col., 2002; Hanniffy y col., 2004; Stanton y col., 2005). Además, se estudia activamente su empleo como vehículos para el suministro de vacunas orales (Wells y Mercenier, 2003; Wells, 2011).

Objetivos

Objetivos

Tradicionalmente los procesos fermentativos se realizaban de forma empírica, siendo los microorganismos presentes en las materias primas o en el entorno de las elaboraciones (herramientas, aditivos, etc.) los responsables de la fermentación. Sin embargo, solo un pequeño número de cepas de estas especies poseen las propiedades requeridas por la industria, incluyendo rápido crecimiento en leche y en los medios de propagación industriales, producción de aromas deseables y resistencia al ataque de los bacteriófagos. Por estos motivos, existe una demanda continua de nuevas cepas con mejores características que puedan complementar o sustituir a las cepas comerciales que están actualmente en uso. También son necesarias nuevas cepas o cepas con nuevas propiedades para emprender fermentaciones novedosas.

Para cubrir estas demandas, los productos tradicionales elaborados con leche cruda sin la adición de fermentos conforman un enorme reservorio de nuevas cepas. Nuestra hipótesis de trabajo plantea que los productos lácteos fermentados contienen especies de BAL y otros tipos microbianos de interés para la industria. Los microorganismos que controlan las fermentaciones tradicionales dependen de la materia prima y de la selección que imponga los procesos tecnológicos de cada producto. Por tanto, para la identificación de especies y/o cepas nuevas es necesario caracterizar productos lácteos tradicionales y en particular los que presentan tecnologías originales de elaboración. Por otra parte, la utilización de técnicas microbiológicas novedosas, como las independientes de cultivo, pueden detectar

tipos distintos a los identificados hasta este momento que pueden requerir un aislamiento selectivo específico. En todo caso, las cepas seleccionadas deben de caracterizarse en profundidad y ensayarse, finalmente, en las condiciones de elaboración para asegurar la idoneidad de los tipos en la mejora del sabor, aroma, propiedades nutritivas y/o de conservación de los productos.

El objetivo final del trabajo se ha perseguido mediante la consecución de los siguientes objetivos parciales:

1.- Caracterización de productos lácteos tradicionales

1.1.- Evaluación y comparación de las técnicas de cultivo y las técnicas cultivo independientes para la caracterización microbiológica de productos lácteos.

1.2.- Estudio de la composición y evolución de la microbiota implicada en la elaboración y maduración del queso Casín tradicional, elaborado en nuestra región con leche cruda sin adición de fermentos.

1.3.- Caracterización de la microbiota implicada en la elaboración y maduración del queso tradicional polaco Oscypek, elaborado en la región de los montes Tatra en Polonia con mezcla de leche cruda de oveja y vaca y sin adición de fermentos.

1.4.- Estudio de la microbiota de una leche fermentada natural de origen desconocido, cuya producción se ha extendido por el noroeste de la Península y que se elabora mediante la adición a la leche de una alícuota de la leche fermentada precedente.

2.- Caracterización de microorganismos de interés tecnológico

2.1.- Análisis fenotípico y genotípico de cepas de la especie *Lactococcus lactis* aisladas de quesos tradicionales.

2.2.- Estudio de la actividad antimicrobiana de cepas salvajes de *Lc. lactis* contra microorganismos patógenos y alterantes.

2.3.- Identificación, tipificación y caracterización de cepas de *Leuconostoc* spp. para su empleo como cultivos adjuntos.

3.- Diseño y ensayo de fermentos diseñados con cepas de BAL de los productos lácteos tradicionales.

3.1.- Diseño de mezclas y evaluación de sus propiedades tecnológicas más importantes.

3.2.- Ensayo experimental de mezclas en elaboraciones piloto de queso.

Objectives

Objectives

Traditionally the processes of milk fermentation were carried out empirically, through the development and action of indigenous microorganisms present in the microbiota associated to the raw materials or others coming from the environment of processing, which includes tools and additives. Nowadays, dairy fermentations are taken over by starters, carefully selected microorganisms added in high numbers to milk in order to control manufacturing and/or ripening stages, or adjunct cultures, microorganisms with a major texture and/or aroma contribution to the final product. However, only a small number of strains possess the required properties that large-scale industrial processes demand, including insensitivity or resistance against the attack of bacteriophages. Therefore, there exists a recognized continuing demand for new strains or strains with improved functionality to complement or replace currently-in-use commercial strains.

In this sense, traditional dairy products made from raw milk without commercial starters have been found to constitute a large phenotypic and genetic reservoir, from which new starter strains could be selected. Beyond replacing starter components, traditional products may further contain strains with novel properties to enhance the overall quality of industrial and traditional milk fermentations and/or to undertake new dairy fermentations. Identification and selection of appropriate starter and adjunct cultures requires the microbial typing of traditional dairy products, the isolation, identification and characterization of large numbers of LAB

strains, and they assay of selected strains in pilot scale manufacture to assure they meet all required properties to be used in dairy. The use of such strains aims the manufacture of dairy products with improved aroma and taste, while increasing their safety and preservation abilities.

The final objective of this work was pursued by the following partial subobjetives.

1.- Characterization of traditional products

1.1.- Evaluation and comparison of culture-dependent and culture-independent methods for the microbial characterization of dairy products.

1.2.- Study of the composition and evolution of the microbiota involved in manufacturing and ripening of the traditional Casín PDO cheese, which is produced in Asturian Principality, Northern Spain, from raw milk without added starters.

1.3.- Microbial description the microbiota involved in manufacturing and ripening of the traditional Oscypek PDO cheese, produced in the Tatra Mountains region of Poland.

1.4.- Microbial typing of a homemade, natural fermented milk from undetermined origin but well spread in the North-West of Spain.

2.- Characterization of microbial populations with technological interest

2.1.- Detailed phenotypic and genetic analysis of *Lactococcus lactis* strains from traditional cheeses.

2.2.- Study of the antimicrobial activity of wild *Lc. lactis* strains against pathogens and spoiling microorganisms.

2.3.- Characterization of *Leuconostoc* spp. strains and evaluation of their suitability as adjunct cultures for dairy.

3.- Design and assay of starter mixtures based on wild LAB strains

3.1.- *In vitro* analysis of starter mixtures for technologically-important properties.

3.2.- Evaluation of the mixtures as cheese starters in pilot-scale, experimental trials.

Trabajo experimetal

Primera parte

PRIMERA PARTE: INTRODUCCIÓN A LAS TÉCNICAS

• Artículo I

Ángel Alegría • Renata González • Mario Díaz • y Baltasar Mayo
Assessment of Microbial Populations Dynamics in a Blue Cheese by Culturing and Denaturing Gradient Gel Electrophoresis
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El principal objetivo de este primer trabajo de la Memoria de Tesis Doctoral consistió en abordar de manera conjunta las técnicas de cultivo tradicionales y las técnicas moleculares; estas últimas se utilizaron abundantemente en los trabajos que se relacionan en capítulos posteriores. La utilización combinada de ambos tipos de técnicas posibilita una comparación más sencilla de los resultados y permite determinar sus ventajas e inconvenientes. Elegimos como modelo un queso azul semi-industrial, elaborado con leche pasteurizada y con la adición de fermentos. Este queso representa un sistema microbiano más sencillo que los asociados a quesos tradicionales elaborados con leche cruda.

El estudio de la composición y evolución microbianas en este queso modelo se llevó a cabo mediante la enumeración de los microorganismos revivificables totales en un medio de cultivo no selectivo (PCAM) y el recuento de diversas poblaciones indicadoras en medios selectivos y diferenciales. Como técnica independiente de cultivo se eligió la electroforesis en geles de gradiente desnaturalizante (DGGE); técnica que ya había sido utilizada con éxito en el grupo de investigación. La DGGE requiere el aislamiento y la purificación del ADN microbiano total de las muestras de queso como paso previo a la amplificación de los genes marcadores. En este trabajo se utilizaron dos parejas de cebadores universales que amplifican, respectivamente, la región variable V3 del gen que codifica el ARNr 16S de los procariotas y el dominio D1 del gen que codifica el ARNr 26S de los eucariotas.

Las técnicas de cultivo con medios selectivos y diferenciales permitieron hacer un seguimiento de nueve grupos microbianos distintos, incluyendo los microorganismos totales y poblaciones subdominantes indicadoras de salubridad como las enterobacterias y los estafilococos. Por su parte la DGGE permitió evaluar la composición y evolución de las poblaciones microbianas de una forma muy sencilla a lo largo de la maduración. Como componente mayoritario del fermento, éstas estaban compuestas por la especies *Lactococcus lactis*. Sin embargo, de forma sorprendente, se observó una población numerosa también de la especie *Streptococcus thermophilus*, bacteria que no estaba presente en el fermento y de cuya existencia no hubiéramos sospechado mediante cultivo.

Los resultados confirmaban que la combinación de técnicas de cultivo y técnicas moleculares como la DGGE, permite obtener un conocimiento más profundo de la diversidad microbiana y su evolución durante los procesos de elaboración y maduración del queso.

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Assessment of Microbial Populations Dynamics in a Blue Cheese by Culturing and Denaturing Gradient Gel Electrophoresis

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Abstract The composition and development of microbial population during the manufacture and ripening of two batches of a blue-veined cheese was examined by culturing and polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) (PCR–DGGE). Nine selective and/or differential media were used to track the cultivable populations of total and indicator microbial groups. For PCR–DGGE, the V3 hyper variable region of the bacterial 16S rRNA gene and the eukaryotic D1 domain of 28S rDNA were amplified with universal primers, specific for prokaryotes and eukaryotes, respectively. Similarities and differences between the results obtained by the culturing and the molecular method were recorded for some populations. Culturing analysis allows minority microbial groups (coliforms, staphylococci) to be monitored, although in this study PCR–DGGE identified a population of *Streptococcus thermophilus* that went undetected by culturing. These results show that the characterization of the microbial populations interacting and evolving during the cheese-making process is improved by combining culturing and molecular methods.

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Introduction

The microbial characterization of dairy products has traditionally been performed using culture-dependent techniques employing non-selective, selective, and differential media; this allows the composition and development of majority and indicator populations present during manufacturing and ripening to be monitored. However, a vast array of culture-independent molecular methods is now being used for the microbial typing of food and food fermentations [10, 11]. Molecular methods overcome culture-associated drawbacks such as the low specificity of selective media and the inability to enumerate cells in a viable but non-cultivable state. Most molecular methods rely on the amplification of rDNA or rRNA sequences by the polymerase chain reaction (PCR) and the subsequent electrophoretic analysis of the amplicons produced [1]. In addition, they are less laborious, faster and cheaper than culture-based analyses. However, these techniques are not free from bias nor without limitations [12, 18] and are generally thought of as complementary to conventional methods, providing a more precise microbial picture of food ecosystems.

Denaturing gradient gel electrophoresis (DGGE) and its relative, temperature gradient gel electrophoresis (TGGE), were developed to analyze microbial communities based on sequence-specific distinctions of 16S rRNA amplicons produced by PCR [14]. Separation is based on the reduced electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels with a linear gradient of denaturing agents (urea and formamide) or temperature. A GC clamp of around 50 bp is attached to the 5′ end of one of the primers, preventing the two DNA strands from undergoing complete disassociation. If the total DNA of a microbial community is used in PCR amplification these techniques can provide the profile of

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the genetic diversity of the dominant populations. If total RNA is used instead, the profiles reveal the metabolically active populations [15]. Both PCR–DGGE and PCR–TGGE have recently been used to study different microbial aspects of food-related environments [5], including the diversity and dynamics of microorganisms present during cheese manufacture and ripening [4–8, 16].

This article reports the use of plate count methods and PCR–DGGE analysis for studying microbial development, diversity, and evolution during the manufacture and ripening of a blue cheese made from pasteurized milk inoculated with commercial starters (mesophilic lactic acid bacteria as acidifiers and *Penicillium roqueforti* spores as the ripening culture). The aim of this study was to compare the results obtained with the culture-dependent and culture-independent methods, assessing the advantages and drawbacks of each.

Materials and Methods

Cheese Manufacture and Sampling Conditions

Two batches of blue-veined cheese were manufactured from pasteurized milk under standard conditions [9]. Acidifying starters (Flora Danica) and ripening cultures (*Penicillium roqueforti* strains PR3 and PRG3) were purchased from Chr. Hansen (Hørsholm, Denmark). Milk, curd, and cheese were sampled according to FIL-IDF standard 50 B and transported to the laboratory under refrigerated conditions. Culturing analyses were performed on the day of sampling. For the isolation of DNA, milk, curd, and cheese samples were stored at −20°C until required.

Determination of Microbial Composition by Plate Counting

Ten milliliters of milk or 10 g of curd and cheese were homogenized with 90 ml of a 2% (w/v) sodium citrate solution at 45°C in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3 × 1 min). Tenfold serial dilutions were made in Maximum Recovery Diluent (Scharlab, Barcelona, Spain) and plated in duplicate onto general and selective media, as follows.

Aerobic mesophilic counts: These were counted on plate count agar supplemented with 0.1% skimmed milk (PCA; Merck, Darmstadt, Germany) after 72 h of incubation in aerobiosis at 30°C.

Lactococci: These were grown on M17 agar (Scharlab) and enumerated after 48 h of incubation at 30°C.

Lactobacilli: These were grown on de Man, Rogosa and Sharpe agar (MRS; Merck), adjusted to pH 5.4 and enumerated after 72 h of incubation at 32°C in a 5% CO_2-enriched incubator.

Leuconostoc spp.: Dextran-producing leuconostocs were grown on Mayeux, Sandine, and Elliker agar (MSE; Biokar Diagnostics, Beauvais, France) and enumerated after 5 days of incubation at 25°C.

Enterococci: These were grown on Slanetz and Bartley agar (S–B; Merck) and enumerated after 24 h of incubation at 44°C.

Enterobacteria and coliforms: These were grown on violet red bile glucose agar (VRBG) and violet red bile lactose agar (VRBL) (both from Merck), respectively, using the pour-plate and overlay technique. In brief, dilutions were mixed with 15 ml of agar and poured onto Petri dishes. After solidification, a second agar layer of 10 ml was added. Bacteria were enumerated after 48 h of incubation at 30°C.

Staphylococci: Dilutions were grown on Baird–Parker agar (B–P; Merck) supplemented with egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearing were recorded after 24 h of incubation at 37°C.

Yeasts and moulds: Dilutions of milk, curd, and cheese samples were plated on yeast-extract glucose chloramphenicol agar (YGC; Merck). Yeasts and moulds were independently recorded after 3–5 days of incubation at 25°C.

Except for the YGC plates, 100 µg/ml of cycloheximide (Merck) was added to all enumeration media to inhibit the growth of moulds and yeasts.

PCR–DGGE Analysis

Extraction of Total Microbial DNA

Milk, cheese, and starter samples homogenized in 2% sodium citrate were used for the isolation of total microbial DNA. DNA extraction was accomplished by using a commercial kit (QIAamp DNA Stool Mini Kit; Quiagen, GmbH, Hilden, Germany), following the supplier's recommendations.

PCR Amplification

Total DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S rRNA gene using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′), to which a 39 bp GC sequence was linked to give rise to GC-F357, and R518 (5′-ATTACCGCGGCTGCTGG-3′) [15]. The D1 domain of the 28S rRNA fungal gene was amplified using primers GC-NL1 (5′-GCCATATCAA TAAGCGGAGGAAAAG-3′) and LS2 (5′-ATTCCCAAA CAACTCGACTC-3′) [3]. PCR was performed in a 50 µl volume using a Taq-DNA polymerase master mix (75 mM Tris–HCl pH 8.5, 20 mM $(NH_4)_2S0_4$, 1.5 mM $MgCl_2$, 0.1% Tween 20®, 0.2 mM of each dNTP, and 1.25 units Taq polymerase (Ampliqon ApS, Skovlunde, Denmark), with 100 ng of extracted DNA and 0.2 mM of each primer.

The amplification conditions for prokaryotic and eukaryotic sequences were those described by Muyzer et al. [15] and Cocolin et al. [3], respectively.

Electrophoresis Conditions

Denaturing gradient gel electrophoresis was performed using a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60°C, employing 8% polyacrylamide gels with a denaturing range 40–60% for bacteria and 30–50% for fungi. Electrophoresis was performed at 75 V for 17 h and at 130 V for 4.5 h for bacterial and fungal amplifications, respectively. Bands were visualized by staining with 0.5 μg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA).

Identification of PCR–DGGE Bands

DNA bands in the polyacrylamide gels were assigned to species either by comparison with a control ladder of known strains [8] or by sequencing and comparison of the sequences after isolation of DNA from the bands and re-amplification with the same primers without the GC-clamps. Sequences with a percentage identity of 97% or greater were considered to belong to the same species [17].

Results and Discussion

Microbial Counts Using Conventional Plate Count Techniques

Tables 1, and 2 show the enumeration results for total and indicator populations in the two batches over manufacturing and ripening. As expected for a cheese made from pasteurized milk, *Enterobacteriaceae*, coliforms, enterococci, and staphylococci counts were always in low numbers, though small variations between batches were recorded. No *Staphylococcus aureus* strains were detected. Lactococci were found in the largest numbers; in M17 the numbers recorded usually matched those of total bacterial aerobic counts in PCA, reaching over 10^9 colony forming units (CFU)/g between days 3 and 7 (Tables 1, 2). Lactobacilli grew from low numbers in milk up to 10^8 CFU/g by day 7, although their final numbers were one log unit lower than those of lactococci. Both lactococci and lactobacilli populations showed a slight decline from day 15 onward. The yeast and mould populations increased throughout ripening, approaching, or surpassing (depending on the batch) 10^7 CFU/g of cheese. Owing to their different morphology on the YGC plates, yeasts and moulds could be recorded separately (Table 1). The two *P. roqueforti* strains were distinguishable from one another owing to their different colored mycelia (PR3 dark green, PRG3 pale gray). Yeasts started at similar numbers in both batches; however, counts for batch 1 increased over those for batch 2 (maximum 6.1×10^7 and 5.75×10^5 CFU/g in batches 1 and 2, respectively).

Variability in the cultivable microbial populations of dairy products is well known, even when they are made from pasteurized milk [2, 20]. Different initial microbial loads (in numbers and types) and post-pasteurization contamination may be responsible for the majority of the differences observed in most studies.

Microbial Composition and Dynamics As Revealed By PCR–DGGE

Figures 1, and 2 show the composition and dynamics of the prokaryotic and eukaryotic populations in batches 1 and 2

Table 1 Bacterial counts in log10 CFU ml or g determined in milk and cheese samples of batch 1 during cheese making and ripening

Microbial group (counting medium)	Stage of manufacture or ripening						
	Milk	Curd	3 day	7 day	15 day	30 day	60 day
Total aerobic counts (PCA)	5.01	8.53	9.13	9.28	8.54	8.41	8.58
Lactococci (M17)	5.80	8.41	9.11	9.20	8.58	8.37	8.57
Lactobacilli (MRS, pH 5.4)	3.03	7.26	8.37	8.49	8.18	7.74	7.48
Leuconostoc (MSE)	<1	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00
Enterobacteriaceae (VRBG)	<1	3.88	3.89	2.40	<2.00	2.00	2.00
Coliforms (VRBL)	<1	4.13	3.90	2.30	<2.00	2.00	2.00
Enterococci (S–B)	<1	<2.00	<2.00	<2.00	<2.00	<2.00	5.67
Staphylococci (B–P)	1.48	2.18	2.74	2.00	<2.00	<2.00	2.30
Yeasts (YGC)	<1	2.00	<3.00	8.66	5.94	7.79	7.72
P. roqueforti PR3 (YGC)	<1	3.74	3.83	<4.00	<3.00	<4.00	4.30
P. roqueforti PRG3 (YGC)	<1	4.08	4.26	7.64	6.88	6.64	5.81

The symbol < is used to indicate that numbers were lower than the detection limit

Table 2 Bacterial counts in log10 CFU ml or g determined in milk and cheese samples of batch 2 during cheese making and ripening

Microbial group (counting medium)	Stage of manufacture or ripening						
	Milk	Curd	3 day	7 day	15 day	30 day	60 day
Total aerobic counts (PCA)	4.60	7.46	9.13	9.25	9.09	8.95	8.60
Lactococci (M17)	3.91	7.75	9.35	9.35	9.36	9.02	8.48
Lactobacilli (MRS, pH 5.4)	<1	6.90	8.39	8.39	7.71	8.43	8.40
Leuconostoc (MSE)	<1	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00
Enterobacteriaceae (VRBG)	<1	2.30	2.60	<2.00	<2.00	<2.00	<2.00
Coliforms (VRBL)	<1	2.30	2.78	<2.00	<2.00	<2.00	<2.00
Enterococci (S–B)	<1	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Staphylococci (B–P)	<1	2.48	<2.00	2.00	<2.00	2.00	<2.00
Yeasts (YGC)	<1	2.00	<3.00	<3.00	5.76	5.00	<5.00
P. roqueforti PR3 (YGC)	<1	3.65	3.70	3.60	4.95	<5.00	<5.00
P. roqueforti PRG3 (YGC)	<1	3.93	4.18	4.11	6.74	6.96	6.53

The symbol < is used to indicate that numbers were lower than the detection limit

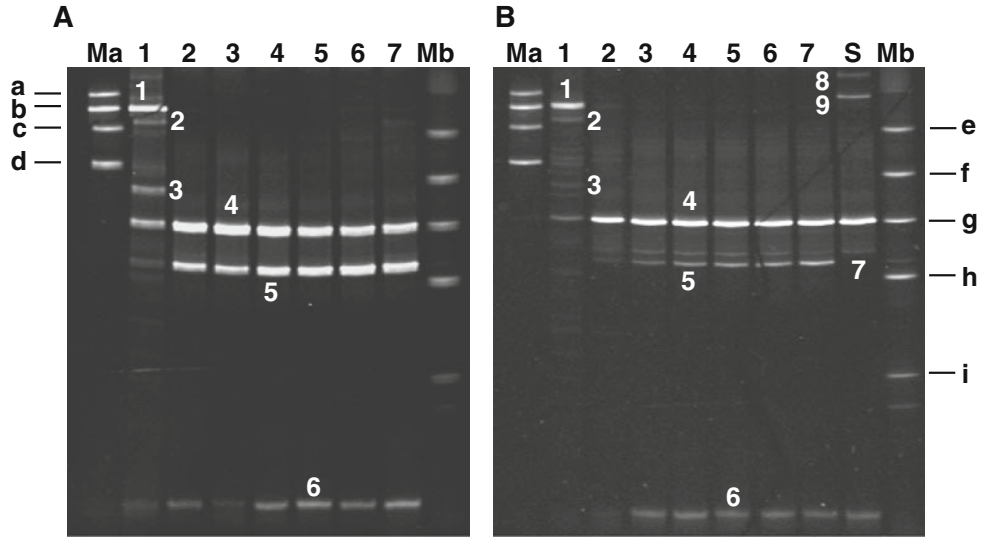

Fig. 1 DGGE profiles of microbial populations from cheese during manufacture and ripening, using amplicons of the V3 variable region of the bacterial 16S rRNA gene. *Lanes Ma and Mb* contain combined amplicons of identified strains used as controls: *a Lactococcus garvieae*, *b Lactobacillus plantarum*, *c Leuconostoc mesenteroides*, *d Streptococcus parauberis*, *e Enterococcus faecium*, *f Enterococcus faecalis*, *g Lactococcus lactis*, *h Escherichia coli*, *i Lactobacillus paracasei*. **A** contains samples from the first batch. Samples: *1* milk, *2* curd, *3*, *4*, *5*, *6*, and *7* cheese at 3, 7, 15, 30, and 60 days of ripening. **B** contains samples from the second batch (same order). *Lane S* shows the DGGE analysis of a sample of the Flora Danica starter. Key to identified bands: *1 L. plantarum*, *2 Weissella cibaria*, *3 Actinobacterium* spp., *4 L. lactis*, *5* and *6 S. thermophilus*, *7 L. lactis*, *8* and *9 L. lactis*-related sequences

determined by PCR–DGGE. Panel A in both figures corresponds to the DGGE patterns of batch 1, panel B to those of batch 2. The profiles of the cheese batches were relatively simple compared to the complex DGGE patterns of cheeses made of raw milk [4, 8, 16]. More bands were seen in the two milk samples, though most of them proved to be very faint. The intensity of an individual band is assumed to be a semi-quantitative measure of the abundance of the corresponding microorganism in the original population [15]. In addition to bands for *L. lactis* (band 4) and *Lactobacillus plantarum* (band 1), bands corresponding to *Weisella cibaria* (band 2) and *Actinobacterium* spp. (band 3) were found in the two milk samples (lane 1 in Figs. 1A, B). As for the cheeses, three and four different bacterial bands were observed in batches 1 and 2, respectively. *L. lactis* was the most prominent band (band 4) in both. In the cheese samples, bands of lactococci were always accompanied by two bands identified as *Streptococcus thermophilus* (bands 5 and 6). In addition, in batch 2 another band located between bands 4 and 5 (band 7) was observed throughout manufacture and cheese ripening. This band was also present in the PCR–DGGE profile of

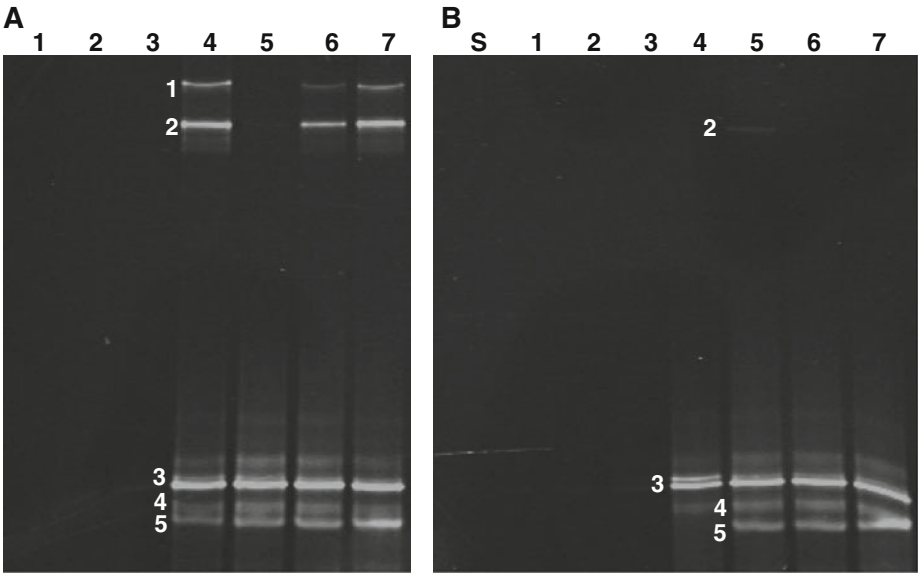

Fig. 2 DGGE profiles of moulds and yeasts using a PCR amplicon of the eukaryotic domain D1 of 26S rDNA. **A** contains samples from the first batch. Samples: *1* milk, *2* curd. *3*, *4*, *5*, *6* and *7* cheese at 3, 7, 15, 30 and 60 days of ripening. **B** contains samples from the second batch. Sample: *1* milk, *2* curd, 3, 4, 5, 6, and 7 cheese at 3, 7, 15, 30, and 60 days of ripening. *Lane S* corresponds to the Flora Danica starter. Key to identified bands: *1* and *2 Debaryomyces hansenii*, *3*, *4*, and *5 Penicillium roqueforti*

the Flora Danica starter (lane S in Fig. 1B). The sequence of this band was identical to that of *L. lactis* (band g). The DGGE pattern of Flora Danica produced two additional bands in the uppermost part of the gel (bands 8 and 9), the DNA sequence of which showed around 98% similarity to *L. lactis* sequences. The presence of double bands corresponding to a single species may be due to heterogeneous copies of rRNA operons, a well-established limiting factor of the PCR–DGGE technique, but also to other artifacts related to the melting and re-association properties of related sequences [12, 18].

Figure 2 shows the DGGE profiles obtained with the primers for amplifying eukaryotic sequences. Five different bands were observed among the samples of the two batches. All were identified by isolation, re-amplification, sequencing and comparison against sequences in databases. The sequences of two bands (bands 1 and 2) were identical to those known for *Debaryomyces hansenii*; these were present in all samples of batch 1 after day 7 (except on day 15; lane 5 in Fig. 2A), while only a faint band was observed in the 15 day sample in batch 2. In the lower part of the gels, three patent bands appeared in samples of both batches from day 7 onwards (bands 3–5). The sequence of these bands matched those of *P. roqueforti*. As expected, PCR–DGGE analysis of Flora Danica with the eukaryotic primers gave no bands (lane S in Fig. 2B).

The bands of both the prokaryotic and eukaryotic populations remained the same (qualitatively and quantitatively) during the entire cheese-making process. Of note is the absence in cheese of bands corresponding to bacteria from milk (except for that of *L. lactis*, a bacterium also present in the starter). Microorganisms in the pasteurized milk may be in a viable but non-cultivable state, and then do not progress into the cheese, or if they grow only small numbers are reached as compared to those obtained after the addition of starters. Although not declared, the *S. thermophilus* population detected may have come from the Flora Danica; as at least one of the two bands observed in the cheese samples was also detected for this undefined starter culture (the lowest band in lane S in Fig. 1B).

Comparison of Plate Count and PCR–DGGE Results

The results obtained by culturing and DGGE showed similarities as well as patent differences. The heterogeneous distribution of microorganisms within the samples is a widely known cause of variation between and among culturing and DGGE results. The molecular method proved to be faster and cheaper in terms of running costs. Further, the analysis of the diversity and development of the microbial populations in the two batches over manufacturing and ripening (14 samples) was performed in a couple of weeks instead of the 4 months needed to finish conventional culturing analysis.

Intriguing is the differential results obtained for the lactobacilli with the two techniques. By conventional culturing, these were shown to form part of the majority populations, reaching numbers similar to those of lactococci (as in batch 2, day 60). However, although bands related to lactobacilli were detected in milk, they were never detected in cheese. Somehow, lactobacilli seem to be under-represented by the PCR–DGGE technique. Lactobacilli are more resistant to lysozyme than other lactic acid bacteria species (and particularly the lactococci), which may account for the differences. Species-specific primers, such as those developed by Walter et al. [19], should be used for accurate tracking of lactobacilli.

In addition, the unexpected population of *S. thermophilus* was not detected by culturing; this bacterium is not declared by Chr. Hansen as a component of Flora Danica, and was not isolated from it by Lodics and Steenson [13]. This technique is, therefore, preferable for a rapid inspection of the diversity and development of the dominant populations during the microbial typing of new products or processes. Once detected, culturing methods can be used for the isolation, identification, and typing of representative organisms. The use of selective and differential media allowed the tracking of multiple microbial populations (nine microbial groups), while only majority species could be followed by the PCR–DGGE technique (3 and 4 bands in batches 1 and 2, respectively). This is particularly important for populations used as hygiene indicators (*S. aureus*, coliforms), which, because of their smaller numbers, might only be detected by selective culturing. In addition, the different colony morphology shown by the two *P. roqueforti* strains allowed their individual enumeration throughout ripening; this would not be possible with the PCR–DGGE technique. On the other hand, counts cannot be directly ascribed to a particular species, while this is possible with DGGE bands.

Conclusion

Classic culturing and molecular methods have been repeatedly reported to provide complementary results; this affirmation is strengthened by the results of this study. Both plate counting and PCR–DGGE analyses can be used for identifying and tracking majority populations throughout cheese manufacture and ripening, and both have their own advantages and drawbacks. They are both easy to perform, but the PCR–DGGE technique still requires dedicated equipment and reagents (thermocycler, nucleotides, polymerase, DGGE apparatus, etc.). Thus, the choice of using one or another ultimately depends on the purpose of the study if all necessary materials and instruments are available. However, the combination of the two undoubtedly improves the microbial characterization of the cheese-making process.

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SEGUNDA PARTE: CARACTERIZACIÓN MICROBIOLÓGICA DE PRODUCTOS LÁCTEOS TRADICIONALES

• Artículo II

Ángel Alegría, Pablo Álvarez-Martín, Noelia Sacristán, Elena Fernández, Susana Delgado y Baltasar Mayo.

Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk.

International Journal of Food Microbiology, 2009. Volumen 136, número 1, páginas 44-51.

• Artículo III

Ángel Alegría, Elena Fernández, Susana Delgado y Baltasar Mayo.

Microbial characterisation and stability of a farmhouse natural fermented milk from Spain

International Journal of Dairy Technology, 2010. Volumen 63, número 3, páginas 423-430.

• Artículo IV

Ángel Alegría, Pawel Szczesny, Baltasar Mayo, Jacek Bardowski y Magdalena Kowalczyk

Biodiversity in Oscypek, a traditional Polish Cheese, determined by culture-dependent and -independent approaches

Applied and Environmental Microbiology, 2012. Volumen 78, número 6, páginas 1890-1898.

Los productos lácteos tradicionales elaborados sin la adición de fermentos resultan una excelente fuente de bacterias lácticas destinadas al diseño de nuevos fermentos iniciadores y cultivos adjuntos. También son especialmente valiosos para la obtención de cepas autóctonas con las que diseñar fermentos específicos. Con el doble objetivo de conocer en profundidad la composición de la microbiota y su evolución a lo largo del tiempo en este tipo de productos, y de obtener aislados con potencial interés tecnológico, se caracterizaron microbiológicamente tres productos de estas características: el queso asturiano Casín, el queso polaco Oscypek y una leche fermentada natural. La elección de estos tres tipos de productos tuvo distintas motivaciones. En primer lugar, el queso Casín es sin duda uno de los quesos tradicionales asturianos más originales desde el punto de vista tecnológico (con un amasado semanal de la cuajada a lo largo de la maduración) y organoléptico (sabor picante producto de una lipolisis intensa). Dado que no existían datos de su microbiología, y dado que ésta ha de estar necesariamente influenciada por su particular tecnología de elaboración, decidimos abordar su estudio para colaborar en la descripción de este queso tan típico, que conseguía además la DOP en el año 2008. El estudio del queso polaco Oscypek se realizó durante una estancia formativa en Polonia con un equipo de investigación interesado en la caracterización microbiana del queso. El Oscypek es un queso muy poco industrializado, con una tecnología a medio camino entre los quesos tipo Gouda y los quesos suizos e italianos, que no había sido estudiado con anterioridad. Finalmente, en la leche fermentada natural nos llamó la atención la estabilidad microbiana que presentaba, puesto que, como se comenta en el artículo, la inoculación de la leche con parte del producto fermentado produjo en nuestras manos un producto agradable durante más de año y medio.

Como resultado más sorprendentemente del estudio microbiológico del queso Casín, se detectó la presencia dominante de la especie *Lactococcus garvieae* durante la etapa de fermentación. En trabajos posteriores que no se recogen en esta memoria, las cepas de esta especie se caracterizaron en profundidad y se compararon con cepas de *Lactococcus lactis,* y. una de las cepas se sometió a secuenciación genómica completa con el objetivo de conocer su inocuidad y sus

características tecnológicas. *Lc. garvieae* era reemplazada por cepas de *Lc. lactis* subsp. *lactis* hacia el final de la maduración. Adicionalmente, la DGGE detectó de nuevo la presencia de ADN perteneciente a la especie *Streptococcus thermophilus* durante todo el proceso de maduración. Esta especie nunca había sido identificada en quesos tradicionales españoles.

El estudio de la diversidad y evolución de las poblaciones del queso Oscypek se llevó a cabo mediante las técnicas ya comentadas, complementadas por una metagenómica filogenética utilizando la secuenciación de amplicones del gen que codifica el ARNr 16 por pirosecuenciación. Este trabajo ha sido uno de los pioneros en aplicar esta última técnica al estudio microbiológico de quesos tradicionales. Como era esperable, diversos géneros y especies de bacterias ácido-lácticas fueron identificados por las tres técnicas utilizadas. Mediante la pirosecuenciación, técnica de secuenciación masiva muy poderosa para detectar componentes microbianos minoritarios de los ecosistemas, se identificaron secuencias correspondientes a varias especies de bifidobacterias; éstas se detectaban por vez primera en un queso. Queda por determinar, para el futuro, si estos microorganismos se encuentran viables en el queso y si tienen algún papel relevante en sus características organolépticas diferenciales.

Finalmente, la leche fermentada natural, mostró una microbiota simple, tanto mediante técnicas de cultivo como por DGGE. Ambas técnicas mostraban que la población microbiana mayoritaria estaba compuesta de forma exclusiva por una cepa de *Lc. lactis* subsp. *lactis* y otra *Lc. lactis* subsp. *cremoris*. En menor concentración (unas tres unidades logarítmicas por debajo) apareció también una única cepa de la especie *Lactobacillus plantarum*. Estas tres bacterias son las responsables de las buenas cualidades organolépticas de la leche fermentada y parecen formar una asociación microbiana muy estable, por lo que podría ser utilizada en su conjunto como cultivo iniciador para la elaboración de esta leche u otros productos lácteos fermentados.

Segunda parte

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Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk

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ABSTRACT

Classical culturing and denaturing gradient gel electrophoresis (DGGE) techniques have been used for studying the microbial diversity and dynamics of the traditional Spanish Casín cheese during manufacturing and ripening. As with other starter-free cheeses made from raw milk, the microbial diversity of Casín was shown to be high by both culturing and DGGE analyses. The culture technique showed that lactic acid bacteria (LAB) species constituted the majority of the microbial populations. Of the 14 bacterial species identified, *Lactococcus garvieae* was predominant in the three-day-old cheese sample, although it was replaced by *Lactococcus lactis* subsp. *lactis* at day 30. As expected, the DGGE profiles obtained were complex, consisting, depending on the sample, in five to ten different amplification bands. Among these, a band corresponding to *Streptococcus thermophilus* was observed throughout the whole manufacturing process. This species had never been identified from traditional Spanish cheeses previously. Culturing and molecular methods showed high populations of undesirable microorganisms, arguing for a required improvement in the hygiene of Casín manufacture. Random amplification of polymorphic DNA (RAPD) profiling suggested that the *L. garvieae* and *L. lactis* populations were composed of one and five strains, respectively. In addition, only a single *L. lactis* RAPD pattern was stably maintained from day three to day 30, indicating high succession of strains along ripening. After a thoroughly characterisation, strains of the two *Lactococcus* species could be used in designing specific starter cultures for Casín. Additional species (such as *Lactobacillus plantarum* and *Corynebacterium variabile*) might be included as adjunct cultures.

1. Introduction

Among the large list of Asturian Principality (Northern Spain) traditional cheeses, Casín, which wears a Protected Designation of Origin (PDO) label as of May 2008, is probably the one with more originality and typicity. Documents referring to this type of cheese date back as far as the XIII century, suggesting that it is amongst the oldest traditional cheeses in Spain. It is manufactured without a starter culture from raw cow's milk of the Casina breed (a kind of Scottish Highlander) in a small rural area surrounded by mountains. In such a secular isolated environment, manufacturers have maintained their traditional process through the ages. Fig. 1 shows a detailed diagram of the manufacturing process of the cheese. In short, Casín cheese is still made by a mixed enzymatic (mostly) and acid curling of evening and morning milk mixtures at 35 °C. The coagulum is then cut into hazelnut-like grains, which are allowed to drain in a cheese cloth for 2–3 days. Then, salting is carried out by applying coarse salt to the cheese surface. Typical of Casín manufacture is a weekly manual kneading, which is maintained up to the end of ripening (Fig. 1). Consequently, the cheese has no crust and its cylindrical or semi-spherical shape (12–15 cm diameter, 5–7 cm height) is formed by hand during the final kneading. At this point the upper surface is decorated for marketing by a wooden manufacturers' stamp.

As microbial studies on Casín have never been performed, the microbial typing of the cheese may serve the purpose of both evaluating its hygienic conditions and aiding in the design of specific starter and/or adjunct cultures. These starters are those respecting all technologically-relevant microorganisms found in traditional cheeses and their relative proportions (Parente and Cogan, 2004). The use of such cultures would insure to reproduce the fermentation in a reliable manner, while preserving to some extent the typical intense flavour of the traditional cheeses (Albenzio et al., 2001).

In addition, interest in the microbiota of raw milk cheeses and other traditional dairy products is further maintained by a recognised need for new LAB strains to complement or replace those currently in-use industrial strains (Hansen, 2002; Wouters et al., 2002). Traditional dairy products harbour a huge recognised reservoir of phenotypic and genetic microbial diversity, which may have many potential biotechnological applications (Wouters et al., 2002; Topisirovic et al., 2006; van Hylckama Vlieg et al., 2006). Traits of LAB species of particular interest to the dairy industry include: bacteriophage resistance (Madera et al., 2003), production of antimicrobial substances (de

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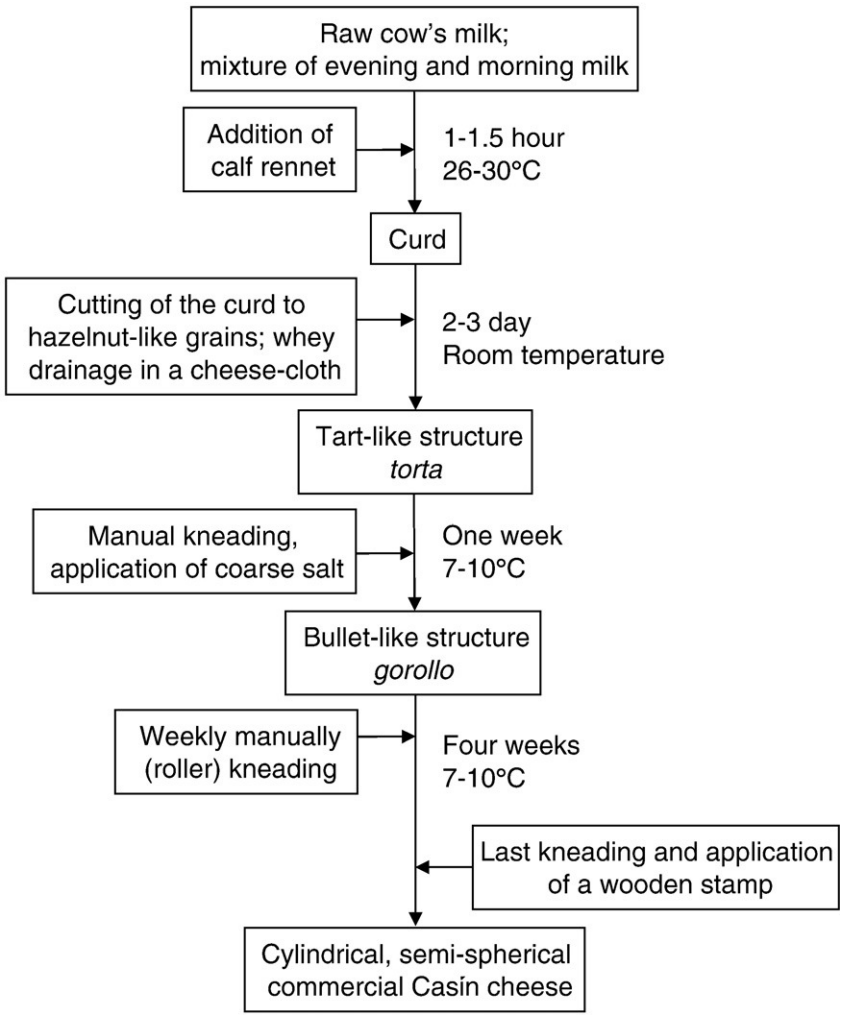

Fig. 1. Flow scheme of the manufacturing process of Casín cheese. Approximate duration of manufacturing steps and temperature through the process is indicated. Words in italics are local terms for the successive forms of the cheese during ripening.

Vuyst and Leroy, 2007) and unique flavour-forming potential (Ayad et al., 2001; Smit et al., 2005). At present, LAB strains are also analysed for their probiotic properties (Collado et al., 2007) and ability to form bioactive compounds (Siragusa et al., 2007; Guglielmetti et al., 2008). Strains with improved or new properties may be useful to fulfil the needs of traditional fermentations or be used for the formulation of new functional dairy products.

The microbial characterisation of dairy ecosystems is currently performed by using conventional culturing and culture-independent molecular techniques, as they both give complementary results (Giraffa and Neviani, 2001). Among the latter techniques, the denaturing gradient gel electrophoresis (DGGE) tracks compositional changes in the microbial communities via sequence-specific separation of PCR-amplified fragments (Muyzer et al., 1993). This technique has been used to characterise the microbial diversity in many dairy environments (Ercolini et al., 2001; Cocolin et al., 2002; Lafarge et al., 2004; Ogier et al., 2004). Moreover, it has also been used to follow the microbial population dynamics throughout manufacture and ripening of several traditional cheeses (Coppola et al., 2001; Randazzo et al., 2002; Ercolini et al., 2004; Flórez and Mayo, 2006).

The aim of the present study was to analyse the microbial diversity of major and indicator populations of traditional Casín cheese and their evolution through manufacturing and ripening by culturing and DGGE. For a complete microbial description of the cheese, predominant microbial species detected by the two techniques were further identified by molecular methods.

2. Material and methods

2.1. Sampling conditions

Two batches of Casín cheese were made by two independent and geographically separated manufacturers in June 2007. Milk, curd and cheese at 3, 7, 15 and 30 days of ripening were sampled according to FIL-IDF standard 50B and transported to the laboratory under refrigerated conditions.

2.2. Microbial counts

Ten gram samples of milk, curd and cheese were homogenised with 90 ml of a 2% (w/v) sodium citrate solution at 45 °C in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3 × 1 min). Serial 10-fold dilutions were made in Maximun Recovery Diluent (Scharlau, Barcelona, Spain) and plated in duplicate on to general and selective media.

2.2.1. Total aerobic mesophilic

Aerobic mesophilic bacteria were grown on Plate Count Milk Agar (PCMA; Merck, Darmstadt, Germany) and enumerated after 72 h of incubation at 30 °C. Counts of total aerobic mesophilic bacteria were also done on PCMA, Brucella Agar (BA, Merck) and Blood Agar (BLA, Merck) after 72 h of incubation in aerobiosis, microaerophilia, and anaerobiosis at 30 °C.

2.2.2. Lactococci

Lactococci were grown on M17 agar (Scharlau) and enumerated after 48 h of incubation at 32 °C.

2.2.3. Lactobacilli

Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRSA; Merck), adjusted to pH 5.4 and enumerated after 72 h of incubation at 32 °C in a Hera Cell 2400 (Thermo Fisher Scientific Inc., Waltham, Ma., USA).

2.2.4. Leuconostocs

Leuconostocs were grown on de Man, Sharpe and Elliker agar (MSEA; Biokar Diagnostics, Beauvais, France) containing 30 µg/mL vancomycin (Sigma) to inhibit lactococci and enterococci, and enumerated after five days of incubation at 25 °C.

2.2.5. Enterococci

Enterococci were grown on Slanetz and Bartley agar (S-BA; Merck) and enumerated after 24 h of incubation at 44 °C.

2.2.6. Enterobacteria and coliforms

Enterobacteria and coliforms were grown on Violet Red Bile Glucose agar (VRBGA) and Violet Red Bile Lactose agar (VRBLA) (both from Merck) respectively, using the pour-plate and overlay technique. In short, dilutions were mixed with 15 ml of agar and poured onto Petri dishes. After solidification, a second agar layer of 10 ml was added. Bacteria were enumerated after 24–48 h of incubation at 30 °C.

2.2.7. Staphylococci

Dilutions were grown on Baird–Parker agar (B–PA; Merck) supplemented with egg yolk tellurite solution (Merck) and black colonies with, or without, egg yolk clearing were recorded after 24 h of incubation at 37 °C.

2.2.8. Yeasts and moulds

Dilutions of milk, curd and cheese samples were plated on Yeast-Extract Glucose Chloramphenicol agar (YGCA; Merck) and yeasts and moulds were enumerated after 3–5 days of incubation at 25 °C.

2.3. Chemical analysis

Standard FIL-IDF methods were used to determine basic chemical parameters. FIL-IDF Standards 21B and 4A were followed for examining total solids in milk and cheese respectively. pH was measured according to FIL-FID Standard 104A, and the NaCl and protein content was measured according to FIL-FID Standards 88A and 20B respectively. The water activity (a_w) was measured in duplicate using an AquaLab apparatus (Decagon Devices Inc., Pullman, Wa., USA).

2.4. Molecular identification of lactic acid bacteria

One hundred and eighty colonies from the PCMA, BA and BLA agar plates were purified by subculturing on the same media and pure cultures were stored frozen at −80 °C until analysis. Cultures were recovered in the corresponding media and isolated colonies were suspended in milliQ water and heated for 10 min at 98 °C. After centrifugation for 10 min at 13,000×*g*, cell free extracts were used as a source of DNA template to amplify a segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique. The PCR primers used, 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), were based on conserved regions of the 16S rRNA gene. Amplicons were purified to remove unincorporated primers and nucleotides using Microcon PCR filters (Millipore, Bedford, Ma., USA) and sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, Ca., USA) with primer 27FYM. An average of 850 bp were obtained per sequence and compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and with those in the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Sequences with a percentage of identity of 97% or higher were allocated to the same species (Stackebrandt and Goebel, 1994; Palys et al., 1997).

2.5. Typing of Lactococcus spp. strains

A representative number of *Lactococcus lactis* (45) and *Lactococcus garvieae* (25) isolates were grouped by RAPD analysis using primer BoxA2R (5′-ACGTGGTTTGAAGAGATTTTCG-3′), as reported by Koeuth et al. (1995). Total genomic DNA was prepared by using a commercial kit (GenElute™ Bacterial GenomiC DNA; Sigma Chemical Co., St. Louis, Miss., USA). The similarity of the patterns was expressed by the Spearman moment correlation coefficient. Clustering was performed by the unweighted pair group method using arithmetic averages (UPGMA).

2.6. DGGE analysis

2.6.1. Extraction of DNA from cheese samples

Homogenised milk, curd and cheese samples in 2% sodium citrate were used for isolation of total microbial DNA. DNA extraction was accomplished essentially as described by Ercolini et al. (2003) but with the following modification: cheese homogenates were treated with pronase (2.5 mg/ml) (Sigma) for 1 h at 37 °C before lysis of the cells.

2.6.2. PCR amplification

DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S rRNA gene by using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′ to which a 39 bp GC sequence was linked to give rise to GC-F357) and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). The D1 domain of the 26S rRNA fungal gene was amplified by using the primers GC-NL1 (5′-GCCATATCAATAAGCG-GAGGAAAAG-3′) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) (Cocolin et al., 2002). PCR was performed in 50 µL volumes containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of the primers, 5 U of Taq-polymerase (Roche Diagnostics, Barcelona, Spain) and 100 ng of extracted DNA. Amplification conditions of prokaryotic and eukaryotic sequences were as described by Muyzer et al. (1993) and Cocolin et al. (2002), respectively.

2.6.3. Electrophoresis conditions

DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, Ca., USA) at 60 °C and 8% polyacrylamide gels with a denaturing range of 40–60% for bacteria and 30–50% for fungi. Electrophoresis was carried out at 75 V for 17 h and at 130 V for 4.5 h for bacterial and fungal amplifications, respectively. Bands were visualised after staining with 0.5 µg/ml ethidium bromide (Sigma).

2.6.4. Identification of DGGE bands

DNA bands in the polyacrylamide gels of the commonest species (*L. lactis, L. plantarum*) were assigned to species by comparison with a control ladder of known strains (Flórez and Mayo, 2006). All others were ascribed to species by sequencing and comparison of the sequences as detailed above, after isolation of DNA from the bands and reamplification with the same primers without the GC-clamps.

3. Results

3.1. Basic microbial and chemical parameters of Casín cheese

The basic microbial and chemical properties of two distinct batches of traditional Casín cheese made from raw milk by independent producers were analysed through manufacturing and ripening. Microbial and chemical values of the two batches were combined because we

Table 1
Average microbial counts (in Log_{10} cfu per g or ml) and standard deviation of diverse microbial groups along manufacturing and ripening stages of two independent batches of Casín cheese.

Microbial group (counting medium)	Stage of manufacturing or ripening					
	Milk	Curd	3 days	7 days	15 days	30 days
Total aerobic counts (PCA)	5.42 ± 0.42	6.42 ± 0.57	8.50 ± 0.11	9.06 ± 0.20	8.84 ± 0.38	8.65 ± 0.20
Lactococci (M17A)	5.01 ± 0.38	6.40 ± 0.57	8.58 ± 0.07	8.93 ± 0.20	8.74 ± 0.32	8.54 ± 0.19
Lactobacilli (MRSA, pH 5.4)	3.00 ± 0.25	3.54 ± 0.31	5.12 ± 0.13	6.19 ± 0.04	8.63 ± 0.11	8.80 ± 0.16
Leuconostoc (MSEA)	nd[a]	nd	3.02 ± 0.02	4.58 ± 0.11	6.44 ± 0.33	6.24 ± 0.52
Enterococci (S-BA)	3.20 ± 0.27	4.91 ± 0.57	4.23 ± 0.15	5.71 ± 0.17	6.56 ± 0.10	6.33 ± 0.21
Staphylococci (B-PA)	3.67 ± 0.52	5.27 ± 0.15	5.62 ± 0.10	6.48 ± 0.31	5.97 ± 0.11	6.07 ± 0.30
Enterobacteriaceae (VRBGA)	5.18 ± 0.19	6.12 ± 0.26	5.79 ± 0.14	6.01 ± 0.19	6.41 ± 0.2	6.52 ± 0.36
Coliforms (VRBLA)	3.73 ± 0.80	5.15 ± 1.20	5.54 ± 0.09	6.13 ± 0.10	6.36 ± 0.18	6.47 ± 0.47
Yeasts and moulds (YGCA)	nd	nd	3.38 ± 0.26[b]	6.13 ± 0.33	7.00 ± 0.12	6.79 ± 0.17

[a] nd, not detected; detection limit Log_{10} 2.0.
[b] These numbers correspond to yeasts, as moulds were never recorder (detection limit two Log_{10} lower than that of yeast counts).

were more interested in discovering canonical aspects of Casín manufacture than in finding differences. Table 1 shows the composition and evolution of the predominant and indicator populations, while chemical gross composition is summarised in Table 2. Surprisingly, counts of the different microbial populations were rather similar between batches (Table 1), as shown by the low standard deviations; coliforms being the most variable population. Counts of total aerobic bacteria reached the highest value at around day seven, as did counts of lactococci, with maximal populations of around 10^9 colony forming units (cfu) per g of cheese. Initial numbers of lactobacilli were around 10^3 cfu/g and attained their highest level at day 30 (near 10^9 cfu/g). Dextran-producing leuconostoc reached maximum numbers at day 15 (2.75×10^6 cfu/g). Numbers of hygienic-indicator populations were high throughout the whole process, reaching their highest levels (averaging 3.15×10^6 cfu/g) from day seven to day 30, depending on the population. Of note was the continued growth of coliforms (which matched the population of *Enterobacteriaceae* from day seven onwards) until the end of ripening. Although numbers of staphylococci were also high, strains of *Staphylococcus aureus* were never detected in the B–PA counting plates. The population of yeasts reached a maximum level at day 15 (10^7 cfu/g), while moulds were never detected (detection limit two Log_{10} units lower than the corresponding yeast counts). Regarding chemical parameters (Table 2), normal trends during ripening were observed for most variables. In agreement with the highest population of lactococci at day seven, pH was the lowest at this time-point, increasing slowly thereafter. As humidity decreases during ripening, so the level of salt in moisture increases. Although this, the final content of salt in moisture was relatively low (2.30% at day 30). The water activity (a_w) also decreases through ripening, but the microbial growth is not compromised even at its lowest level (0.96).

3.2. Microbial diversity and dynamics of Casín cheese by DGGE

Samples of curd and cheese at days 3, 7, 15 and 30 of ripening of the two batches were analysed by DGGE. In the ripened cheese, separated samples of the cheese interior and cheese surface (a rind of 0.3–0.5 cm) were examined. As an example, the results obtained for one of the batches are presented in Fig. 2. Between five and ten different bands corresponding to the prokaryotic V3 variable region of the 16S rRNA were observed in the distinct samples (Fig. 2A). In total, 14 different bands were encountered, of which 13 were identified by either comparison to bands from control strains or by isolation, reamplification, sequencing and comparison against sequences in databases. The most prominent band in all samples was that of *L. lactis* (band i). Two other bands were also present during both manufacture and ripening; these corresponded to *Streptococcus parauberis* (band h) and *Streptococcus thermophilus* (band k). In samples from curd and 3 day-old cheese, a weak band was observed in the upper part of the gel, which was identified as *L. garvieae* (band a). Bands corresponding to *Lactobacillus plantarum* (bands b) and *Enterococcus faecium* (band d) were clearly visible in curd and the three- and seven-day-old cheese samples. At around day seven, five bands appeared, which corresponded to *Streptococcus uberis*/*Streptococcus iniae* (band f), *Enterobacter* spp. (band l), *Corynebacterium variabile* (band m), and *Lactobacillus casei*/*Lactobacillus paracasei* (bands n). Finally, a band matching the sequence of *Macrococcus caseolyticus* (band e) was identified in the sample corresponding to the cheese surface at day 30 (line 6 in Fig. 2A). Similarly, nine bands were observed for the yeast D1 variable domain of the 26S rDNA (Fig. 2B); these corresponded to only four species, as the sequences of five bands matched those of a single species, *Geotrichum candidum* (bands a), and two bands belonged to *Kluyveromyces lactis*/*Kuyveromyces marxianus* (bands b). Furthermore, a faint band present in the seven-day-old sample related to *Saccharomyces* species (band c) and a weak band identified as *Trichosporum gracile* (band d) was observed from day seven onwards.

Bacterial and yeast DGGE profiles of cheeses from the second producer were shown to be highly similar, and a majority of identified bands coincided in the two batches. The exception was the presence of a prominent band corresponding to *Acinetobacter johnsonii* in the

Table 2
Average gross composition and physicochemical parameters of two independent batches of Casín cheese throughout manufacturing and ripening.

Chemical parameter	Stage of manufacturing or ripening					
	Milk	Curd	3 days	7 days	15 days	30 days
Total solids (%)	11.28 ± 0.87	35.37 ± 0.56	53.19 ± 0.83	55.28 ± 1.29	58.47 ± 1.80	61.85 ± 1.35
Fat (%)	4,35 ± 1.34	20.54 ± 0.63	29.16 ± 2.28	30.94 ± 2.19	31.25 ± 2.33	32.98 ± 2.25
Total protein (%)	3.30 ± 0.53	12.07 ± 0.46	19.30 ± 0.65	21.68 ± 0.58	24.18 ± 0.28	25.14 ± 0.41
pH	6.64 ± 0.13	6.38 ± 0.10	5.22 ± 0.16	5.17 ± 0.02	5.23 ± 0.09	5.25 ± 0.21
Salt in moisture (%)	0.13 ± 0.03	0.18 ± 0.04	1.65 ± 0.07	1.68 ± 0.04	1.85 ± 0.09	2.29 ± 0.12
a_w	0.999 ± 0.01	0.997 ± 0.02	0.993 ± 0.02	0.987 ± 0.03	0.983 ± 0.02	0.962 ± 0.03

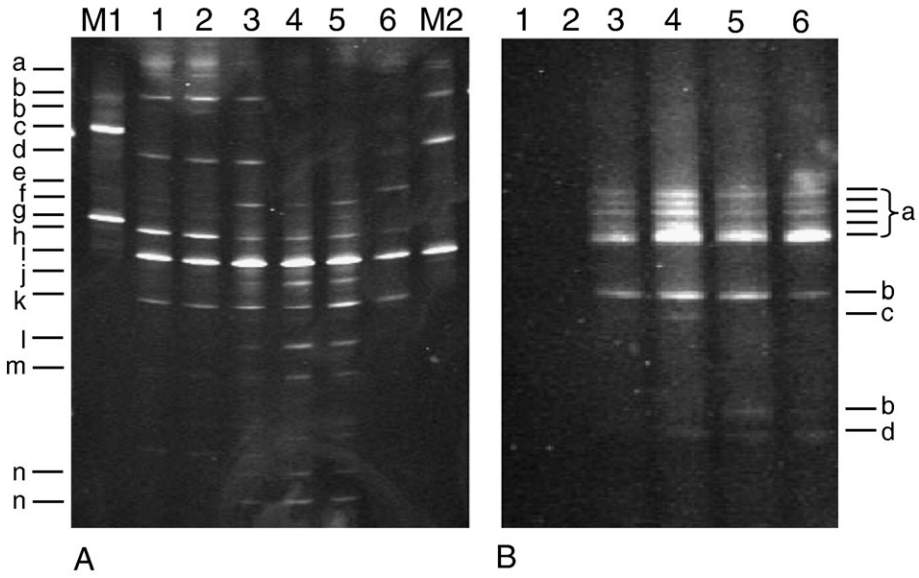

Fig. 2. DGGE profiles of microbial populations from Casín cheese during manufacturing and ripening. Samples: 1, curd; 2, 3, 4, and 5, cheeses of 3, 7, 15 and 30 days of ripening; 6, cheese surface at day 30. Panel A: DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene. M, combined amplicons of identified strains used as a control: M1, *Leuconostoc citreum* (c), *Lactobacillus brevis* (g); M2, *Lactobacillus plantarum* (b), *Enterococcus faecium* (d), *Lactococcus lactis* (i). Key of identified sequences different to those from the controls: a, *Lactococcus garvieae;* e, *Macrococcus caseolyticus;* f, *Streptococcus uberis/Streptococcus iniae;* h, *Streptococcus parauberis;* j, unidentified band; k, *Streptococcus thermophilus;* l, *Enterobacter* spp.; m, *Corynebacterium variabile;* n, *Lactobacillus casei/Lactobacillus paracasei.* Panel B: DGGE profiles of PCR amplicons of the eukaryotic domain D1 of 26S rDNA. Key of identified sequences: a, *Geotrichum candidum;* b, *Kluyveromyces lactis/Kluyveromyces marxianus*; c, *Saccharomyces* spp.; d, *Trichosporon gracile.*

three-day-old cheese sample from the second producer. Similarly, the bands of *K. lactis/K. marxianus* were more prominent and those of *G. candidum* were weaker (data not shown).

3.3. Microbial diversity and dynamics of Casín cheese by culturing

In order to maximize the recovery of microorganisms from Casín, which would improve the microbial description of the cheese, three different culture media (PCMA, BA, and BLA) and three different culture conditions (aerobiosis, microaerophilia, and anaerobisis) were assayed. Although statistically not significant, BA and BLA showed higher recovery numbers than PCMA from two seven-day-old cheese samples of two independent batches, particularly under anaerobic conditions (data not shown).

Three- and 30-day-old cheese samples from one of the producers were inoculated in these three media and incubated anaerobically at 30 °C for 72 h. These two sampling points were considered essential, since they roughly correspond to the end of acidification and the time at which the cheese is marketed. In total, 180 colonies (86 from day three and 94 from day 30) isolated from the different media were purified by subculturing and identified by molecular methods, as reported. The results are summarised in Table 3. Isolates of 14 different microbial types were detected, of which 11 could be identified to the species level. Despite different recovery rates, dominant species were detected in all three media; except for *Staphylococcus saprophyticus* and *Lb. plantarum,* which were only isolated from BA and BLA plates. The small number of isolates of most species makes it difficult to ascertain whether they have preferential recovery in the three distinct media used.

L. garvieae was shown to be the dominant species at day three (46 isolates), followed by *L. lactis* subsp. *lactis* (15 isolates), *S. saprophyticus* (12 isolates) and *Klebsiella* spp. (7 isolates). The species distribution in this sample contrasts with that found in the mature cheese (30 day-old sample), in which *L. lactis* isolates were dominant (82 isolates), followed by small numbers of *L. plantarum* (5 isolates) and *Micrococcus luteus* (two isolates).

To assess the intra-species diversity, a representative group of *L. lactis* (45) and *L. garvieae* (25) isolates were analysed by the RAPD typing technique. As *L. lactis* came from both the three-day (15 isolates) and 30-day samples (30 isolates), the RAPD analysis may also serve to address the evolution and/or stability of the *L. lactis* population during Casín ripening. A single RAPD profile was obtained with primer BoxA2R for all *L. garvieae* isolates, indicating that the acidification process was dominated by one strain. In contrast, eight distinct RAPD patterns were found among the *L. lactis* isolates (Fig. 3). Some of them resulted to be related (Fig. 3B), but as differences are shown in prominent bands (Fig. 3A) they could still belong to different strains. RAPD profiles from day three are different to those

Table 3
Majority microorganisms identified from Casín samples of 3 and 30 day-old cheeses isolated in three different culture media.

Species[a]	Stage of manufacturing and media of isolation						Total
	3 day-old cheese			30 day-old cheese			
	PCA	BA	BLA	PCA	BA	BLA	
Lactococcus lactis subsp. *lactis*	1	9	5	24	24	34	97
Lactococcus garvieae	17	20	9		1		47
Staphylococcus saprophyticus		3	9				12
Klebsiella spp.	3	3	1				7
Lactobacillus plantarum					1	4	5
Escherichia coli	1	1					2
Micrococos luteus						2	2
Streptococcus spp.			1	1			2
Corynebacterium variabilis					1		1
Flavobacterium spp.			1				1
Leuconostoc mesenteroides			1				1
Microbacterium oxydans				1			1
Musa acuminata				1			1
Staphylococcus pasteuri		1					1
Total	22	37	27	27	27	40	180

[a] Isolates were all identified by partial amplification and sequencing of their 16 S rRNA genes. Identical homology to two or more species impeded in some cases the accurately ascription of isolates to a specific species.

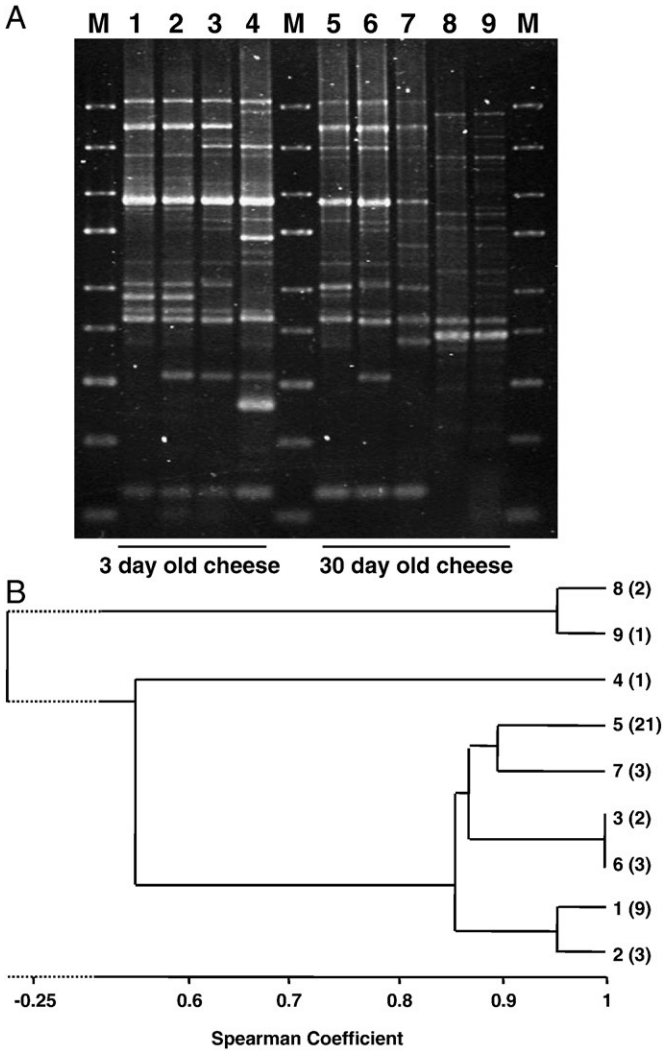

Fig. 3. Genotypic relationships among the *Lactococcus lactis* isolates from Casín cheese at day three (end of acidification) and day 30 (ripened cheese). Panel A: Distinct rapid amplification polymorphic DNA (RAPD) patterns obtained by PCR of 45 *L. lactis* isolates with primer BoxA2R (Koeuth et al., 1995). M, 100 bp molecular weight ruler (Bio-Rad, Richmond, CA., USA). Panel B: Dendogram of similarity of the RAPD patterns of all 45 strains clustered by the UPGMA method using the Spearman coefficient. In parenthesis, number of isolates having identical RAPD profiles.

from day 30, suggesting a certain degree of strain evolution. However, two isolates from day three (Fig. 3A, line 3) and three isolates from day 30 (Fig. 3A, line 6) showed identical patterns, which indicates that some *L. lactis* strains might be well adapted to the whole cheese-making process.

4. Discussion

The sensorial properties of cheeses depend on a large number of factors, among which the qualitative and quantitative microbial composition is paramount (Smit et al., 2005). Microbial types further determine hygienic conditions and shelf-life (Guinane et al., 2005). Thus, control of the microorganisms through manufacturing and ripening is thought to be essential in cheese-making. Not surprisingly, modern cheese manufacture relies upon pasteurisation and the deliberate addition of carefully selected microorganisms. Depending on the main function, added microorganisms are referred to as starters or primary cultures (if they participate in the initial acidification) and adjunct, maturing or secondary cultures (if they influence flavour, aroma and maturing activities) (Parente and Cogan, 2004). Primary and secondary cultures are mainly composed of well-characterised strains of LAB species.

In this study, the basic microbial and chemical properties of two independent batches of Casín cheese made by its traditional technology were analysed during manufacture and ripening. Small differences were observed between batches in most variables measured, which may reflect variations in uncontrolled environmental conditions, as well as differences in milk composition and microbial load and composition among batches from the two producers. A certain level of variation is typical of most artisan products, particularly in cheeses made from raw milk without the addition of starters cultures (Poznanski et al., 2004; Flórez et al., 2006; Randazzo et al., 2006; El-Baradei et al., 2007; Dolci et al., 2008).

Both conventional culturing and DGGE analysis were used in this work for the microbial characterisation of Casín cheese. The combined use of culturing and culture-independent techniques for the typing of complex microbial environments, including those of traditional food fermentations, has been found to supply complementary data, as shown by the results obtained in this work and those reported by others (Randazzo et al., 2002; Poznanski et al., 2004; Flórez and Mayo, 2006). Therefore, the use of both approaches is considered more comprehensive for a full description of the microbial populations in these environments. The microbial diversity found using both techniques in Casín cheese was similar. At least 14 different bacterial types were determined from the 180 colonies identified from the culture plates, and twelve distinct bands were identified by the DGGE technique. However, as repeatedly reported for other cheeses (Randazzo et al., 2002; Ercolini et al., 2003, 2004; Flórez and Mayo, 2006; El-Baradei et al., 2007), discrepancies in the microorganisms detected by culture-dependent and culture-independent methods were also noted. These differences could be attributed to some of the limitations of these two techniques. On one hand, the presence of bacterial types in viable but not cultivable states and an excessive selectivity of some media can cause a poor recovery of certain microorganisms by culturing. On the other hand, differential lysis of the microbial populations, presence of amplifiable DNA from dead microorganisms and differential amplification of some sequences can bias the molecular culture-independent results.

The bacterial and fungal species detected by culturing and DGGE in Casín cheese have all previously been isolated from dairy-related environments including traditional cheeses (Randazzo et al., 2002; Ercolini et al., 2003, 2004; Flórez and Mayo, 2006; El-Baradei et al., 2007). Despite this, the microbial characterisation of Casín cheese has provided many differences in microbial composition and evolution as compared to other traditional cheeses. It was surprising to find *L. garvieae* as the dominant species during acidification. In agreement with culturing data, a noticeable (but diffuse) band corresponding to *L. garvieae* was observed by DGGE in samples of curd and three-day-old cheese (band a in Fig. 2A). This band, however, was absent in all other subsequent cheese samples; in accordance again with culturing. *L. garvieae* is a well-recognised fish pathogen (Eyngor et al., 2004), and has also been retrieved from subclinical mastitis in water buffalos (Teixeira et al., 1996) and from many clinical human specimens (Fefer et al., 1998). Recently, *L. garvieae* has further been reported as a common component of the autochthonous microbiota of dairy products manufactured from raw milk (Fortina et al., 2007). Furthermore, DGGE analysis of Casín from different producers has unambiguously determined the presence of *L. garvieae* strains in the cheese milk (unpublished results). *L. garvieae* isolates from different sources have proven to be genetically unrelated (Foschino et al., 2008), suggesting that niche-driven adaptations allow this species to develop and persist in diverse environments. The study of several dairy strains and their comparison to pathogenic counterparts has shown that the former do not usually harbour virulence determinants (Fortina et al., 2007). It can therefore be deduced that the presence of *L. garvieae* strains in artisan cheeses do not pose a serious health

hazard. In agreement, consumption of Casín and other similar cheeses has never been associated with a food-borne disease. Furthermore, *L. garvieae* dairy strains have been found to present a series of desirable technological properties and some authors propose the use of characterised strains as part of the starter culture (Fortina et al., 2007), provided the absence of virulence factors and pathogenicity has been unequivocally determined. *L. garvieae* cheese isolates have been shown to present a slow rate of acidification (Fortina et al., 2007), but this is comparable to wild lactococcal isolates from other cheeses (Delgado et al., 2002).

At day three, *L. lactis* isolates (15 isolates) constitute less than 18% of the dominant population, while more than 53% of the microorganisms are *L. garvieae*. However, *L. lactis* strains are clearly dominant at day 30, at which time only a single *L. garvieae* isolate was found. This replacement in populations suggests that *L. garvieae* strains are more susceptible to the stressful conditions (acidity, low temperature) of ripening. In this study, only one of the batches was sampled by culturing, which raises the question of whether the data are representative. However, the agreement between culturing analysis of one batch and DGGE analysis of the two batches indicates that the data are likely to be typical.

Four different RAPD profiles were observed among the *L. lactis* isolates at day three and five profiles were observed at day 30 (Fig. 3). One of the profiles was present in the two samples (day three and day 30), suggesting that at least some strains persist throughout manufacture and ripening. High genetic variability in lactococcal strains from traditional cheeses has been reported elsewhere (Corroler et al., 1998; Mannu et al., 2000; Delgado and Mayo, 2004). In order to include unrelated strains in the design of specific starter cultures, strains presenting early and late RAPD patterns will be selected. Less genetic variability was observed in this study among the *L. garvieae* isolates, which showed a single RAPD profile only. Although this, two clearly distinct strains could be distinguished by phenotypic tests (unpublished data).

Of note from our findings is the presence of a DGGE band corresponding to *S. thermophilus*, which was visible in the two batches analysed in this study and in batches from other producers (data not shown). This species has never been isolated from traditional Spanish cheeses (Cogan et al., 1997). The cultivation conditions used in this work (30 °C, 72 h) did not allow *S. thermophilus* to form visible colonies on counting media after 72 h incubation. Work is currently in progress to selectively isolate this species from Casín.

Also of interest is the presence of micrococci, staphylococci, microbacteria and corynebacteria species within the cheese matrix, which might be a consequence of the repeated kneading of the cheese mass, internalising surface-associated bacteria. Species from these groups have recently been shown to dominate the surface microbial composition of smear-ripened cheeses (Mounier et al., 2005), where they develop in higher numbers than those attained by deliberately inoculated of commercial cultures (Goerges et al., 2008). The typical flavour of Casín cheese is strong, pungent and spicy, indicative of a strong lipolysis. Lipolysis may result from the action of native milk enzymes liberated from the fat globule during kneading, but it can further be enhanced by the action of microbial lipases. Strains of these species may certainly be useful as adjunct and maturing cultures.

The presence of high numbers of coliforms, enterococci and related organisms is also typical of cheeses made from raw milk. Species of these microbial types have been detected by both culturing and culture-independent techniques in this and many other raw milk cheeses (Poznanski et al., 2004; Flórez et al., 2006; Dolci et al., 2008). These populations are considered as indicators of faecal contamination and therefore also indicate poor manufacturing practices. The high counts of species supposed to be opportunistic pathogens observed in this work (such as *Staph. saprophyticus* and *Klebsiella* spp.), reinforces the need for improvement in hygiene conditions throughout Casín manufacture. However, it is worth noting that, as shown in Table 3, these undesirable microorganisms are not found among the major populations at day 30.

The results of this study present the first data on the microbial composition of Casín cheese and the dynamics of microbial diversity throughout ripening. A large collection of microorganisms have been gathered from two critical steps within the cheese manufacturing process (the end of acidification and ripened cheese). The technological characterisation of such isolates should permit the selection of appropriate strains for specific starter and adjunct cultures, which may be of help for standardisation and improvement of the overall cheese quality and safety.

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Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches

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Oscypek is a traditional Polish scalded-smoked cheese, with a protected-designation-of-origin (PDO) status, manufactured from raw sheep's milk without starter cultures in the Tatra Mountains region of Poland. This study was undertaken in order to gain insight into the microbiota that develops and evolves during the manufacture and ripening stages of Oscypek. To this end, we made use of both culturing and the culture-independent methods of PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE) and pyrosequencing of 16S rRNA gene amplicons. The culture-dependent technique and PCR-DGGE fingerprinting detected the predominant microorganisms in traditional Oscypek, whereas the next-generation sequencing technique (454 pyrosequencing) revealed greater bacterial diversity. Besides members of the most abundant bacterial genera in dairy products, e.g., *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus*, identified by all three methods, other, subdominant bacteria belonging to the families *Bifidobacteriaceae* and *Moraxellaceae* (mostly *Enhydrobacter*), as well as various minor bacteria, were identified by pyrosequencing. The presence of bifidobacterial sequences in a cheese system is reported for the first time. In addition to bacteria, a great diversity of yeast species was demonstrated in Oscypek by the PCR-DGGE method. Culturing methods enabled the determination of a number of viable microorganisms from different microbial groups and their isolation for potential future applications in specific cheese starter cultures.

Protected-designation-of-origin (PDO) labels are granted to traditional agricultural products and foodstuffs originating in a specific area whose quality or characteristics are essentially or exclusively due to particular geographic environments (with their inherent natural and human factors) and whose production, processing, and preparation take place in a defined region (19). The quality of PDO cheeses is based on particular species and herds, grazing pastures, and ancient technologies developed and maintained in a shared manner by the human communities within the PDO area. All these factors select for specific microorganisms that have evolved through the ages and whose activity plays a pivotal role in the sensorial, safety, and preservative properties of the products (22, 46). Therefore, identification, typing, and characterization of these microorganisms are essential for selecting specific starters to control the fermentation while preserving the original sensory profiles.

Oscypek is a traditional Polish scalded-smoked cheese that has had PDO status since 2008. The cheese has the shape of a spindle, with a beautiful characteristic pattern imprinted by the carved wooden molds that give the cheese its final form. It is manufactured from raw sheep's milk without starter cultures in the Tatra Mountains region of Poland, by the process diagramed in Fig. 1. The Oscypek cheese is ready for consumption after smoking without additional ripening, although it can be stored for several weeks. The traditional manufacturing process maintained throughout centuries may have selected appropriate species and/or strains of lactic acid bacteria (LAB) that could be used as specific starters. The new cultures can also be used to complement or replace the starters currently used by the large-scale dairy industry (5). In addition, traditional cheese ecosystems may harbor LAB strains showing unique flavor-forming capabilities (4), enhanced bacteriophage resistance (31), or the production of new, broad-range antimicrobial agents (1).

In addition to conventional microbial characterization, a vast array of culture-independent molecular techniques is now available to address the diversity and evolution of microbial populations throughout cheese manufacture and ripening (25). Among others, techniques such as denaturing gradient gel electrophoresis (DGGE) (40), single-strand conformation polymorphism (SSCP) (14), fluorescent *in situ* hybridization (FISH) (18), length heterogeneity-PCR (LH-PCR) (29), quantitative real-time PCR (qPCR) (21), and terminal-restriction fragment length polymorphism (T-RFLP) (3) complement classic culturing techniques, providing a more complete picture of the cheese ecosystem. Investigators have recently begun to apply next-generation sequencing techniques, such as pyrosequencing (35), to study the diversity and dynamics of the microbial populations in natural food fermentations (24, 41, 26) and in fresh foods during storage (17). This method enables rapid insight into the population structure, dynamics, gene content, and metabolic potential of microbial communities.

In this study, both culturing and the culture-independent methods of DGGE and pyrosequencing of segments of the 16S rRNA gene were used to type major and indicator microbial populations in traditional Oscypek cheeses. This allowed us to identify the dominant cultivable bacterial species and to assess microbial diversity and dynamics during the manufacture and ripening of several cheese batches.

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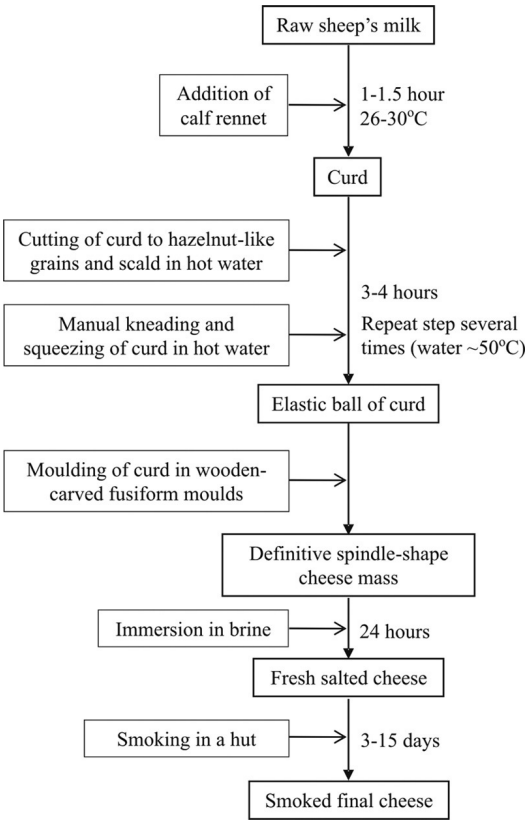

FIG 1 Flowchart of manufacturing and ripening stages of the traditional Polish cheese Oscypek.

MATERIALS AND METHODS

Cheese samples and sampling conditions. Cheeses manufactured by four independent cheese makers from the Tatra Mountains in Poland were sampled in September 2009. Microbiological and culture-independent molecular analyses were performed on four batches representing traditional Oscypek cheeses produced mostly from raw ewe's milk in shepherds' huts. Curd, fresh cheese (1 day), and smoked cheese that had not been ripened after smoking (3 days) were sampled according to FIL-IDF standard 50B and were kept under refrigeration until analysis.

Microbial analysis by culturing. Twenty-gram samples of curd or cheese were mixed with 180 ml of physiological salt (PS; 0.9% sodium chloride solution) at 37°C and were homogenized in a laboratory homogenizer (H500 Pol-Eko-Aparatura; Wodzisław Sąski, Poland) for 5 min at 24,000 rpm. Serial 10-fold dilutions in PS were then plated in duplicate onto seven rich and selective media for counting different microbial groups, as follows.

Mesophilic bacteria were counted on plate count agar supplemented with 0.1% skim milk (PCMA; Merck, Darmstadt, Germany) after 72 h of incubation under both aerobic and anaerobic (in 2.5-liter jars with AnaeroGen; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) conditions at 30°C. In addition, brain heart infusion (BHI; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) agar plates supplemented with 0.2% cysteine (BHIAC) were used to count total anaerobic bacteria after incubation at 37°C for 72 h in anaerobiosis. Lactococci were grown on M17 (Oxoid) agar supplemented with 10 g lactose/liter (LM17A) and were enumerated after 48 h of incubation at 30°C. Lactobacilli were grown on de Man, Rogosa, and Sharpe agar (MRSA; Merck) adjusted to pH 5.4 and were counted after 72 h of incubation under aerobic and anaerobic conditions at 37°C. Dextran-producing leuconostocs were grown on sucrose-enriched agar supplemented with vancomycin (LMVA, containing 10 g tryptone/liter, 5 g yeast extract/liter, 10 g sucrose/liter, 20 g $CaCO_3$/liter, 15 g agar/liter, and 200 μg vancomycin/ml) and were counted after incubation for 5 days at 21°C. Enterobacteria and coliforms were grown on MacConkey agar (MCA; Merck) and were counted after 48 h of incubation at 37°C. Finally, dilutions of the samples were plated on yeast extract-glucose-chloramphenicol agar (YGCA, containing 5 g yeast extract/liter, 20 g glucose/liter, 15 g agar/liter, and 0.1 g chloramphenicol/liter), and yeasts and filamentous molds were independently counted after 5 days of incubation at 28°C.

Molecular identification of lactic acid bacteria. After incubation, plates were photographed, and single colonies of the different morphologies for each medium were randomly selected for identification. Bacteria were isolated from the PCMA, LM17A, BHIA, MRSA, and LMVA at different dilutions, from 10^{-3} to 10^{-6}, depending on the medium. In every case, two plates of each dilution with well-separated colonies were analyzed. Isolates were purified by subculturing and were stored at −80°C in fresh medium with 15% (vol/vol) glycerol. Cryoprotected cultures were recovered in a medium corresponding to that in which they were isolated, and colonies were used as a source of DNA, which was subsequently used in PCR amplifications. Cell extracts were obtained by a mechanical procedure in a Mini-Beadbeater apparatus (BioSpec Products, Inc., Bartlesville, OK) after suspension of a single colony in 100 μl of sterile water and mixing with 50 mg of sterile glass beads (Sigma-Aldrich, Inc., St. Louis, MO). Cell extracts were separated from cellular debris by centrifugation at 13,000 × *g* for 10 min. Supernatants were used as a source of template DNA for PCR amplification of a large segment of the 16S rRNA gene with the universal bacterial primer 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and the universal prokaryotic primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (28).

Amplicons were purified using GenElute PCR Clean-Up columns (Sigma-Aldrich) and were sequenced with an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) using primer 27F. On average, 850 bp was obtained and was compared with sequences in the GenBank database, using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and in the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Sequences were assigned to a given species if they showed a similarity equal to or higher than 97% to the sequences of that species (47, 38).

Isolation of total microbial DNA. Curd and cheese samples were homogenized in PS. Two-milliliter samples of homogenates were centrifuged at 9,000 × *g* for 1 min. For the isolation of bacterial DNA, the pellets were resuspended in 300 μl of TES buffer (25 mM Tris, 10 mM EDTA, 50 mM sucrose) containing 20 mg of lysozyme/ml (catalog no. 62971; Fluka, Sigma-Aldrich) and 15 μl of mutanolysin at 1 U/μl (catalog no. M9901; Sigma-Aldrich). Then the samples were incubated for 1 h at 37°C. After centrifugation at 9,000 × *g* for 1 min, the pellets were resuspended in 100 μl of Tris buffer (10 mM Tris HCl [pH 8.5]), and DNA was isolated by using the commercial Genomic Mini DNA purification kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's recommendations. The isolation of DNA from fungi was based on the method of DNA isolation for *Saccharomyces cerevisiae* (45).

PCR amplification and DGGE analysis. Purified DNA was used as a template for PCR amplification of the V3 variable region of the prokaryotic 16S rRNA gene with primer F357 (5′-TACGGGAGGCAGCAG-3′), to which a 39-bp GC sequence was linked to give rise to GC-F357, and primer R518 (5′-ATTACCGCGGCTGCTGG-3′) (36). Group-specific amplification of 16S rRNA for the lactococcus-enterococcus-streptococcus group and the lactobacillus-leuconostoc-pediococcus group was performed with the primer pairs LB2-GC (5′-GATTYCACCG CTACACATG-3′) with LAC3 (5′-AGCAGTAGGGAATCTTCGG-3′) and LB2-GC with LB1 (5′-AGCAGTAGGGAATCTTCCA-3′), respectively (16). Finally, the D1 domain of the 26S rRNA gene of fungi was amplified with primers NL1-GC (5′-GCCATATCAATAAGCGGAGGAA AAG-3′) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) (9). PCR amplification conditions were carried out as reported previously (9, 16, 36). Amplicons were analyzed electrophoretically in 8% polyacrylamide gels

TABLE 1 Counts of distinct microbial groups estimated under different incubation conditions during the manufacturing and ripening stages of traditional Oscypek cheeses made by four independent producers

Microbial group (culture medium)	Count (log_{10} CFU/g) in the indicated samples from producer:								
	I		II			III			IV
	Fresh cheese	Smoked cheese	Fresh cheese	Fresh salted cheese	Smoked cheese	Curd	Fresh cheese	Smoked cheese	Smoked cheese
Total mesophilic bacteria (PCMA)									
Under aerobic conditions	8.92	9.00	9.45	9.53	9.34	9.60	9.26	9.37	9.49
Under anaerobic conditions	9.03	8.82	9.43	9.47	8.42	9.40	9.29	9.01	9.49
Total mesophilic bacteria (BHIAC)	9.37	8.82	9.36	9.18	8.26	9.37	9.26	9.30	9.37
Lactococci (LM17A)	9.37	8.92	9.52	9.12	8.59	9.44	9.45	9.49	9.51
Lactobacilli (MRSA)									
Under aerobic conditions	7.91	7.03	7.18	7.30	7.37	8.42	8.27	8.10	7.98
Under anaerobic conditions	8.32	7.15	8.41	7.98	7.77	8.34	8.20	8.32	7.52
Leuconostocs (LMVA)	>7.00	>6.00	6.64	6.63	5.72	>7.00	>7.00	7.82	7.45
Yeasts and molds (YGCA)	5.27	5.34	5.11	6.56	5.79	5.65	6.27	5.76	4.69
Enterobacteria (MCA)	5.36	4.92	5.61	5.91	4.58	5.43	5.41	3.30	<3.00

with 40 to 60% and 30 to 50% formamide denaturing gradients for bacteria and fungi, respectively, by using a DCode device (Bio-Rad, Richmond, CA). Electrophoresis proceeded at 75 V for 17 h for bacteria and at 130 V for 4.5 h for yeasts and molds. Bands were identified by their migration behavior compared to that on a control ladder (20), as well as by DNA isolation, reamplification with the same primers without the GC clamps, sequencing, and comparison of the sequences with the databases.

Amplicon preparation for pyrosequencing. Primers for the target area were selected to span a region of 250 to 500 bp (a combination of the average mean read length and the maximum amplicon size for a GS FLX amplicon sequencing run as recommended by 454 Life Sciences, Roche Applied Sciences). A 294-nucleotide sequence of the V5 and V6 regions of the 16S rRNA gene (with respect to *Escherichia coli* 16S rRNA gene positions 786 to 1079) was amplified from the isolated DNA by PCR.

Fusion primers were designed in which a proprietary primer sequence (Adaptor) of the Roche GS FLX sequencing technology and a sample-specific 10-nucleotide key sequence (Multiplex Identifier [MID]) (italicized) were included in order to differentiate between distinct samples. The forward primer was 5′-CCTATCCCCTGTGTGCCTTG*GCAGTCTC AG*GATTAGATACCCTGGTAGT-3′ (where the underlined sequence corresponds to the forward primer E786F) (10), and the reverse primer was 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG*ATATCGCGAG* TCACGACACGAGCTGACGAC-3′ (where the underlined sequence is the primer equivalent of *E. coli* positions 1061 to 1079 in *Lactococcus lactis* IL1403).

For each sample, a 50-μl PCR mixture was prepared containing 1× PCR buffer, 200 μM deoxynucleoside triphosphate mixture (Fermentas, St. Leon-Rot, Germany), 0.4 μM each primer, and 1.25 U of Ex *Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan). To each reaction mixture, 1 μl of the extracted template DNA was added. The PCR conditions used were 95°C for 5 min and 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by one cycle at 72°C for 7 min.

Amplicon quality check and quantity measurement. Amplicon samples were purified using Ampure XP beads (Beckman Coulter Inc., High Wycombe, United Kingdom) and were run on an Agilent Bioanalyzer, model 2100, to check sample quality. The quantity of amplicon DNA was estimated using a Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA). Prior to sequencing, the samples were diluted to the same concentration and were pooled.

Ultradeep amplicon sequencing on a 454 GS FLX pyrosequencer. DNA amplicons were sequenced on a GS FLX pyrosequencer (454, Roche). Samples were clonally amplified by emulsion PCR. DNA-carrying beads were loaded onto the medium region of a picotiter plate (PTP) (1/16 of a plate for each sample) and were sequenced in one direction on a GS FLX Titanium instrument.

Sequence reads for each amplicon were extracted from the resulting standard flowgram files (SFF) into FASTA format and were split into separate pools based on the MIDs by using the *sfffile* command.

Sequences obtained from pyrosequencing were then processed with Mothur software, version 1.7.2 (44), in which the algorithms mentioned below are implemented. In the first step, reads were trimmed so as to analyze only regions with average scores over 50 bases and a window of at least 35 bases. Then reads either shorter than 150 bases, or with an ambiguous base call (an "N"), or containing a homopolymeric track longer than 8 bases were removed. Afterwards, unique reads were aligned to a SILVA-compatible alignment database. Based on this alignment, potential chimeric sequences were removed using the ChimeraSlayer algorithm (23). Finally, the remaining sequences were clustered into operational taxonomic units (OTUs) and were assigned to taxonomic branches.

The structures of the communities of all samples were compared using a weighted and an unweighted Unifrac algorithm (30) and the Yue and Clayton measure (50).

RESULTS

Basic microbial analysis. The results of the counting of majority and indicator microbial populations of the different traditional Oscypek cheese samples analyzed in this work are summarized in Table 1. Counts of total mesophilic bacteria under aerobic (PCMA) and anaerobic (PCMA and BHIAC) conditions showed no statistical differences, similarly to counts of lactobacilli in aerobiosis and anaerobiosis (both in MRSA). In general, mesophilic bacterial counts matched those obtained in LM17A, suggesting that *Lactococcus* spp. were the dominant microorganisms in all cheeses (at a level near 1.0×10^9 CFU/g). High numbers of *Lactobacillus* species were also observed in all cheese samples, though at levels 1 or 2 logarithmic units lower than those of lactococci. Dextran-producing *Leuconostoc* spp. were present in all samples at variable levels (ranging from 5.72 to 7.82 log_{10} CFU per g). Yeasts and molds were also present in all cheese samples in numbers ranging from 5 to 6 log_{10} CFU per g. Finally, enterobacterial populations were present at levels similar to those of yeasts and molds, and their number showed a tendency to decrease during cheese processing from curd to smoked-cheese samples.

Identification of isolates. From the counting plates of total

TABLE 2 Identification of isolates from different counting culture media

Species	No. of isolates obtained on the following medium:						Total no. of isolates
	PCMA[a]		LM17A	BHIAC	MRSA[b]	LMVA	
	O_2	An					
Lactococcus lactis subsp. *lactis*	38	40	42	46	1		167
Lactobacillus casei	1	1	8	6	31		47
Leuconostoc citreum	7		4	6	3	9	29
Lactobacillus plantarum			1	1	13	1	16
Leuconostoc lactis		2			2	2	6
Leuconostoc mesenteroides						4	4
Streptococcus thermophilus	1			2			3
Enterococcus faecalis		2		1			3
Enterococcus durans			2				2
Enterococcus italicus	1						1
Leuconostoc pseudomesenteroides		1					1
Lactococcus lactis subsp. *cremoris*			1				1
Bacillus simplex				1			1
Lactobacillus parabuchneri					1		1
Lactobacillus brevis					1		1
Enterobacter kobei						1	1
Total	48	46	58	63	52	17	284

[a] O_2 and An indicate incubation under aerobic and anaerobic conditions, respectively.
[b] For isolates identified on MRSA, only the numbers identified under aerobic conditions are shown.

mesophilic bacteria and those for the different LAB populations, 284 colonies were selected at random as representative of the different sizes and morphologies. The media used for isolation of the colonies and the identification results are summarized in Table 2. In agreement with the counting results, nearly 60% of the isolates were identified as *Lactococcus lactis* subsp. *lactis.* All these isolates but one came from counting plates of the PCMA, BHIAC, and LM17A media. As a subdominant population, lactobacilli, comprising mostly *Lactobacillus casei* and *Lactobacillus plantarum*, were identified. Most lactobacilli came from the MRSA plates, although a few were isolated from all other counting media (Table 2). *Leuconostoc citreum*, *Leuconostoc lactis*, and *Leuconostoc mesenteroides* constituted the majority of dextran-producing *Leuconostoc* species in Oscypek. The LMVA medium was rather selective for species of this genus, but surprisingly, *Leuconostoc* spp. were also isolated from both rich (PCMA, BHIAC) and group-selective (LM17A, MRSA) counting plates, indicating that they reach cell densities similar to those attained by lactobacilli in Oscypek. Among the minority components, three *Streptococcus thermophilus* isolates, one *Lactococcus lactis* subsp. *cremoris* isolate, and several isolates of distinct *Enterococcus* species were recovered.

DGGE analysis. In addition to the culture-dependent approach, DGGE analysis of Oscypek cheese was performed in order to analyze the diversity of the microbial populations in the cheese by a culture-independent technique and to follow their dynamics through manufacture and ripening. The results obtained with universal and group-specific primers are all shown in Fig. 2. Universal primers were used to track the bacterial populations (Fig. 2A) and eukaryotic organisms (Fig. 2B), while specific primers were used for both the lactococcus-enterococcus-streptococcus group (Fig. 2C) and the lactobacillus-leuconostoc-pediococcus group (Fig. 2D). All the bands were identified by reamplification and sequencing (Table 3).

As many as 13 different bands corresponding to the bacterial V3 variable region of the 16S rRNA gene were observed in the different cheese samples (Fig. 2A). It should be noted that the cheese samples analyzed came from four batches produced in different households by independent producers. The main band in all of the samples belonged to *Lactococcus lactis* (band g). In agreement with counting and identification results, bands of *Lactobacillus plantarum* (band b) and *Leuconostoc citreum/Leuconostoc mesenteroides* (band c) were clearly present in almost all samples. In addition, bands corresponding to *Lactococcus garvieae* (band 1), *Streptococcus vestibularis* (bands 4 and 5), *Tetragenococcus halophilus* (band 7), *Streptococcus thermophilus* (bands 8 and 10), and *Streptococcus salivarius* (band 9) were observed in some cheese samples.

The composition and evolution of yeast and mold populations, analyzed by DGGE with primers NL1-GC and LS2, are shown in Fig. 2B. All the bands were identified by reamplification and sequencing. A great variability in both the number and the intensity of the bands was observed among samples from different batches and producers. Thick bands corresponding to *Saccharomyces* spp. (band 14) and *Debaryomyces hansenii* (bands 19, 20, and 21) were observed in some samples. In addition, other dairy-associated yeasts (such as *Candida pararugosa*, *Geotrichum silvicola*, *Candida zeylanoides*, *Yarrowia lipolytica*, and *Kluyveromyces marxianus*) were occasionally present in some other samples (Fig. 2B).

The DGGE results corresponding to the LAB groups of lactococci-enterococci-streptococci and lactobacilli-leuconostocs-pediococci are presented in Fig. 2C and D, respectively. Bands of *Lactococcus lactis* and *Lactococcus garvieae* were present in all samples. It is worth noting that the *Lactococcus garvieae* band was shown to be more intense than other bands in most of the samples. The latter species, which was also shown with the universal primers, was never identified among the isolates. In addition to these two *Lactococcus* species, a band belonging to *Lactococcus raffinolactis* (band 24) was identified in cheese samples from producers I and II. With primers LB2-GC and LB1, three out of the five bands produced be-

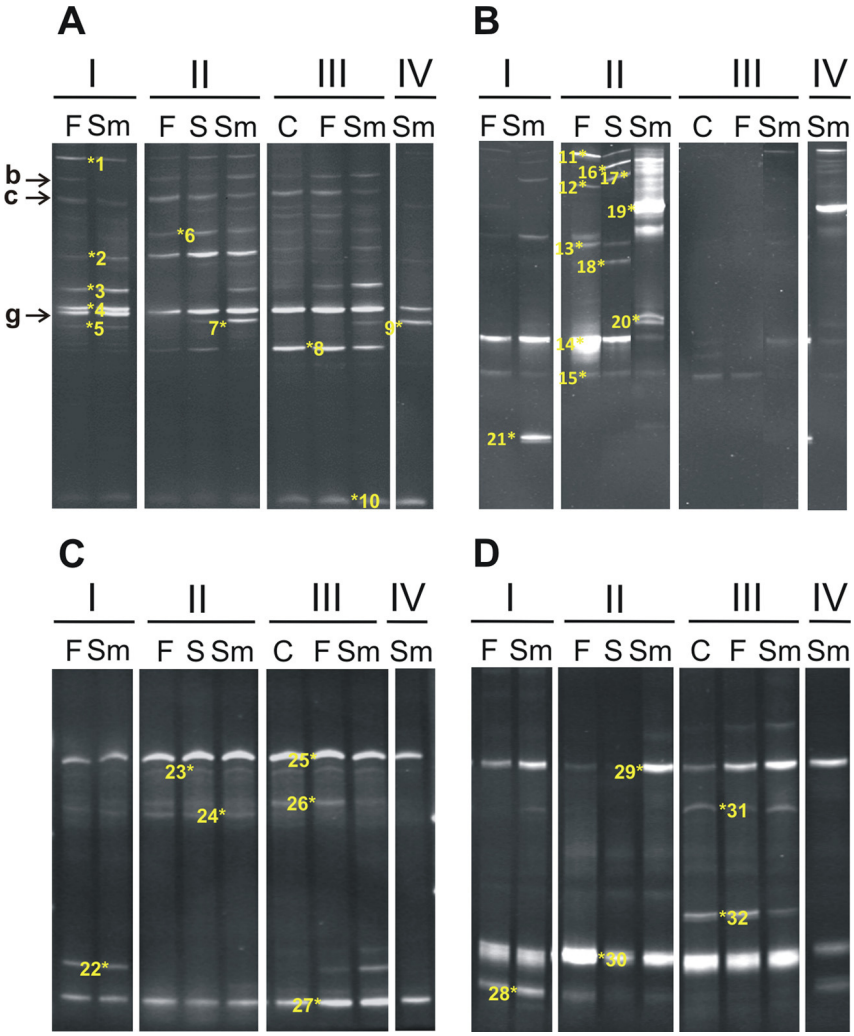

FIG 2 Diversity and dynamics of bacterial, fungal, and LAB (represented by lactococci and lactobacilli) populations, as shown by analysis of their DGGE profiles. The 16S rRNA genes of bacteria and the D1 domains of the 26S rRNA genes of fungi were amplified by using universal prokaryotic (A) and eukaryotic (B) primers and specific primers for the lactococcus-enterococcus-streptococcus group (C) and the lactobacillus-leuconostoc-pediococcus group (D). The order of cheese samples is the same in all panels and corresponds to the order of samples in Table 1. Capital roman numerals represent four different producers. F, fresh; Sm, smoked; S, fresh salted; C, curd. Numbered bands were purified and identified by reamplification, sequencing, and sequence comparison. The identities of the bands are summarized in Table 3.

longed to the *Leuconostoc* species. Bands of *Leuconostoc lactis* (band 30) and *Lactobacillus plantarum/Lactobacillus paraplantarum* (band 29) were present in all curd and cheese samples, while bands of *Leuconostoc mesenteroides/Leuconostoc pseudomesenteroides* (band 28), *Leuconostoc citreum* (band 31), and *Lactobacillus helveticus/Lactobacillus crispatus* (band 32) were observed only occasionally.

16S rRNA-based pyrosequencing analysis of Oscypek. Pooled 16S rRNA gene amplicons of bacterial communities from three cheese samples were sequenced. The numbers of reads after sequencing were 35,179 for curd, 59,451 for fresh cheese, and 46,894 for smoked cheese, all from producer III. These reads were later cleaned, and putative chimeric sequences were removed (14.2% of all cleaned reads). Further processing involved clustering and assignment to taxonomic branches. Good's coverage estimator was 99% for all three samples, indicating that we managed to capture the majority of the bacterial biodiversity in each sample.

Reads were assigned to four different phyla: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* (see Table S1 in the supplemental material). Out of 40 genera, we have identified 9 major genera or taxonomic groups represented by at least 0.4% of the total pool across the samples (Fig. 3). Six of them belong to *Lactobacillales* (*Firmicutes*), constituting 97% of all clean reads. The remaining three were unclassified *Bacilli*, unclassified *Bifidobacteriaceae* (*Actinobacteria*), and *Enhydrobacter* (*Proteobacteria*). These were represented by ca. 2.5% of all clean reads. The majority of the taxonomic spectrum identified in the samples consisted of auxiliary genera represented by at most tens of reads. By use of pyrosequencing data, no deeper assignment (i.e., to the level of individual species) was possible; however, it should be noted that ca. 20% of all sequences were assigned to "unclassified" branches. This indicates the strong presence of strains that have not yet been thoroughly annotated.

Statistical comparison of the three individual samples from producer III showed very high similarity between the memberships and structures of the samples. The Yue and Clayton measure in all-

TABLE 3 Bacterial and eukaryotic species identified from Oscypek cheese by the DGGE technique

Panel	Band no.	Microorganism	GenBank accession no. of closest relative(s)[a]	Identity (%)
Bacteria				
A	1	*Lactococcus garvieae*	AP009333	100
A	b	*Lactobacillus plantarum*	AB362982	100
A	c	*Leuconostoc citreum/Leuconstoc mesenteroides*	AB362721/AB596940	99
A	2, 6	*Streptococcus uberis/Streptococcus parauberis*	AM946015/CP002471	99/99
A	3, g	*Lactococcus lactis*	NC_013656	100
A	4, 5	*Streptococcus vestibularis*	AEVI01000085	98
A	7	*Tetragenococcus halophilus*	AP012046	99
A	8, 10	*Streptococcus thermophilus*	FR875178	100
A	9	*Streptococcus salivarius*	CP002888	100
C	22, 23, 26, 27	*Lactococcus lactis*	CP003132/JF733789	100
C	24	*Lactococcus raffinolactis*	NR_044359	99
C	25	*Lactococcus garvieae*	NC_015930	100
D	28	*Leuconostoc mesenteroides/Leuconostoc pseudomesenteroides*	AB671574/AF515228	98
D	29	*Lactobacillus plantarum/Lactobacillus paraplantarum*	NC_014554/HE600693	100
D	30	*Leuconostoc lactis*	AB548870	100
D	31	*Leuconostoc citreum*	AB682757	98
D	32	*Lactobacillus helveticus/Lactobacillus crispatus*	NC_010080/NC_014106	99/99
Yeasts and molds				
B	11	*Candida pararugosa*	GQ222346	99
B	12	*Geotrichum silvicola*	DQ377646	100
B	13	*Torulaspora* spp.	EF063125	95
B	14	*Saccharomyces* spp.	EU441887	97
B	15	*Phialemonium* spp.	AB278184	96
B	16	*Candida zeylanoides*	EU879957	98
B	17	*Yarrowia lipolytica*	EU327102	100
B	18	*Kluyveromyces marxianus*	EU669470	100
B	19, 20, 21	*Debaryomyces hansenii*	GQ458041	100

[a] BLAST analysis against the nonredundant nucleotide collection of the GenBank database was performed.

versus-all comparison is near its maximum value (that is, 1); however, in agreement with the experiments conducted, the curd and the fresh cheese, and the fresh and smoked cheeses, are slightly more similar to each other than are the curd and the smoked cheese.

As seen in Fig. 3, during processing, shifts in the microbial community structure of Oscypek were found in all four major groups: *Lactococcus*, unclassified *Lactobacillales*, *Streptococcus*, and *Leuconostoc*. The number of *Lactococcus* spp. increased at the ex-

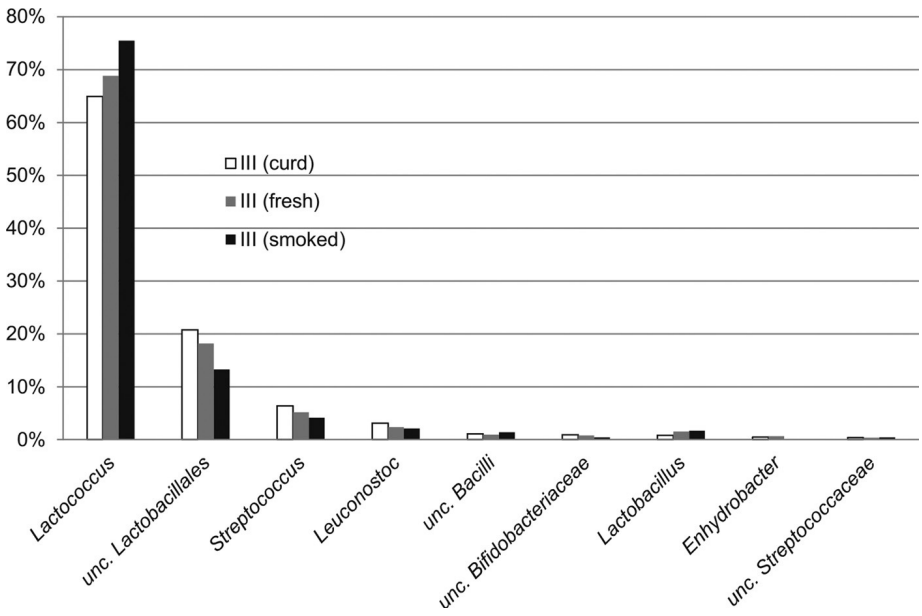

FIG 3 Major taxonomic groups in traditional Oscypek by the percentage of assigned reads. Only genus-level reads that represented at least 0.4% of the total pool of reads across the three samples are shown. unc., unclassified.

pense of the latter three bacterial groups. Additional differences were more subtle.

DISCUSSION

An understanding of the microbial composition of cheeses made from raw milk is essential for the quality and safety of the final product, key factors in PDO cheeses. Besides culturing, a vast array of molecular techniques is now applied to address the question of microbial diversity and population dynamics throughout the cheese manufacturing and ripening processes (25). However, despite the increasing number of polyphasic studies, including both culture-dependent and culture-independent approaches, to our knowledge no data on 16S rRNA-based pyrosequencing of the microbiome of traditional cheeses have been published. Moreover, a single report on the microbial analysis of kefir by a high-throughput sequencing approach has been released recently (13).

In addition, little has been demonstrated concerning the microbial communities responsible for the ripening of traditional Oscypek cheese. In fact, a single report on the microbiological analysis of Oscypek and its intermediary products has been published (32). This study was performed only by culture-dependent techniques, which included determination of total microbial counts using a broth medium and selective media for lactobacilli, lactococci, streptococci, enterococci, and yeasts.

In the present work, the biodiversity of traditional Oscypek cheeses was analyzed by culturing, PCR-DGGE fingerprinting, and 454 pyrosequencing. The first two methods revealed the predominant microorganisms, belonging to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus*. Besides *Lactococcus lactis*, strains of lactobacilli and leuconostoc species could be considered possible adjunct-culture candidates for the design of specific starter cultures for Oscypek cheese. There were also microorganisms identified by one method only, either the culture-dependent method (single isolates of *Enterococcus durans*, *Enterococcus italicus*, *Lactobacillus parabuchneri*, *Bacillus simplex*, and *Enterobacter kobei*) or the culture-independent DGGE approach (*Lactococcus garvieae*, several *Streptococcus* species other than *S. thermophilus*, and *Tetragenococcus halophilus*, *Lactococcus raffinolactis*, and *Lactobacillus helveticus/Lactobacillus crispatus*). Although the use of different selective media has been shown to increase the chance of identification of particular organisms (12), culturing methods are limited by the fact that a great number of microorganisms are difficult or impossible to cultivate (2). This nonrecoverable microbiota can be detected by culture-independent techniques, such as DGGE. The sensitivity of the DGGE technique can be slightly improved by specific primers detecting less-abundant bacteria (43), which was the case in this study, where *Lactococcus raffinolactis* was identified only with primers specific for the lactococcus-enterococcus-streptococcus group, while *Lactobacillus helveticus/Lactobacillus crispatus*, *Leuconostoc citreum*, *Leuconstoc mesenteroides/Leuconostoc pseudomesenteroides*, and *Leuconostoc lactis* were identified exclusively with primers specific for the lactobacillus-leuconostoc-pediococcus group. However, one of the drawbacks of the DGGE technique is the fact that the number of rRNA genes per chromosome equivalent differs widely in bacteria (27). In addition, heterogeneous copies of rRNA gene operons exist for both bacteria (37) and fungi (49), so that different bands may belong to the same species.

Simultaneously, three samples of traditional Oscypek from producer III were analyzed by a high-throughput next-generation sequencing technique (454 pyrosequencing). In addition to the most abundant bacterial groups (e.g., *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus*) found by culture-dependent and -independent methods, other bacteria, belonging to the families *Bifidobacteriaceae* and *Moraxellaceae* (mostly *Enhydrobacter*), were identified. They represented, respectively, 0.71% or 0.45% of the total pool of reads; thus, they are minor but significant elements of the bacterial community of Oscypek, since the contributions of the majority of other branches did not exceed 0.1%. Taking into account the traditional conditions of Oscypek processing (raw milk, production in shepherds' huts), it is not surprising to find bacteria known to belong to gut microbiota or the manufacturing environment (workers' hands, water, etc.). *Actinobacteria* and *Proteobacteria*, which are widely distributed in nature, have been found using pyrosequencing of tagged 16S rRNA gene amplicons in traditionally fermented food such as pearl millet slurries (24).

Interestingly, a relatively high ratio (ca. 20%) of reads was assigned to "unclassified" taxonomic branches. This result indicates a strong presence of strains that have not yet been sequenced or annotated but at the same time reveals the weakness in the current state of the field. In our studies, taxonomic assignment was possible only up to the genus level because of two factors: the shortness of the fragment sequenced (294 bp) and the limits of database classification (48). Further advances, including strain-level resolution in taxonomic assignment, will depend on whole-genome sequencing strategies (39).

Compared with the predominant bacteria, other groups identified in traditional Oscypek were detected at relatively low abundances. Among the organisms that were revealed or whose percentages of assigned reads increased in the fresh cheese (immersed in brine) from producer III, there were some salt-tolerant bacteria related to marine environments, such as *Sanguibacter*, *Flavobacteriaceae*, *Tetragenococcus*, or *Chromohalobacter* spp. Some of these groups include recognized pathogens, suggesting the necessity of improvement of the safety conditions of the cheese. The family *Flavobacteriaceae* includes many marine species (6). Different marine bacterial species have already been identified in other traditional cheese ecosystems by culture-independent approaches (34, 15). However, genera belonging to the family *Flavobacteriaceae*, except for the genus *Flavobacterium*, are generally adapted to nonmarine ecosystems, including the soil, water, and animal habitats (7). *Flavobacteria* have been shown to be associated with the spoilage of food and food products (11). It is worth noting that the percentage of reads assigned to *Flavobacteriaceae* decreased slightly in the smoked cheese compared to the fresh sample from producer III. Also of note is the percentage of reads for *Actinobacteria* (0.987%), which seemed to be members of the family *Bifidobacteriaceae* and possibly to belonging to *Bifidobacterium* species. To our knowledge, this is the first report of bifidobacteria from a traditional cheese. Since they might have great industrial potential or probiotic significance, efforts will be made in the near future at the selective recovery in culture of bifidobacteria from Oscypek.

The counts of microbial populations of different Oscypek cheese samples analyzed in this work demonstrated that the number of enterobacteria decreased from curd to smoked-cheese samples. Thus, our data suggest that the smoking process increases the quality and safety of the traditional Oscypek. These results are supported by the observation that cold smoking reduces the

amount of *Listeria monocytogenes* in smoked salmon (42). During warm smoking of Oscypek cheese, phenolic compounds are produced from polyphenols present in the wood used by shepherds for smoking (33). Because of the bacteriostatic and/or bactericidal properties of volatile phenols, these compounds are assumed to be responsible for decreasing the microbial population during the smoking process (32). Another probable explanation, taking into account the selective reduction in the levels of particular bacterial groups, could be the effect of microbial competition, due particularly to acidification and/or the production of antimicrobial compounds by LAB species.

Changes in microbial composition during the manufacture and ripening of Oscypek cheese were also observed among the predominant bacterial groups. The percentage of reads representing unclassified members of *Lactobacillales*, *Streptococcus*, *Leuconostoc*, and *Bifidobacteriaceae* diminished after brining and smoking, whereas that for *Lactococcus* increased. However, in our culture-dependent studies, we did not observe significant changes in microbial counts for lactococci, lactobacilli, or streptococci during the manufacturing process. It is worth remembering that the two approaches, pyrosequencing and culturing, differ in their levels of sensitivity. In contrast, a general decrease in the levels of most bacterial groups, including lactococci, lactobacilli, streptococci, and enterococci, during Oscypek cheese preparation has recently been reported by Majcher and coworkers (32). This discrepancy may be due to the individual characteristics of various cheeses and/or to the different media and culturing conditions used in the two studies. We also observed approximately 5-fold differences in the levels of yeasts and molds from Majcher's results. However, this could be explained by the fact that the experiments were performed on different culture media, Czapek agar in Majcher's work and YGCA in our studies. The great diversity of yeasts demonstrated by the PCR-DGGE method in our studies has been observed previously in various artisanal dairy products (9, 8, 21).

In conclusion, culturing and culture-independent methods, such as DGGE and pyrosequencing of the 16S rRNA gene fragments, were successfully applied to reveal the microbial composition and its evolution during the manufacture of traditional Oscypek cheese. Our results confirmed the usefulness of polyphasic molecular methods for the identification of cultivable, noncultivable, abundant, and scarce microorganisms in complex dairy food products. The culture-independent DGGE technique allowed identification and tracking of the majority of the population throughout manufacturing, while the pyrosequencing technique produced a complete inventory of the bacterial species encountered within the Oscypek ecosystem. It is expected that each bacterial species present in Oscypek cheese may contribute somehow to the ripening process. Finally, culturing methods enabled the estimation of the number of CFU within various microbial groups and the isolation of microorganisms for potential future applications in cheese starter cultures. On the basis of the counting results, bacteria belonging to lactococci, lactobacilli, and leuconostocs were proposed as possible candidates for starter cultures for Oscypek production. The high bacterial biodiversity in the different batches revealed by all culturing and culture-independent approaches may be partially explained by the heterogeneous production of traditional Oscypek.

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ORIGINAL RESEARCH

Microbial characterisation and stability of a farmhouse natural fermented milk from Spain

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This work reports the microbial characterisation of a farmhouse natural fermented milk (NFM) with good sensorial properties produced in Spain. Culturing and denaturing gradient gel electrophoresis (DGGE) analyses showed that Lactococcus lactis *subsp.* lactis *and* L. lactis *subsp.* cremoris *(approximate levels of 10^9 cfu/mL) were dominant in this NFM, while* Lactobacillus plantarum *appeared at a lower level (10^6–10^7 cfu/mL). Repetitive extragenic palindromic (REP)-PCR typing of the isolates identified single strains each of* Lc. lactis *subsp.* lactis, Lc. lactis *subsp.* cremoris *and* Lb. plantarum*. These three strains formed a stable microbial association which has been maintained for at least some decades.*

Keywords Natural fermented milk, Lactic acid bacteria, Traditional dairy products, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*.

INTRODUCTION

Milk can be consumed in its fluid form or transformed into a variety of different products, of which fermented milks are among the most important (de Ramesh *et al.* 2006; Robinson and Tamime 2007). The first fermented milks were produced by accident via the development – under serendipitously appropriate conditions – of indigenous lactic acid bacteria (LAB). It is difficult to establish when the purposeful practise of fermenting milk began, but it can be assumed that it was soon after the first human populations settled in the Middle East some 15 000 years ago (Tamime 2002; Robinson and Tamime 2007). The production of natural fermented milk (NFM) from raw milk later spread all over the world. Evidence of NFMs can still be found in large areas of Africa, Middle East, Asia, and even in Europe, such as *ergo* from Ethiopia, *amasi* (also known as *hodzeko* and *mukaka wakakora*) from Zimbabwe, *roub* from The Sudan, *rayeb*, *lben*, *laban*, *kad*, *zabady* and *zeer* from the Magreg, and *filmjölk* and *långfil* from Sweden (Kosikowski and Mistry 1997; Robinson and Tamime 2007; Tamang 2010). Traditional NFMs (such as *leite callado* and *lleche presa*) are also still produced in rural areas of North-western Spain at a farmhouse scale. In total, more than 400 generic names of NFMs are registered throughout the world (Kurmann *et al.* 1992), although the number of distinct varieties may be shorter (Robinson and Tamime 1990, 2007; Tamime 2002).

NFM relies on the growth of mesophilic LAB species, which lower the pH and produce the most typical sensorial compounds of the products (FAO/WHO 2003). Two different classes of NFM can be distinguished: inoculated and noninoculated (Kosikowski and Mistry 1997; Robinson and Tamime 2007). Noninoculated NFMs are made by leaving the raw milk at room temperature until it becomes sufficiently acidic for the coagulum to appear. Inoculated NFMs are manufactured by adding a portion of a previous NFM batch to a new milk substrate (backslopping). In either case, *Lc. lactis* strains are among the dominant microbiota (Gonfa *et al.* 2001; Mathara *et al.* 2004; Patrignani *et al.* 2006). In traditional products manufactured from raw milk it is also common to find species of mesophilic lactobacilli such as *Lb. plantarum* and *Lactobacillus casei/Lactobacillus paracasei*, as well as *Leuconostoc*, *Enterococcus* and *Pediococcus* species (Gonfa *et al.* 2001; Mathara *et al.* 2004; Patrignani *et al.* 2006). In warm climates, other lactobacilli such as *Lactobacillus helveticus*, *Lactobacillus fermentum* and/or *Lactobacillus acidophilus* may also develop. Moderate to high (up to 10^8 cfu/g) numbers of yeast species are also usually present in NFMs (Gadaga *et al.* 2000; Gonfa *et al.* 2001; Benkerroum and Tamime 2004); different types of yeast may cause its spoilage or enhance its flavour. The dominant yeast species include *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Candida lusitaniae* (Gadaga *et al.* 2000; Benkerroum and Tamime 2004). Micrococci, coliforms and pathogens

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(*Staphylococcus aureus*, *Bacillus cereus*) are occasionally found in NFMs (Gonfa *et al.* 2001), stressing the need for improving the microbial safety of these products. Natural fermented milks can also be manufactured from pasteurised and/or sterilized milk (at both the artisanal and industrial scale), which renders products safer. Industrial NFMs are inoculated with acidifying and aromatic starter cultures, while artisanal products are usually inoculated via backslopping techniques. Such transfers impose conditions that select strongly for strains that grow rapidly in milk and that show strong resistance to high levels of lactic acid.

This study reports the microbial characterisation and evolution of a stable, farmhouse NFM with good sensorial properties and commercial potential, produced by a number of families in Northwestern Spain. The original batch of NFM was of uncertain origin, but the current product was the result of backslopping inoculation of sterilised UHT-treated milk every 3–5 days followed by incubation at room temperature (20–25°C) for 18–24 h. Once coagulated, the milk was stored at 7°C for consumption until the manufacture of a new batch. The NFM was maintained for several years in the household from which it was acquired without losing its original activity or sensorial properties. Following acquisition and continued production at the laboratory using the same artisanal methods, samples were analysed at 0, 3, 6, 12 and 15 months. The stable sensorial properties of the product suggested the presence of a stable microbial community with potential use as an industrial starter.

MATERIALS AND METHODS

Sampling conditions

Six batches of the NFM were sampled at the time of consumption (1–3 days after production) according to IDF Standard 50B (IDF, 1985) and transported to the laboratory under refrigerated conditions. The pH of the milk before and after fermentation was measured according to IDF Standard 104A (IDF, 1984). Duplicate culturing analyses were performed on the day of sampling. For the isolation of DNA, samples were stored at −20°C until required.

Microbial analyses

Ten millilitre of NFM were homogenised with 90 mL of a 2% (w/v) sodium citrate solution at 45°C in a Colworth Stomacher 400 (Seward Ltd, London, UK) (for 3×1 min). Ten-fold serial dilutions were made in Maximum Recovery Diluent (Scharlau, Barcelona, Spain) and plated in duplicate on general and selective media as follows.

Aerobic mesophilic bacteria

Aerobic mesophilic bacteria were enumerated on plate count agar with 1% skimmed milk (PCAM; Merck, Darmstadt, Germany) after 72 h incubation under aerobiosis at 30°C.

Lactococci

Lactococci were grown on M17 agar (M17A; Scharlau) and enumerated after 48 h incubation at 30°C.

Lactobacilli

Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRSA; Merck) adjusted to pH 5.4, and enumerated after 72 h incubation at 32°C in a 5% CO_2 atmosphere in a Hera Cell 2400 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Leuconostoc *spp.*

Dextran-producing leuconostocs were grown on Mayeux, Sandine and Elliker agar (MSEA; Biokar Diagnostics, Beauvais, France) and enumerated after 5 days incubation at 25°C.

Enterococci

Enterococci were grown on Slanetz and Bartley agar (SBA; Merck) and enumerated after 24 h incubation at 44°C.

Enterobacteria and coliforms

Enterobacteria and coliforms were grown on violet red bile glucose agar (VRBGA) and violet red bile lactose agar (VRBLA) (both from Merck), respectively, using the pour-plate and overlay technique. Dilutions were mixed with 15 mL of agar and poured onto Petri dishes. After solidification a second agar layer of 10 mL was added. Bacteria were enumerated after 48 h incubation at 30°C.

Staphylococci

Dilutions were grown on Baird-Parker agar (BPA; Merck) supplemented with egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearing were recorded after 24 h incubation at 37°C.

Yeasts and moulds

Dilutions of acidified milk samples were plated on yeast extract glucose chloramphenicol agar (YGCA; Merck) and yeasts and moulds enumerated after 3–5 days incubation at 25°C.

Molecular identification

Molecular identification of bacteria

From the different NFM samples, 104 colonies from the M17 (54), MRS (39) and MSE (11)

agar plates were purified by subculturing on the same medium from which they were collected. Pure cultures were stored frozen at −80°C until analysis. Total genomic DNA from isolates was purified from overnight cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich Inc., St. Louis, MO, USA) following the manufacturer's recommendations. Total DNA from isolates was employed as a template to amplify a segment of the 16S rRNA gene via the polymerase chain reaction (PCR) using the universal prokaryotic primers S-D-Bact-0008-a-S-20 (27F) (5′-AGAGTTTGATCCTGGCTCAG-3′) and S-*-Univ-1492R-b-A-21 (1492R) (5′-GGTTACCTTGTTACGACTT-3′). Polymerase chain reaction was performed in 50 μL volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of the primers, 1.5 U of Taq-polymerase (Ampliqon, Skovlunde, Denmark) and 100 ng of extracted DNA. Purified amplicons were digested with *Hae*III and *Hha*I restriction enzymes (Invitrogen Ltd, Paisley, UK) and electrophoresed in 2% agarose gels. These were visualised with ethidium bromide (0.5 μg/mL) (Sigma-Aldrich) and photographed under UV light.

Molecular identification of yeasts

Cell-free extract of yeasts, obtained by suspending a colony in water, boiling for 10 min and centrifugation, were used as a template in PCR reactions to amplify a segment of the eukaryotic rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2 using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′). The PCR conditions used were those reported by White *et al.* (1990).

Sequencing and comparison of sequences

Selected amplicons of bacteria and yeasts were purified in GenElute PCR Clean-Up columns (Sigma-Aldrich) and sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using primer 27F or ITS5, respectively. On average, 800 bp were obtained per sequence. These were compared with those in the GenBank database using the BLAST program (National Center for Biotechnology Information, 2009) and with those in the Ribosomal Database Project (RDP, 2009). Sequences with a percentage similarity of 97% or higher were allocated to the same species (Stackebrandt and Goebel 1994; Palys *et al.* 1997).

Typing of isolates

Isolates were grouped by repetitive extragenic palindromic PCR (REP-PCR) typing using primer BoxA2R (5′-ACGTGGTTTGAAGAGATTTTCG-3′), as reported by Koeuth *et al.* (1995). Repetitive extragenic palindromic PCR products were purified and electrophoresed in 1% agarose gels as discussed above.

Denaturing gradient gel electrophoresis (DGGE)

Extraction and purification of DNA

Homogenised NFM samples in 2% sodium citrate were used for isolation of total microbial DNA. DNA extraction was accomplished using a commercial kit (QIAamp DNA Stool Mini Kit; Quiagen, GmbH, Hilden, Germany) following the manufacturer's recommendations. To confirm and quantify the bacterial populations identified by DGGE in the NFM, DGGE analyses were also made of the total DNA extracted from enrichment cultures (on M17 and MRS) of the 10-fold dilutions used for enumeration purposes. The cells were pelleted by centrifugation and total microbial DNA isolated as reported above for the purified cultures.

PCR amplification

DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S rRNA gene using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′), to which a 39 bp GC sequence was linked to give rise to GC-F357, and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al.* 1993). The D1 domain of the 26S rRNA fungal gene was amplified using primers GC-NL1 (5′-GCCATATCAATAAGCGGAGGAAAAG-3′) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) (Cocolin *et al.* 2002). The amplification conditions for prokaryotic and eukaryotic sequences were those described by Muyzer *et al.* (1993) and Cocolin *et al.* (2002), respectively.

Electrophoresis conditions

DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60°C and employing 8% polyacrylamide gels with a denaturing range of 40–60% for bacteria and 30–50% for fungi. Electrophoresis was performed at 75 V for 17 h and at 130 V for 4.5 h for bacterial and fungal amplifications respectively. Bands were visualised after staining with ethidium bromide.

Identification of DGGE bands

DNA bands in the polyacrylamide gels were assigned to species by comparison with a control ladder of known strains (Flórez and Mayo 2006), or, following isolation of DNA from the bands and reamplification with the same primers without the GC-clamps, by sequencing and comparison of the sequences as described above.

RESULTS

The pH of the NFM samples ranged from 4.1 to 4.4. Table 1 shows counts for the majority and indicator microbial populations at the six sampling points, together with a diagram indicating the origin of the different samples analysed. The total cultivatable aerobic counts in PCMA matched those obtained in M17A for all six samples. This indicates that lactococci were the dominant population, reaching around 1.0×10^9 cfu/mL. Lactobacilli numbers were usually two logarithmic units lower than those for lactococci, although sample-to-sample variations were noted (Table 1). Dextran producing leuconostocs were occasionally observed at the very limit of detection (around 10^3 cfu/mL). No staphylococci, enterococci, enterobacteria or coliforms were detected in any of the samples, except for small numbers of the last three groups at the 6 month sample ($t = 6$). In addition, a homogeneous yeast population was recorded at 6 and 12a month samples, with counts of 2.8 and 5.0×10^5 cfu/mL respectively.

Because of the relatively simple microbial composition of the samples, the DGGE profiles were also expected to be rather simple. In fact, these varied between one or two bands. A patent band corresponding to *Lc. lactis* was always present, while that of *Lb. plantarum* was barely visible at time zero and 6 months. Figure 1 shows the DGGE results obtained at 3 months ($t = 3$), in which its DGGE profile is shown in line 1. As for the NFM, the profiles obtained from the cultures of the 10-fold dilutions involved either one or two bands. Cultures from the MRS plates (growth was up to the −3 dilution) gave a single band which migrated to the position of the *Lb. plantarum* control band. In contrast, profiles from the enrichment cultures grown on M17A showed two bands corresponding to *Lb. plantarum* and *Lc. lactis* up to the −3 dilution, followed by a band for *Lc. lactis* alone for the −4, −5 and −6 dilutions (Figure 1).

No DGGE profiles for eukaryotic organisms were recorded at time zero or at 3, 12b, and 15 months, while two bands whose sequence showed identity to both *Kazachstania unispora* and *Saccharomyces servazzii* were recorded at 6 and 12a months (data not shown).

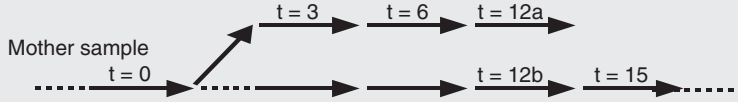
For the microbial characterisation of the NFM, 104 colonies from the M17A (54), MRSA (39) and MSEA (11) plates isolated from the six time points were purified by subculturing, and identified by molecular methods. All colonies were subjected to PCR amplification of the 16S rRNA genes with primers 27F and 1492R, followed by amplified ribosomal DNA restriction analysis (ARDRA) with the restriction enzymes *Hae*III and *Hha*I. Only two distinct ARDRA profiles were obtained with either *Hae*III or *Hha*I. As an example, Figure 2 shows the profiles obtained with *Hae*III. All isolates from the M17A plates gave the profile depicted in Figure 2(a), while Figure 2(b) shows that recorded for isolates from the MRSA plates. Two isolates from the MSEA plates showed an ARDRA profile identical to that shown by isolates from the M17A plates, while the remaining nine showed a profile identical to that shown by the cultures from MRSA plates. These results strongly

Table 1 Diagram of sampling and average microbial counts (in $\log_{10}$ cfu/mL) of different microbial groups in the natural fermented milk at four sampling times, and diagram of the sampling

	Sample[a] (month)					
Microbial group (counting medium)	*t = 0*	*t = 3*	*t = 6*	*t = 12a*	*t = 12b*	*t = 15*
Total aerobic counts (PCAM)	8.90 ± 0.31	8.49 ± 0.42	9.18 ± 0.28	8.65 ± 0.18	9.03 ± 0.35	8.68 ± 0.41
Lactococci (M17A)	8.94 ± 0.16	8.59 ± 0.23	9.10 ± 0.34	8.74 ± 0.10	8.95 ± 0.34	8.76 ± 0.21
Lactobacilli (MRSA, pH 5.4)	7.04 ± 0.36	5.70 ± 0.67	7.40 ± 0.46	5.84 ± 0.68	6.94 ± 0.72	6.54 ± 0.56
Leuconostoc (MSEA)	3.00[b]	3.25 ± 0.12	3.00[b]	3.00[b]	3.00[b]	3.46 ± 0.15
Enterococci (Slanetz-Bartley, SBA)	1.00[b]	1.00[b]	1.90 ± 0.12[b]	1.00[b]	1.00[b]	1.00[b]
Staphylococci (Baird-Parker, BPA)	1.00[b]	1.00[b]	1.00[b]	1.00[b]	1.00[b]	1.00[b]
Enterobacteriaceae (VRBGA)	nd	nd	1.30 ± 0.23	nd	nd	nd
Coliforms (VRBLA)	nd	nd	1.08 ± 0.18	nd	nd	nd
Yeasts and moulds (YGCA)	1.00[b]	1.00[b]	4.65 ± 0.46	4.69 ± 0.41	1.00[b]	1.00[b]

[a]Two replicates of each sample were analysed, from which counts were analysed in duplicate. Average results and standard deviation are indicated.

[b]The colonies with the typical morphology of the microbial groups to be counted were not detected. If these were present, they must have been below the detection limit.



nd, not detected.

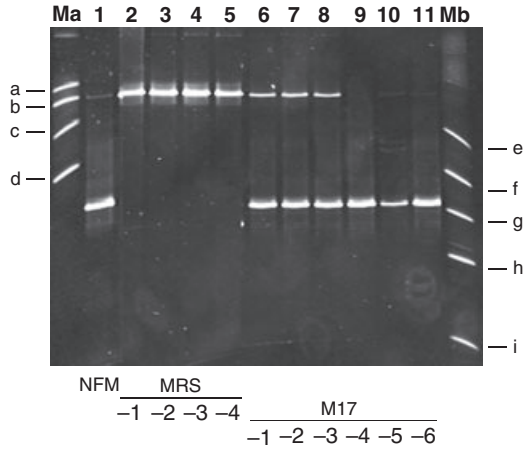


Figure 1 Denaturing gradient gel electrophoresis profiles of microbial populations from the natural fermented milk (NFM) at 3 months (lane 1) and those recovered after growth of the corresponding 10-fold dilutions on MRS and M17. M, combined amplicons of identified strains used as a control: Ma, *Lactococcus garvieae* (a), *Lactobacillus plantarum* (b), *Leuconostoc mesenteroides* subsp. *mesenteroides* (c), and *Streptococcus parauberis* (d). Mb, *Enterococcus faecium* (e), *Enterococcus faecalis* (f), *Lactococcus lactis* (g), *Escherichia coli* (h), and *Lactobacillus paracasei* (i).

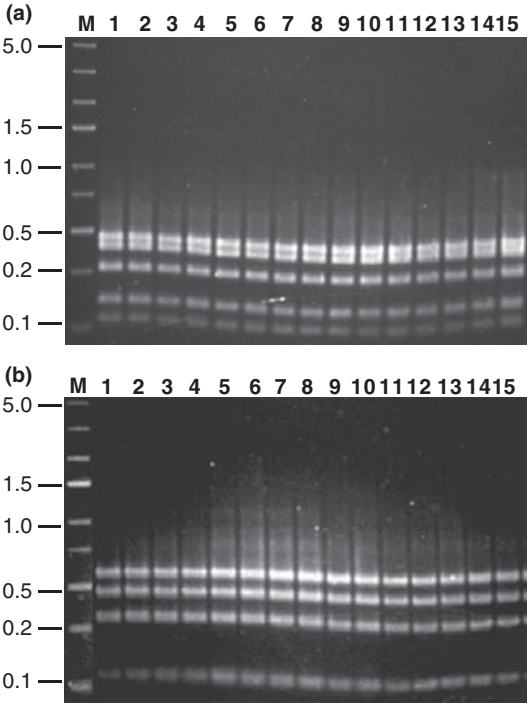


Figure 2 Partial amplified ribosomal DNA restriction analysis (ARDRA) profiles of 15 colonies isolated from the natural fermented milk on M17A (Panel a) and MRSA (Panel b). The 16S rRNA gene was amplified using primers S-D-Bact-0008-a-S-20 (27F) and S-*-Univ-1492R-b-A-21 (1492R) and digested with the restriction enzyme *Hae*III. M, molecular weight marker (GeneRuler1 kbp ladder; Fermentas GmbH, St. Leon-Rot, Germany).

suggest that the majority populations of the NFM recorded in M17A and MRSA plates were represented by a small number of bacterial species.

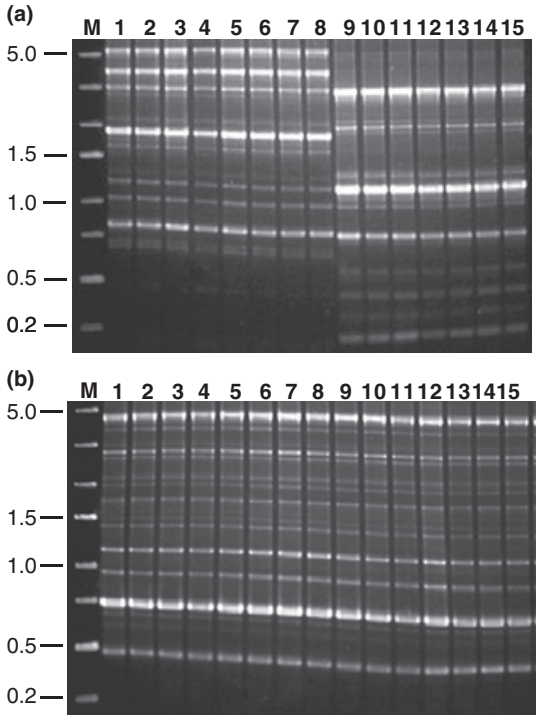


Figure 3 REP- PCR typing of lactococci and lactobacilli isolates from the natural fermented milk with primer BoxA2R. Panel a: Lanes 1-8, *Lc. lactis* subsp. *lactis* isolates; lanes 9-15 *Lc. lactis* subps. *cremoris* isolates. Panel b: Lane 1-15 *Lb. plantarum* isolates. M, GeneRuler molecular weight marker (Fermentas).

Sequencing of 15 randomly-chosen amplicons of colonies isolated from the three media identified these as *Lc. lactis* and *Lb. plantarum* respectively. It is noteworthy that for *Lc. lactis*, members of both the *lactis* and *cremoris* subspecies were detected during analysis of the sequences. Indeed, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* genotypes were represented as two distinct colony morphotypes on the M17A plates. This allowed the differential enumeration of *lactis* and *cremoris* subspecies in all NFM samples analysed. The two subspecies were encountered in every sample, with dominance alternating between the *lactis* and *cremoris*; e.g. percentages of *Lc. lactis* subsp. *lactis* ranged from 11% to 66%.

At 6 and 12a month samples, four yeast colonies each were identified using the eukaryotic-specific primers ITS4 and ITS5. As expected from the single morphotype observed on the enumeration plates, all sequences analysed corresponded to a single species – *K. unispora* (formerly known as *Saccharomyces unisporus*).

All 94 bacterial isolates were subjected to REP-PCR typing to assess the strain diversity of the *Lb. plantarum, Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. Surprisingly, a single REP profile was obtained for each species (Figure 3), which indicated a rather low genetic diversity at the strain level, similarly to that found at the species level.

DISCUSSION

Natural fermentation is one of the oldest methods of extending the shelf life of milk, and it is still widely practised in many parts of the world. This work describes the microbial characterisation of an NFM, analysed at six time points over one and a half years. The acquired NFM of the present study was produced by backslopping inoculation of UHT-sterilised milk, culturing at room temperature for 18–24 h, and storing under refrigeration until use. Depending on consumption, the process was repeated every 3–5 days. Both the temperature of incubation and the majority microorganisms identified (see below) classify this NFM as of the mesophilic type (Robinson and Tamime 1990). This is probably the largest group of fermented milks, into which fall many traditional products such as cultured *buttermilk, filmjölk, långfil,* and many ethnic products from Africa, the Middle East and Asia (Gadaga *et al.* 2000; Beukes *et al.* 2001; Gonfa *et al.* 2001; Benkerroum and Tamime 2004; Tamang 2010). The original batch of this NFM could be not traced back. It may well have come from somewhere in the Middle-East or the Balkans, but it has been passed from one family to another in the producing area for more than 10 years now, and it is well appreciated for its agreeable sensory properties.

The dominant microorganisms on the culture plates were identified by partial ARDRA and sequencing of the ribosomal amplicons, which where then compared against sequences held in public databases. A large population of a single strain each of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* formed the dominant cultivable population in the analysed NFM. Some kind of proto-cooperation might maintain the two *Lc. lactis* strains at similar numbers over consecutive inoculations. A single *Lb. plantarum* strain at around two $\log_{10}$ units lower numbers accompanied the two lactococcal strains. The same species were found by the culture-independent technique of DGGE, by which no other organisms were identified even after enrichment of the dilutions in M17 and MRS. These three strains were shown to be present throughout the entire study period (15 months), suggesting they are compatible and well adapted to one another. They also appear to be well adapted to the restrictive conditions imposed by the manufacturing process; cells have to attain high cell densities rapidly and to be resistant to the low pH (4.1–4.3) of the coagulated milk. These restrictive conditions are surely responsible for the low species and strain diversity found in this NFM.

Lc. lactis – both the *lactis* and *cremoris* subspecies – are among the dominant microbiota of most mesophilic NFM types (Gonfa *et al.* 2001; Mathara *et al.* 2004; Patrignani *et al.* 2006; Dewan and Tamang 2007). This is also the case of *Lb. plantarum,* which has been reported in the literature as a usual component of traditional NFMs manufactured from raw milk (Gonfa *et al.* 2001; Mathara *et al.* 2004; Dewan and Tamang 2007; El-Baradei *et al.* 2008). Growth of these three LAB types to high cell densities during fermentation produces lactic acid from lactose, causing the coagulation of milk when the isoelectric point of the caseins is reached (around pH 4.6). In addition, LAB metabolism modifies milk constituents (protein and fats) through their complex proteolytic and lipolytic systems (Leroy and de Vuyst 2004; Topisirovic *et al.* 2006). These activities contribute to the final sensorial characteristics of NFMs. Lactic acid further improves stability and safety of NFMs by inhibiting spoilage and pathogenic microorganisms (Topisirovic *et al.* 2006).

A large population of *K. unispora* was observed in the 6 month sample, which was maintained in the subsequent analysis at 12 months (t = 12a). This nonlactose fermenting species has been reported a common inhabitant of many dairy products, including kefir and cheese (Callon *et al.* 2006; Wang *et al.* 2008). It is unknown how this yeast species entered the present laboratory-produced NFM. At 6 months, small populations of enterococci and coliforms were also noted, which disappeared at 12 months. All of these 'new microorganisms' may have arise from a contaminated UHT-milk sample. Microbial analysis of the mother NFM sample at 12 and 15 month (t = 12b and t = 15 respectively) showed no yeasts, which reinforces the contamination hypothesis. In any event, in the laboratory-made NFM, the yeast seems to become a stable component of the microbial association without perturbing the relationships between the other members or impoverishing the sensorial properties of the NFM.

CONCLUSION

The results of this work provide a microbial characterisation of an undefined NFM, the fermentation of which appears to be accomplished by single strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* accompanied by a strain of *Lb. plantarum*. The isolation and characterisation of the component strains would allow a specific starter culture to be produced, which would further allow manufacture of this NFM at an industrial scale. This type of manufacture would contribute to the standardisation and marketing of the product while assuring its safety. Because of its high activity and stability, the bacterial combination might additionally be used as a starter culture for the manufacture of cheese and other dairy products. Stable consortia of LAB and/or yeasts with a potential industrial use as starter and/or adjunct cultures may be found in other NFMs.

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Tercera parte

TERCERA PARTE: CARACTERIZACIÓN DE MICROORGANISMOS DE INTERÉS TECNOLÓGICO

• Artículo V

Elena Fernández, Ángel Alegría, Susana Delgado, María Cruz Martín y Baltasar Mayo.
Comparative Phenotypic and Molecular Genetic Profiling of Wild *Lactococcus lactis* subsp. *lactis* Strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* Genotypes, Isolated from Starter-Free Cheeses Made of Raw Milk.
Applied and Environmental Microbiology, 2011. Volumen 77, número 15, páginas 5324-5335.

• Artículo VI

Ángel Alegría, Susana Delgado, Clara Roces, Beatriz López y Baltasar Mayo.
Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk.
International Journal of Food Microbiology. 2010. Volumen 143, número 1-2, páginas 60-66

• Artículo VII

Ángel Alegría, Susana Delgado, Ana Belén Flórez y Baltasar Mayo.
Identification, typing and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses.
Dairy Science and Technology, (Aceptado, DOI 10.1007/s13594-013-0128-3)

Para el diseño de nuevos fermentos, es esencial llevar a cabo un proceso de identificación tipificación y caracterización de un gran número de aislados de bacterias lácticas, con el fin de encontrar las más apropiadas para cada aplicación. De los trabajos precedentes, se consiguió una colección muy grande de aislados pertenecientes a distintos géneros y especies de BAL. Las cepas así obtenidas, y otras procedentes de estudios anteriores en nuestro laboratorio, constituyeron el material de estudio de este apartado. Para evaluar la adecuación de las distintas cepas como componentes de fermentos, se estudiaron las características tecnológicas (crecimiento y acidificación de la leche, producción de aromas deseables, producción de antimicrobianos, etc. y de seguridad (resistencias atípicas a antibióticos, producción de aminas biógenas) más importantes.

El primer grupo estudiado fueron veinte cepas de *Lactococcus lactis* aisladas de cinco quesos tradicionales asturianos elaborados con leche cruda sin adición de fermentos. Todos los aislados presentaban un fenotipo correspondiente a *Lc. Lactis* subsp. *lactis*, pero sus genotipos se correspondían con *Lc. lactis* subsp. *lactis* y con *Lc. lactis* subsp. *cremoris* (diez cepas con cada uno). Ya se ha mencionado en la introducción cómo *Lc. lactis* subsp. *cremoris* parece ser un fermento más adecuado para la elaboración de algunos tipos de queso; también la polémica que existe en torno a la existencia de dos fenotipos y dos genotipos en *Lc. lactis* sin correspondencia entre ellos, y la imposibilidad de aislar nuevas cepas de *Lc. lactis* subsp. *cremoris* en productos lácteos tradicionales fuera del entorno de su aislamiento original en el norte de Europa. En este contexto, este trabajo se centró en determinar las características fenotípicas y genéticas de las cepas pertenecientes a cada uno de los genotipos procedentes de nuestros quesos tradicionales. Los resultados obtenidos respaldan la idea de que estos dos genotipos, *lactis* y *cremoris,* se corresponden con verdaderas subespecies que pueden distinguirse por pruebas fenotípicas y genéticas, aunque no mediante las pruebas clásicas. Los ensayos realizados indican, además, que algunas cepas de las dos subespecies presentan buenas aptitudes para formar parte de cultivos iniciadores.

En un segundo trabajo se determinó la producción de compuestos antimicrobianos en cepas de *Lc. lactis* de nuestra colección. Este trabajo tuvo dos

finalidades: (i) las cepas que integren un fermento han de ser compatibles, sin que exista la posibilidad de que una cepa produzca sustancias que inhiban o maten a las otras, y (ii) las cepas productoras de bacteriocinas, tal y como se comentó en la introducción, pudieran ser utilizadas de manera independiente como cultivos protectores, dada la condición que tienen estos compuestos como conservadores naturales. Entre los 305 aislados analizados, se identificaron 11 cepas de *Lc. Lactis* subsp. *lactis* productoras de nisina, la bacteriocina más potente producida por una bacteria láctica y la única autorizada como aditivo alimentario en determinados productos lácteos. Encontramos también otras cinco cepas de *Lc. Lactis* subsp. *lactis* productoras de lactococina 972, de la que solo se conocía la cepa productora original (*Lc. lactis* subsp. *lactis* IPLA 972), y una única cepa de *Lc. Lactis* subsp. *cremoris* que producía una variante de la lactococina G/Q. Dada su potencial aplicación, algunas de estas cepas se han transferido a una empresa de fermentos para que evalúe a escala industrial su utilidad en sistemas alimentarios frente a microorganismos patógenos y alterantes.

En el último trabajo que se relaciona, se estudiaron un conjunto de 42 aislados de *Leuconostoc* procedentes de diversos quesos tradicionales. Mediante técnicas moleculares, los aislados se asignaron a una de las siguientes especies: *Leuconostoc citreum* (24), *Leuconostoc mesenteroides* (13) y *Leuconostoc lactis* (5). Tras la identificación, se estudió la variación intraespecífica de los aislados mediante técnicas de tipificación basadas en la PCR. Tras una selección preliminar basada en su crecimiento en leche, se estudiaron en mayor profundidad las características tecnológicas y de seguridad de 14 cepas distintas de las tres especies, lo que nos permitió identificar un apropiado grupo de ellas como integrantes de cultivos adjuntos.

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Comparative Phenotypic and Molecular Genetic Profiling of Wild *Lactococcus lactis* subsp. *lactis* Strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* Genotypes, Isolated from Starter-Free Cheeses Made of Raw Milk▽

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Twenty *Lactococcus lactis* strains with an *L. lactis* subsp. *lactis* phenotype isolated from five traditional cheeses made of raw milk with no added starters belonging to the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* genotypes (*lactis* and *cremoris* genotypes, respectively; 10 strains each) were subjected to a series of phenotypic and genetic typing methods, with the aims of determining their phylogenetic relationships and suitability as starters. Pulsed-field gel electrophoresis (PFGE) analysis of intact genomes digested with SalI and SmaI proved that all strains were different except for three isolates of the *cremoris* genotype, which showed identical PFGE profiles. Multilocus sequence typing (MLST) analysis using internal sequences of seven loci (namely, *atpA*, *rpoA*, *pheS*, *pepN*, *bcaT*, *pepX*, and 16S rRNA gene) revealed considerable intergenotype nucleotide polymorphism, although deduced amino acid changes were scarce. Analysis of the MLST data for the present strains and others from other dairy and nondairy sources showed that all of them clustered into the *cremoris* or *lactis* genotype group, by using both independent and combined gene sequences. These two groups of strains also showed distinctive carbohydrate fermentation and enzyme activity profiles, with the strains in the *cremoris* group showing broader profiles. However, the profiles of resistance/susceptibility to 16 antibiotics were very similar, showing no atypical resistance, except for tetracycline resistance in three identical *cremoris* genotype isolates. The numbers and concentrations of volatile compounds produced in milk by the strains belonging to these two groups were clearly different, with the *cremoris* genotype strains producing higher concentrations of more branched-chain, derived compounds. Together, the present results support the idea that the *lactis* and *cremoris* genotypes of phenotypic *Lactococcus lactis* subsp. *lactis* actually represent true subspecies. Some strains of the two subspecies in this study appear to be good starter candidates.

Lactococcus lactis is a lactic acid bacterium (LAB) commonly dominant in milk and fermented dairy products. Not surprisingly, carefully selected strains of *L. lactis* are majority components of starter cultures for dairy fermentations (38). Worldwide, over 100 million metric tons of milk is transformed annually into dairy products using *L. lactis* starters, reflecting the industrial and thus economic importance of this organism (38). The growth of *L. lactis* in milk is associated with the rapid production of lactic acid, which provides flavor, assists in curd formation, prevents the growth of pathogenic and spoilage bacteria, and creates optimal biochemical conditions for ripening. Via their proteolytic and amino acid conversion pathways, lactococci further contribute to the final texture (moisture, softness) and flavor of dairy products (47). These functions determine the sensory quality, safety, and shelf life of fermented dairy products. *L. lactis* includes three subspecies (*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *hordniae*) plus a diacetyl-forming biovariety (*L. lactis* subsp. *lactis* biovar diacetylactis). The lactose-negative *L. lactis* subsp. *hordniae* (45) has never been found in dairy products. *L. lactis* subsp. *lactis* is distinguished from *L. lactis* subsp. *cremoris* according to five phenotypic criteria: the ability to grow at 40°C, in 4% NaCl, and at pH 9.2, the ability to ferment maltose, and the capacity to deaminate arginine (25, 45), for all of which *L. lactis* subsp. *cremoris* strains are reported to be negative. In addition, *L. lactis* subsp. *lactis* biovar diacetylactis is distinguished by its ability to assimilate citrate, which is converted into diacetyl, a potent odorous compound.

Current dairy lactococcal starters are thought to be derived from a small number of well-adapted, genetically related lineages showing similar genetic profiles and phenotypic properties (24, 39, 55). Therefore, there is a great demand for new strains to solve technological problems such as insufficient acid production, frequent culture failure resulting from the attack of bacteriophages, and the development of undesirable flavors (28, 33, 37, 54). In addition, interest is spurred by the continuing search for strains that harbor unique flavor-forming activities (3) or that produce novel, broad-range antimicrobial agents (6). Strains with these properties might be of use in traditional fermentations but also might allow new processes to be developed.

For some cheese types, *L. lactis* subsp. *cremoris* strains are the preferred starter since their growth response in milk is better and because of the typical aroma profiles associated with

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them (42, 54). Although found in dairy environments, the natural niche of this subspecies remains elusive, and claims of having isolated novel *L. lactis* subsp. *cremoris* strains from milk and naturally fermented products are regarded as controversial (30, 33, 42, 54). Furthermore, distinction between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* is difficult since it is based on a set of phenotypic characteristics that may show strain-to-strain variation. Further, some strains of *L. lactis* showing an *L. lactis* subsp. *lactis* phenotype according to the classical distinction criteria have long been known to show a *L. lactis* subsp. *cremoris* genotype (*cremoris* genotype) (27). Recently, phenotypic *L. lactis* subsp. *cremoris* showing a *L. lactis* subsp. *lactis* genotype (*lactis* genotype) have also been reported (30, 52). Therefore, the *L. lactis* species has an unusual structure with two phenotypically distinct groups defining *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, which may belong to two distinct genotype groups (30, 36, 40, 52, 54). This makes the accurate identification of new isolates very difficult, yet this is a crucial first step in the development of new cultures. In addition, the phenotypic and genetic relationships between the subspecies of *L. lactis* and even within subspecies remain unclear.

As the use of molecular genetic techniques became universal, strains with an *L. lactis* subsp. *cremoris* genotype have been isolated from many sources, including vegetables and plants (28, 37) and milk and dairy products (11, 15, 18, 34, 43, 54). A few strains have also been isolated from Spanish traditional, starter-free cheeses made from raw milk (14, 21, 35).

This work reports a comparative phenotypic, genotypic, and technological characterization of 20 strains with an *L. lactis* subsp. *lactis* phenotype, 10 each belonging to the *lactis* and *cremoris* genotypes. To compare their properties and assess their functionality, the strains were subjected to genetic fingerprinting, carbohydrate fermentation tests, enzyme activity profiling, and antibiotic resistance-susceptibility assays. Growth and production of volatile compounds in milk were also examined. These studies allowed the molecular genetic and phenotypic profiles of the strains belonging to the two genotypes to be compared, to make comparisons with results in the literature, and to propose a new classification for the members of this species.

MATERIALS AND METHODS

Strains, media, and culture conditions. The bacteria studied were *L. lactis* subsp. *lactis* strains, 10 belonging to the *lactis* genotype and 10 belonging to the *cremoris* genotype. They were all isolated during the manufacturing and ripening stages of five traditional, Spanish cheeses made from raw milk without the deliberate addition of commercial starter cultures, which implies that the isolates were all wild strains. The cheese origins of the different strains are as follows: Cabrales (*L. lactis* subsp. *lactis* L39, 1AA59, 3AA15, 2BA36, and 4AA10 of the *lactis* genotype and *L. lactis* subsp. *lactis* 1AA23, 3AA9, 3AA11, 3AA23, and 4AA28 of the *cremoris* genotype), Peñamellera (*L. lactis* subsp. *lactis* 1A38 and 2A83 of the *lactis* genotype and *L. lactis* subsp. *lactis* 2A5, 2A22, and 2A27 of the *cremoris* genotype), Genestoso (*L. lactis* subsp. *lactis* GE-1 of the *lactis* genotype and *L. lactis* subsp. *lactis* GE2-14 of the *cremoris* genotype), and Casín (*L. lactis* subsp. *lactis* CAS3 and Q1-6 of the *lactis* genotype and *L. lactis* subsp. *lactis* LC44 of the *cremoris* genotype). These strains were previously identified by the sequencing of the 16S rRNA gene and comparison of the sequences against those in the GenBank and Ribosomal Database Project II databases (1).

L. lactis subsp. *lactis* CECT 185^T (ATCC 19435^T) (*lactis* genotype, *L. lactis* subsp. *lactis* phenotype), *L. lactis* subsp. *cremoris* CECT 967^T (NCDO 607^T) (*cremoris* genotype, *L. lactis* subsp. *cremoris* phenotype), *L. lactis* subsp. *lactis* MG 1363 (*L. lactis* subsp. *lactis* phenotype, *cremoris* genotype), and *L. lactis* subsp. *lactis* IL 1403 (ex-phenotype *L. lactis* subsp. *lactis* biovar diacetylactis, *lactis* genotype) strains were used as controls throughout this study. Unless otherwise stated, strains were grown statically in M17 (Scharlab, Barcelona, Spain) broth at 30°C for 18 to 24 h.

Molecular identification of strains. The identifications of the isolates were verified by molecular methods, which included partial amplified rRNA gene restriction analysis (ARDRA), sequencing, and sequence comparison. For this, total genomic DNA was purified from overnight cultures using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the DNA was stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. ARDRA was performed after amplification of the 16S rRNA genes with the bacteria-specific universal primer 27F (S-D-Bact-0008-a-S-20; 5′-AGAGTTTGATCCTGGCTCAG-3′) and the bacteria/archaea-specific primer 1492R (S-*-Univ-1492R-b-A-21; 5′-GGTTACCTTGTTACGACTT-3′). Amplicons were purified using GenElute PCR clean-up columns (Sigma-Aldrich), digested with the restriction enzymes HaeIII and HhaI (Invitrogen Ltd., Paisley, United Kingdom), and electrophoresed as described above. Both strands of the amplicons were sequenced using both 27F and 1942R primers; the sequences were then aligned and compared to those in databases.

Restriction fragment length polymorphism (RFLP) typing by PFGE. Intact genomic DNA from *L. lactis* strains was isolated and digested in agarose plugs as described by Howard et al. (26). Purified DNA was digested independently with 20 U of the restriction enzymes SmaI and SalI (Boehringer Mannheim, Mannheim, Germany) for 18 h at 37°C in the restriction buffer recommended by the manufacturer. DNA digests were separated in a contour-clamped homogeneous electric field (CHEF) in a CHEF-DRII apparatus (Bio-Rad, Richmond, CA). Low-range and bacteriophage lambda ladder pulsed-field gel electrophoresis (PFGE) markers were obtained from New England BioLabs (Ipswich, MA). Electrophoresis was carried out in 1% FastLane agarose gels (FMC Corporation, Philadelphia, PA) in 0.5× TBE (Tris-borate-EDTA) for 20 to 24 h at 14°C and 6 V cm^{-1}. Pulse times ranged from 0.5 to 25 s for 12 h and from 25 to 50 s for 6 h for the SmaI digests and from 0.5 to 5 s for 12 h and from 5 to 30 s for 8 h for the SalI digests. Similarity clustering was performed with the MultiVariate Statistical Package (MVSP; Kovach Computing Services, Anglesey, Wales, United Kingdom) using the unweighted-pair group method with arithmetic averages and Sorensen's correlation.

MLST analysis. DNA sequence analysis of 350 to 861 bp of intragenic regions of the genes encoding the ATP synthase alpha subunit (*atpA*), the phenylalanyl-tRNA synthase alpha subunit (*pheS*), the RNA polymerase alpha subunit (*rpoA*), the branched-chain aminotransferase (*bcaT*), the peptidase N (*pepN*), and the X-prolyl dipeptidyl aminopeptidase (*pepX*) was performed employing the oligonucleotides and PCR conditions reported by Rademaker et al. (40). For multilocus sequence typing (MLST) analysis, forward and reverse sequences were trimmed, aligned, and analyzed using MEGA (version 4) software (51). Sequences were then compared to one another, and the similarities of the patterns were analyzed by the neighbor-joining method.

Phenotypic characterization. Phenotypic analysis of the strains was done in filter-sterilized medium, as follows. Growth at 40 and 45°C was tested in Elliker broth (Scharlau, Barcelona, Spain) and examined daily for up to 5 days. Similarly, growth in 4% and 6.5% NaCl and at pH 9.2 and 9.6 was assayed in Elliker broth at 30°C and checked daily for up to 5 days. To test for arginine hydrolase activity, strains were grown for 48 h at 30°C in an arginine broth composed of peptone (5%), tryptone (0.5%), yeast extract (0.5%), K_2HPO_4 (0.2%), L-arginine (0.5%), dextrose (0.05%), $MgSO_4$ (250 mg/liter), and ascorbic acid (0.5 g/liter), pH 7.0. After incubation, cells were removed by centrifugation and 10 μl of the supernatant was mixed with a drop of Nessler's reagent (KI [5 g], $HgCl_2$ [5 g], NaOH [4 g], and 100 ml of filter-sterilized H_2O). Strains were recorded as negative or weakly or strongly positive, judged by the intensity of their orange coloration.

The carbohydrate fermentation profiles of the isolates and control strains were determined using the commercial PhenePlate system (Bactus, Stockholm, Sweden) as recommended by the supplier. Additionally, strains were examined using the API 20 Strep kit following the manufacturer's recommendations (bioMérieux, Montalieu-Vercieu, France).

In addition to the Voges-Proskauer test of the API 20 Strep system, acetoin (acetylmethylcarbinol) production was further analyzed in Clark and Lubs medium (casein and meat peptone [3.5 g/liter each], dextrose [5 g/liter], potassium phosphate [5 g/liter], pH 6.9) with incubation at 30°C for 72 h. To a 2.5-ml aliquot of the cultures, 0.6 ml of Barritt's reagent A (5% [wt/vol] α-naphthol in absolute ethanol) was added, followed by 0.2 ml of reagent B (40% [wt/vol] KOH in

water). Reagents were mixed, and the contents of the tubes were left to settle for 10 min. Strains were recorded as negative or weakly or strongly positive, judged by the intensity of their red coloration.

Citrate assimilation is also included among the API 20 Strep tests. Citrate utilization was further assayed in Kempler and McKay medium (31) under anaerobic conditions in the dark at 30°C for 44 to 72 h.

Enzyme activities were measured using the commercial, semiquantitative API ZYM system (bioMérieux) following the manufacturer's recommendations. Sixty-five microliters of a cell suspension corresponding to McFarland standard 5 (spectrophotometric equivalent, 3×10^9 CFU ml^{-1}) was inoculated into each well of the API ZYM strips, and the strips were incubated for 4 h at 30°C and developed as recommended.

MICs of antibiotics were determined by microdilution in VetMIC plates for LAB (National Veterinary Institute of Sweden, Uppsala, Sweden) containing 2-fold serial dilutions of 16 antibiotics. Colonies grown on LAB susceptibility test medium (LSM) (32) agar plates were suspended in 2 ml of sterile saline solution (Oxoid, Basingstoke, Hampshire, United Kingdom) to obtain a density corresponding to McFarland standard 1 (spectrophotometric equivalent, 3×10^8 CFU ml^{-1}). The suspension was further diluted 1:1,000 with LSM (final cell concentration, 3×10^5 CFU ml^{-1}). One hundred microliters of this inoculum was added to each well of the VetMIC plate, which was incubated at 28°C for 48 h. The MICs were defined as the lowest antibiotic concentration at which no visible growth was observed. The presence of tetracycline resistance genes was checked by PCR using the universal primers for genes encoding the ribosomal protection proteins DI (5′-GAYACICCIGGICAYRTIGAYTT-3′) and DII (5′-GCCCAR WAIGGRTTIGGIGGIACYTC-3′) (10) and using the PCR conditions described by the latter authors. Amplicons were purified and sequenced, and the sequences were compared against those in GenBank.

Growth and acidification of milk. Acid production was determined in UHT milk (Corporacion Alimentaria Peñasanta, S.A. [CAPSA], Siero, Spain). A 1% inoculum from an overnight M17 culture was washed in sterile water and used to inoculate the milk, which was then incubated at 22°C; samples were scored for clotting at 15, 18, and 24 h. The pH was measured at 24 h using a pH meter (Crison Instruments S.A., Barcelona, Spain). The appearance of the coagulum (whey drainage, curd firmness, presence of gas bubbles, curd breaking) was also recorded by visual inspection.

Production of volatile compounds in milk. The volatile compounds produced in milk were determined after growth of the strains in 10 ml of UHT milk (CAPSA) at 30°C for 2, 5, and 21 days. Cultures were grown in screw-cap tubes with a rubber liner to prevent the escape of volatiles, supplied with 100 μl of internal standard (cyclohexanone, 0.36 mg ml^{-1}), and stored at −80°C until analysis. The separation and quantification of volatile compounds were carried out by headspace (HS)/gas chromatography (GC)/mass spectrometry (MS) analysis using a combined system composed of the units G 1888 HS, 6890 GC, and 5975B MSD, respectively (Agilent Technologies, Wilmington, DE), equipped with an HP-Innovax capillary column (60 m by 0.25 μm; Agilent). Sample preparation and gas chromatographic separation were performed as described by Salazar et al. (44). Peaks were quantified as the relative total ionic count abundance with respect to the internal standard. The concentrations (μg/ml) of some volatile compounds (acetaldehyde, diacetyl, 2-propanone, acetic acid, 2-butanone, and ethanol) were calculated, using linear regression equations ($R^2 \geq 0.99$), from the standard curve obtained using five representative concentrations.

Nucleotide sequence accession numbers. The partial sequences of the seven genes examined in the MLST analysis were deposited in the GenBank database under accession numbers JF297335 through JF297474.

RESULTS

Partial ARDRA with the restriction enzymes HaeIII and HhaI, followed by sequencing, unequivocally identified all 20 strains as belonging to *L. lactis.* As expected from previous *in silico* analyses, 10 showed the *lactis* and 10 showed the *cremoris* genotype. The digestion profiles of amplicons of both genotypes were identical with HaeIII, but they gave distinct banding patterns with HhaI. The ribosomal sequences of the strains showing a *lactis* genotype were mostly identical, matching the 16S rRNA sequence of the *L. lactis* subsp. *lactis* type strain (ATCC 19435^T) and the 16S rRNA sequences of the sequenced strains *L. lactis* subsp. *lactis* IL 1403 and *L. lactis* subsp. *lactis* KF147, except for a single nucleotide change in the sequences of strains GE-1 and 2BA36 (adenine for guanine at positions 91 and 465 of the IL 1403 numbering, respectively). The sequences of the strains with the *cremoris* genotype differed in 10 positions from those of the *lactis* genotype: positions 70, 76, 82, 87, 91, 93, 95, 98, 183, and 195. Two distinct sequences were found among the strains with the *cremoris* genotypes. The sequences varied in a single nucleotide at position 183, corresponding to cytosine in six strains (2A5, 2A22, 2A27, 3AA9, 3AA23, and 4AA28) and to thymine in four strains (1AA23, 3AA11, GE2-14, and LC44).

To assess the genetic diversity and relatedness of the strains, all were subjected to RFLP-PFGE analysis. *L. lactis* subsp. *lactis* MG 1363 (*L. lactis* subsp. *lactis* phenotype, *cremoris* genotype) and *L. lactis* subsp. *lactis* IL 1403 (ex-phenotype *L. lactis* subsp. *lactis* biovar diacetylactis, *lactis* genotype) were included as a control. Figure 1 shows the RFLP profiles obtained with the enzyme SalI and the clustering of the strains in terms of the Sorensen's coefficient of similarity. All *L. lactis* isolates of the *lactis* genotype were shown to be unrelated, as they shared a low similarity index. On the contrary, two isolates of the *cremoris* genotype from the same cheese sample (3AA9 and 3AA23) proved to be related (similarity index, 0.89). Moreover, three other *cremoris* genotype isolates (2A5, 2A22, and 2A27) from a single cheese batch showed identical digestion profiles, which indicates that all three could be replicates of the same strain. Similar PFGE results were obtained after digestion with SmaI (data not shown). However, the DNA of three strains (one belonging to the *lactis* genotype, GE-1, and two of the *cremoris* genotype, 3AA9 and 3AA23) was shown to be resistant to digestion with the last enzyme, which prevented a proper strain comparison. SmaI digestions allowed the estimation of the size of the chromosomes, which were shown to range approximately from 2,250 to 2,600 kbp for the *lactis* genotypes and 2,400 to 2,650 kbp for the *cremoris* genotypes. In conclusion, all 10 strains with the *lactis* genotype and at least 8 strains with the *cremoris* genotype could be considered different. In spite of this, isolates were all independently subjected to further genetic analyses and biochemical tests.

The genetic diversity of the *lactis* and *cremoris* genotype strains was further evaluated by MLST using partial nucleotide sequences of six genes, *atpA*, *rpoA*, *pheS*, *pepN*, *bcaT*, and *pepX* and the gene coding for 16S rRNA (Table 1). Amplicons and sequences from all these genes of all strains were obtained and aligned. Nucleotide positions of the sequences showing ambiguities were excluded from the analysis. Unique sequence types (STs) were obtained for every strain. After individual analysis of the seven genes and all genes together, two consistent and distinct clusters with similar topologies were obtained for the *lactis* and *cremoris* genotype strains (Fig. 2). The number of polymorphic sites varied strongly from gene to gene, ranging from 12 in 16S rRNA genes to 94 in *pepX* (Table 1). However, most of these nucleotide variations corresponded to nucleotide differences among strains of the *lactis* and *cremoris* genotypes, indicating that the actual number of alleles in the two species is much lower (from one to six; Table 1). Further, a majority of the substitutions were synonymous (they did not result in amino acid changes), as inferred from the low ratio of the number of nonsynonymous substitutions by the number of synonymous substitutions (*dN*/*dS* ratio; it was particularly low

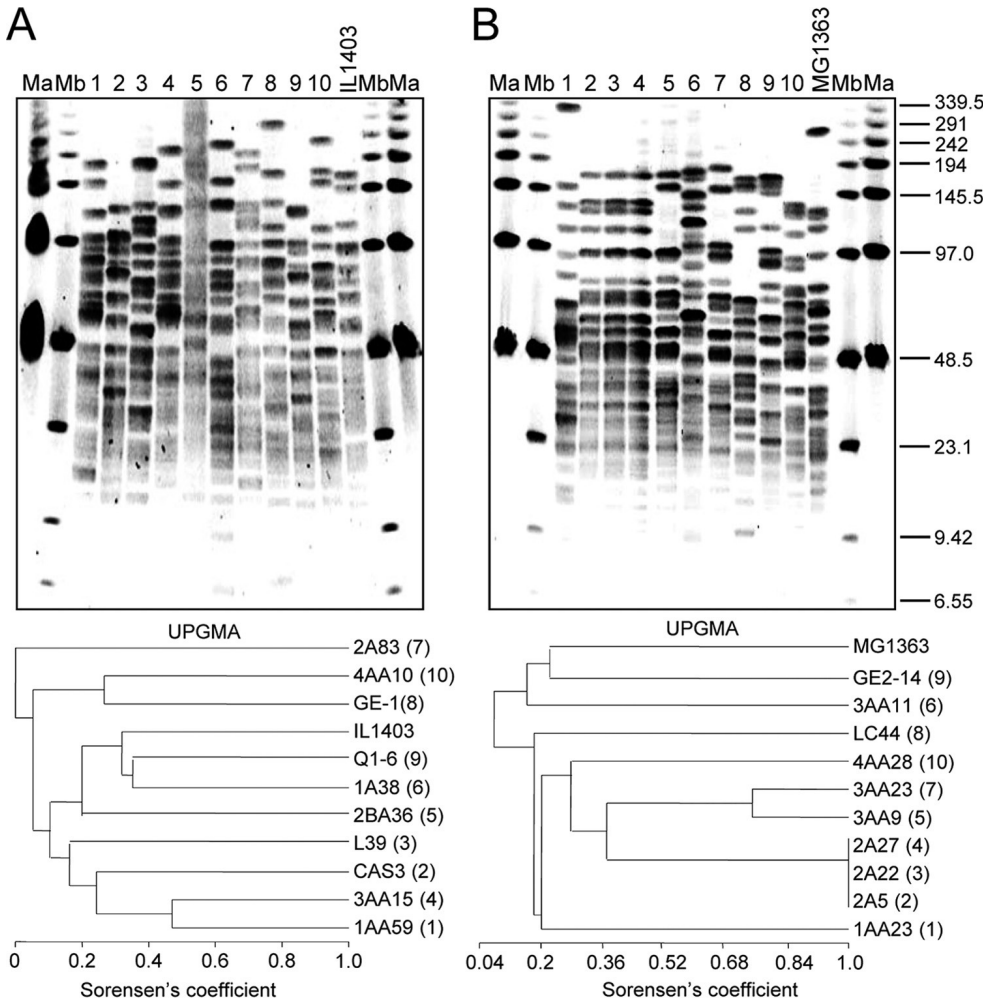

FIG. 1. PFGE patterns of SalI-digested genomic DNA from *L. lactis* isolated from starter-free cheeses made of raw milk of *lactis* (A) and *cremoris* (B) genotypes. (A) Lanes 1 to 10, *L. lactis* genotype *lactis* 1AA59, CAS3, L39, 3AA15, 2BA36, 1A38, 2A83, GE-1, Q1-6, and 4AA10, respectively. (B) Lanes 11 to 20, *L. lactis* genotype *cremoris* 1AA23, 2A22, 2A27, 2A5, 3AA23, 3AA11, 3AA9, LC44, GE2-14, and 4AA28, respectively. IL 1403, *L. lactis* genotype *lactis* IL 1403; MG 1363, *L. lactis* genotype *cremoris* MG 1363. Lanes Ma and Mb, low-range and bacteriophage lambda ladder PFGE markers (New England BioLabs), respectively. Dendrograms of the similarity of the profiles of the strains in the respective panel expressed by the Sorensen's coefficient are shown below the panels. Clustering was performed by the unweighted-pair group method using arithmetic averages (UPGMA). Figures in parentheses after the strain code indicate the lane in the gels.

for the *bcaT* gene), which indicates conservation during evolution of amino acid sequences between members of the two subspecies. Together, the above results strongly suggest that the two clusters are composed of individual organisms showing a high degree of intracluster genetic similarity.

Since the same gene stretches as those reported by Rademaker et al. (40) were amplified and sequenced, the sequences obtained in the present work were trimmed in the same manner used by the latter authors. Sequences were then submitted to a recently developed MLST database for *L. lactis* (http://www-mlst.biotoul.fr/). Composite sequences of all seven genes were aligned with those of representative strains from the study of Rademaker et al (40). This allowed the relatedness of the present cheese isolates to be compared at the DNA level with those of lactococci from dairy and nondairy sources (Fig. 3). In the present study, most strains of both the *lactis* and *cremoris* genotypes clustered together, indicating greater similarity among themselves than to the dairy strains of Rademaker et al (40). Strains from different traditional cheeses grouped together, even though they were isolated from geographical areas more than 200 km apart, indicating that related STs are widespread. At the same time, some other strains from the same cheese batch clustered separately, suggesting the presence of unrelated STs. The *cremoris* genotype strains were split into two groups, one of which seems to be related to *L. lactis* subsp. *lactis* MG 1363 and some other strains with the *L. lactis* subsp. *lactis* phenotype and *cremoris* genotype (Fig. 3), and one of which is apparently closer to the true (phenotypic) *L. lactis* subsp. *cremoris* strains.

Table 2 summarizes the conventional tests used to distinguish the subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and the results obtained. All the strains studied hydrolyzed arginine and grew in 4% NaCl and at 40°C. All but four of the strains hydrolyzed hippurate, and all but the genotype *cremoris* type strain (*L. lactis* subsp. *cremoris* CECT 967^T) grew at pH 9.2. In contrast, none of the strains used citrate,

TABLE 1. Genetic diversity at seven loci based on the nucleotide sequences used for MLST analysis and that of the 16S rRNA genes of 20 *L. lactis* subsp. *lactis* strains of both *lactis* and *cremoris* genotypes (10 each) used in this study

Locus	Length (bp)			G+C content (%)	No. of polymorphic sites	No. alleles by genotype		*dN*/*dS* ratio[a]
	Gene	Amplified fragment	Analyzed fragment			*lactis*	*cremoris*	
atpA	1,503	1,141	861	42	49	4	1	0.30
rpoA	939	814	721	39	15	4	4	0.26
pheS	2,533	618	477	42	45	6	3	0.13
bcaT	1,047	493	350	42	50	3	2	0.02
pepN	1,023	482	473	37	46	1	2	0.10
pepX	2,269	602	508	39	94	4	2	0.20
16S rRNA gene	1,548	1,465	605	49	12	3	2	NA[b]

[a] The *dN*/*dS* ratio was calculated by dividing the number of nonsynonymous substitutions by the number of synonymous substitutions.
[b] NA, not applicable.

and single strains grew well at 45°C and in 6.5% NaCl. Strains did not give a positive reaction in the Voges-Proskauer test, except for a weak reaction of the *L. lactis* subsp. *cremoris* type strain, suggesting that none of the strains belonged to the biovar diacetylactis. Few phenotypic differences were seen between strains belonging to the *lactis* and *cremoris* genotypes; the most notable was growth at pH 9.6, for which all *cremoris* genotypes proved to be positive (although growth was weak), whereas only two strains of the *lactis* genotype were positive. In conclusion, according to the classical phenotypic criteria, the biochemical assays identified all 20 *L. lactis* cheese strains as belonging to the *L. lactis* subsp. *lactis* subspecies.

Carbohydrate fermentation profiles were analyzed by the combined use of the PhenePlate and API 20 Strep systems, in which the utilization of 51 sugar and polyalcohols was examined. The two systems always showed concordant results with the carbohydrates tested. Table 3 shows the combined results. Strain-to-strain variations were found among both the *lactis* and *cremoris* genotypes. However, the fermentation profiles shown by the strains of the *cremoris* genotype were wider owing

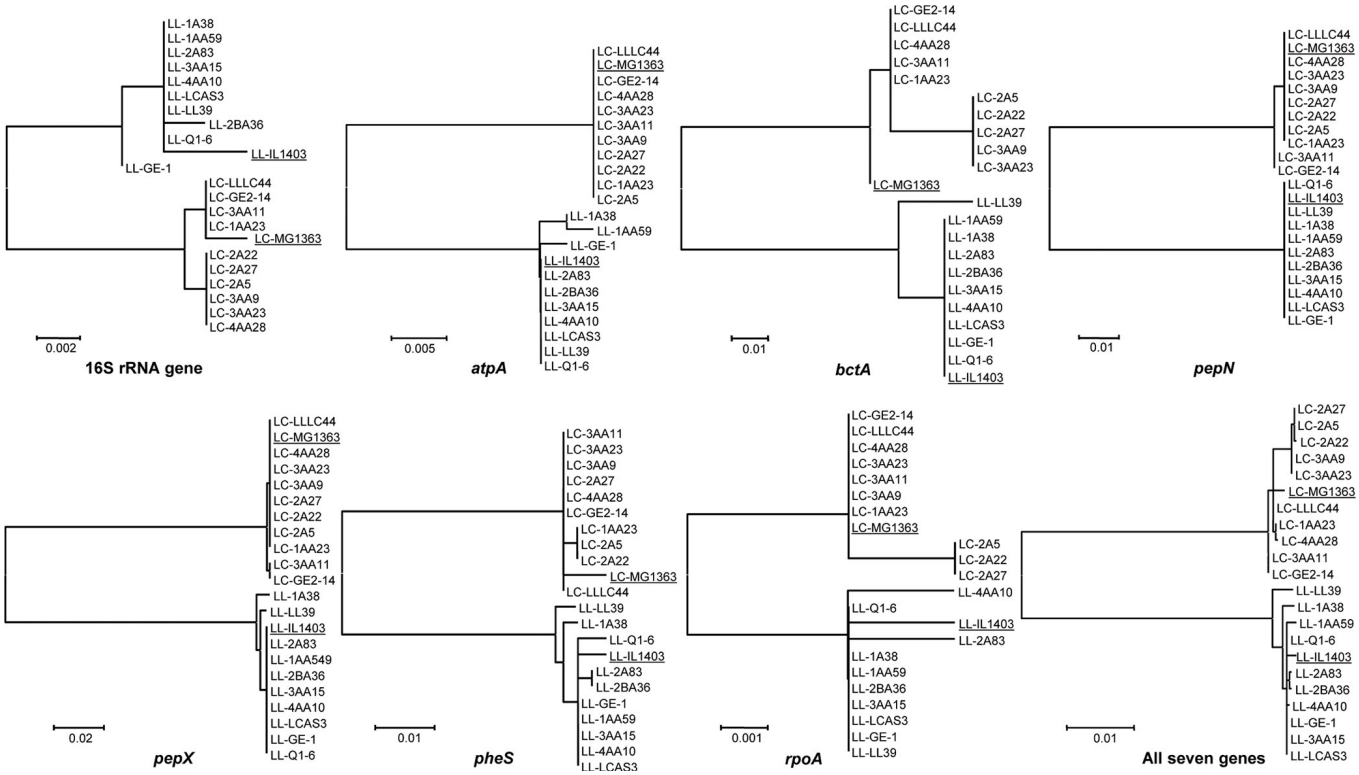

FIG. 2. Neighbor-joining cluster analysis of individual partial DNA sequences of the genes coding for 16S rRNA and the housekeeping protein-encoding genes *atpA*, *rpoA*, *bacT*, *pepN*, *pepX*, and *pheS* from 10 wild strains of *L. lactis* of the *lactis* genotype and 10 wild strains *L. lactis* of the *cremoris* genotype, as well as a seven-locus MLST analysis based on the composite data set for the seven genes. Bootstrap percentages (≥50) after 500 simulations are shown for single and composite sequence analyses. Sequences of the genome-sequenced *L. lactis* IL 1403 (*lactis* genotype) and MG 1363 (*cremoris* genotype) strains were used as a control and are underlined. LL and LC, *L. lactis* subsp. *lactis* having a *lactis* genotype and a *cremoris* genotype, respectively.

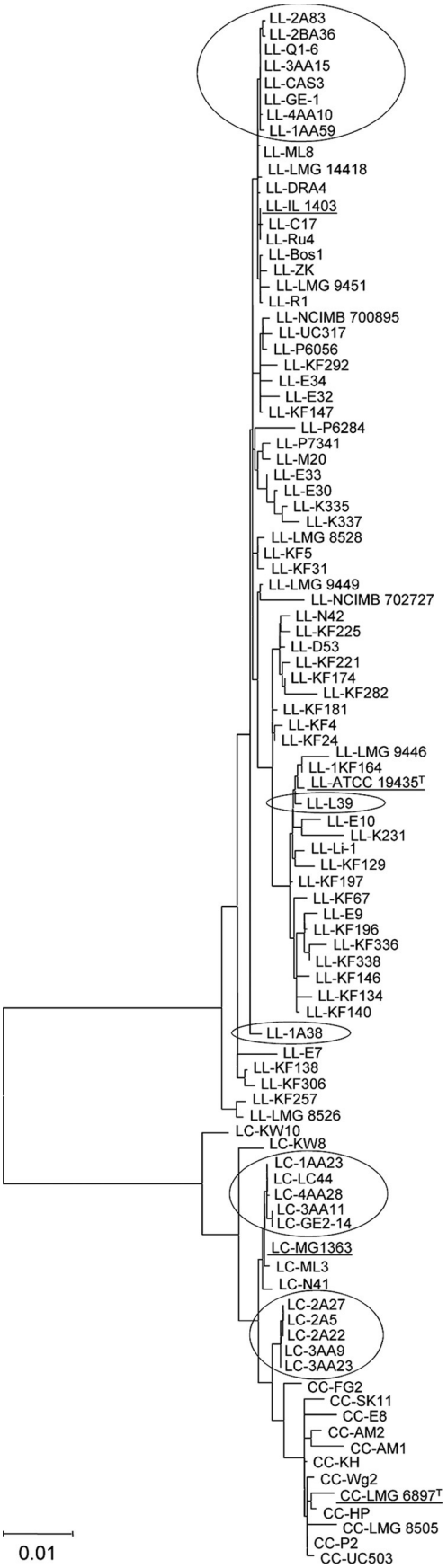

FIG. 3. Diversity analysis of the wild *L. lactis* subsp. *lactis* strains of the *lactis* and *cremoris* genotypes studied in this work compared with *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains of dairy (starter) and nondairy origin (40). Neighbor-joining cluster analysis of a seven-locus MLST analysis based on a composite data set of partial DNA sequences of the 16S rRNA, *atpA*, *rpoA*, *bacT*, *pepN*, *pepX*, and *pheS* genes. For sequence analysis, bootstrap percentages (≥50) after 500 simulations are shown. LL, LC, and CC, *L. lactis* subsp. *lactis* of the *lactis* genotype, the *L. lactis* subsp. *lactis* of the *cremoris* genotype, and *L. lactis* subsp. *cremoris*, respectively. The codes for the strains of this study appear enclosed in ovals on the diagram; type strains of both subspecies, *L. lactis* subsp. *lactis* ATCC 19435^T (CECT 185^T) and *L. lactis* subsp. *cremoris* LMG 6897^T (CECT 967^T), and *L. lactis* laboratory strains IL 1403 (genotype *lactis*) and MG 1363 (genotype *cremoris*) are underlined.

to their use of L-arabinose, arbutin, glycerol, inosine, mannitol, starch, and D-xylose. Key sugars used for distinguishing strains belonging to the *L. lactis* subsp. *lactis* (positive) and *L. lactis* subsp. *cremoris* (negative) subspecies were in fact equally fermented by all the present strains (such as maltose and ribose) or were fermented by more strains of the *cremoris* genotype (mannitol).

To further compare the biochemical properties of the strains, all were subjected to phenotypic profiling for enzyme activities using the API ZYM and API 20 Strep systems and for antibiotic resistance-susceptibility via determination of the MICs for 16 antibiotics using the VetMIC system. The results are shown in Tables 4 and 5, respectively. Only 10 of the 20 enzymes whose activities were assayed with the two kits were positive for the strains studied. Activities showed high variability among the strains and between genotypes (Table 4). The enzyme profiles of the *cremoris* genotype were usually greater and/or the level of activity was higher than those of the *lactis* genotype; these showed moderate esterase (C_4) activity and high esterase-lipase (C_8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase activities. In contrast β-galactosidase and β-glucosidase activities were usually stronger for the *lactis* genotype strains.

Few differences, if any, were observed in terms of the antibiotic MIC profiles among the strains of the *lactis* and *cremoris* genotypes (Table 5). High MICs were observed in both the *lactis* and *cremoris* genotypes for antibiotics to which lactococci have been reported to be intrinsically resistant (aminoglycosides, trimethoprim, and rifampin) (2). As an exception, the three identical strains of the *cremoris* genotype, 2A5, 2A22, and 2A27, showed atypical tetracycline MICs, compatible with the presence of dedicated resistance mechanisms. Following standard gene amplification, sequencing, and sequence analysis, all three strains were shown to harbor a *tet*(S) gene, which is thought to be responsible for strong resistance to this antibiotic.

Except for three *lactis* genotype strains (2BA36, 2A83, and 4AA10), all coagulated UHT milk at 22°C, reaching a final pH at 24 h ranging from 4.89 to 4.14 (Table 2). After 48 h in milk at 30°C, 11 volatile compounds were detected by HS/GC/MS, of which four were quantified (Table 6). The repeatability of this analysis was high; the coefficients of variation for the different volatile compounds and strains varied from 1 to 8%. Large strain-to-strain variations in either absolute or relative

TABLE 2. Phenotypic and biochemical properties of wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes isolated from starter-free, raw milk cheeses

Genotype/strain[a]	Presence of phenotypic property										
	pH in milk, 22°C, 24 h	Hydrolysis of:		Citrate utilization	Acetoin production	Growth at or in:					
		Hippurate	Arginine			40°C	45°C	4% NaCl	6.5 NaCl	pH 9.2	pH 9.6
Genotype *lactis*											
1AA59	4.19	−	+	−	−	+	−	+	−	w[b]	−
CAS3	4.14	+	+	−	−	+	w	+	+	+	−
L39	4.69	+	+	−	−	+	−	+	−	+	w
3AA15	4.14	+	+	−	−	+	w	+	w	+	−
2BA36	6.17	+	+	−	−	+	+	+	−	+	−
1A38	4.89	+	+	−	−	+	−	+	w	+	+
2A83	6.37	+	+	−	−	+	−	+	−	+	−
GE-1	4.17	+	+	−	−	+	−	+	−	+	−
Q1-6	4.78	−	+	−	−	+	−	+	−	+	−
4AA10	5.76	−	+	−	−	+	−	+	−	w	−
CECT 185^T	4.24	−	+	−	−	+	−	+	−	+	−
Genotype *cremoris*											
1AA23	4.24	+	+	−	−	+	−	+	w	+	w
2A5	4.30	+	+	−	−	+	−	+	w	+	+
2A22	4.29	+	w	−	−	+	−	+	w	+	w
2A27	4.27	+	+	−	−	+	−	+	−	+	w
3AA9	4.32	w	w	−	−	+	−	+	−	+	w
3AA11	4.23	+	+	−	−	+	−	+	−	+	w
3AA23	4.19	−	+	−	−	+	−	+	−	+	+
LC44	4.23	+	+	−	−	+	−	+	−	+	w
GE2-14	4.25	+	+	−	−	+	−	+	−	+	w
4AA28	4.25	+	+	−	−	+	−	+	−	+	w
CECT 967^T	4.28	+	−	−	w	−	−	−	−	−	−

[a] CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
[b] w, weak reaction or growth.

abundance of most of the volatile compounds were observed in strains of both the *lactis* and *cremoris* genotypes, especially with respect to the production of acetaldehyde, ethanol, and aldehyde- and alcohol-derived compounds from the catabolism of branched-chain amino acids. The volatile compound profiles of the *lactis* and *cremoris* genotype strains were qualitatively and quantitatively different. The *lactis* genotype strains produced more diacetyl and marginal levels of acetoin, while the *cremoris* genotype strains produced higher levels of acetaldehyde and all the known amino acid-derived flavor compounds, especially 2-methyl propanal, 3-methyl butanal, and 2- and 3-methyl butanol. Prolonged incubation of milk for 5 and 21 days did not show a significant difference either in the volatile profiles of the strains or in the abundance of the volatile compounds detected (data not shown).

DISCUSSION

Traditional cheeses and nondairy fermented products are considered a potential source of new *L. lactis* strains with novel properties that might be able to replace or complement currently used dairy starters (29, 30, 37, 54). Additionally, wild strains are a source of phenotypic and genetic variability (13, 48) that might be used through genetic engineering to enhance the activity and performance of current starter strains (55). Phenotypically, the 20 strains of this study belonged to *L. lactis* subsp. *lactis*, which agrees well with the species identification of wild strains from milk (11, 18, 33) and traditional cheeses (14, 19, 34), as does the low percentage of *cremoris* genotypes.

In the past, the identification of *Lactococcus* species and subspecies was based entirely on phenotypic tests, primarily because species and subspecies are defined by their phenotypes (20). Phenotypic assays are sometimes ambiguous, can provide different results over the growth phase, and are dependent on culture conditions (19, 42, 52, 54). However, since the 1990s, identification has relied mostly on molecular genetic analyses (22, 42, 49), fuelled by the development of simple PCR-based methods (19, 36, 56). The use of molecular genetic techniques alone, however, has introduced some additional confusion into the taxonomy of *L. lactis*, complicating the unusual structure of this species (30, 40). Molecular genetic techniques allowed the recovery of strains with a *cremoris* genotype from different sources, including milk, dairy products, and plant material. It is not clear whether these new *cremoris* isolates have biochemical properties similar to those of isolates used as starters in the dairy industry. In fact, the phenotypic and technological characterization of *cremoris* genotype isolates and the comparison of their properties with those of recognized starter strains of both the *lactis* and *cremoris* subspecies have only rarely been undertaken (18, 37, 43, 54). Such studies are critical, however, for the selection of the most suitable strains for each application. Replacing the unreliable, traditional phenotypic tests with other phenotypic assays and molecular genetic techniques such as those used in the pres-

TABLE 3. Carbohydrate fermentation profiles of the wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes of this study

Genotype/strain[a]	Fermentation of carbohydrate[b]																	
	Adonitol	Amigdalin	L-Arabinose	Arbutin	Gentibiose	Gluconate	Glycerol	Inosine	5-keto-gluconate	Maltose	Mannitol	β-Methyl glucoside	Ribose	Salicine	Starch	Sucrose	D-Xylose
Genotype *lactis*																	
1AA59	−	−	−	−	−	−	−	−	−	+	−	−	+	+	w	+	−
CAS3	−	+	−	−	+	−	−	w	−	+	+	+	+	+	+	+	−
L39	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	−
3AA15	−	+	−	−	+	−	w	−	−	+	−	+	+	+	w	+	−
2BA36	−	+	−	−	+	−	−	+	−	+	w	+	+	−	+	+	−
1A38	w	−	−	−	+	w	−	−	−	+	−	+	+	+	w	w	−
2A83	−	−	−	−	+	−	−	−	−	+	−	+	+	+	w	−	−
GE-1	−	+	−	w	+	−	+	−	−	+	w	+	+	+	w	+	−
Q1-6	−	+	−	−	−	−	−	−	−	+	w	+	+	+	−	−	−
4AA10	−	+	−	w	+	−	w	−	−	+	−	+	+	+	w	+	−
CECT 185[T]	−	−	−	−	+	w	−	−	−	+	−	+	+	+	w	−	−
Genotype *cremoris*																	
1AA23	−	+	w	−	+	−	w	w	−	+	+	+	+	+	+	+	+
2A22	−	+	w	w	+	−	w	w	−	+	+	+	+	+	+	+	+
2A27	−	+	−	w	+	−	−	+	−	+	+	+	+	+	+	+	+
2A5	−	+	−	w	+	−	w	w	−	+	+	+	+	+	+	+	+
3AA23	−	−	−	w	+	−	−	−	−	+	+	+	+	+	+	−	+
3AA11	−	−	+	w	w	−	w	w	w	+	+	w	+	w	+	+	−
3AA9	−	+	−	w	+	−	w	w	−	+	+	+	+	+	w	+	+
LC44	−	+	−	−	+	−	w	w	−	+	w	+	−	+	+	−	−
GE2-14	−	−	−	−	−	−	−	−	−	+	w	−	+	+	w	−	−
4AA28	−	−	+	−	−	−	−	−	−	+	w	−	+	+	w	−	−
CECT 967[T]	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−

[a] CECT 185[T] and CECT 976[T] are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.

[b] All strains fermented glucose, galactose, lactose, and lactulose, and all but *L. lactis* subps. *cremoris* CECT 967[T] fermented cellobiose and trehalose. None of the strains fermented D-arabitol, deoxyribose, doxyglucose, dulcitol, D-fucose, L-fucose, fumarate, galacturonic-lactone, glycogen, inositol, inulin, malinate, malonate, malitiol, mannonic acid lactone, melbionate, melezitose, melibiose, ornithine, palatinose, pyruvate, raffinose, rhamnose, sorbitol, sorbose, tagatose, L-tartrate, or urea under the assay conditions. w, weak reaction.

TABLE 4. Enzymatic activities measured with the API ZYM and 20 Strep systems in wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes isolated from raw milk, starter-free cheeses

Genotype/ strain[a]	Enzymatic activity[b]									
	Alkaline phosphatase	Esterase (C_4)	Esterase lipase (C_8)	Pyrrolidonyl-arylamidase	Leucine arylamidase	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	β-Galactosidase	α-Glucosidase	β-Glucosidase
Genotype *lactis*										
1AA59	2.5	0	5	30	10	2.5	0	40	2.5	0
CAS3	2.5	0	2.5	2.5	10	15	2.5	10	0	30
L39	2.5	0	5	30	15	20	10	2.5	15	40
3AA15	2.5	0	2.5	30	5	0	2.5	40	2.5	0
2BA36	2.5	0	5	20	5	5	5	40	5	0
1A38	2.5	0	5	2.5	20	10	2.5	0	0	40
2A83	2.5	0	5	5	5	5	5	0	0	0
GE-1	0	0	5	20	40	15	2.5	20	0	40
Q1-6	2.5	0	5	30	20	10	5	2.5	0	40
4AA10	2.5	0	5	20	10	10	2.5	0	0	15
CECT 185^T	2.5	0	5	20	30	20	5	2.5	40	40
Genotype *cremoris*										
1AA23	2.5	2.5	5	20	10	30	10	2.5	10	0
2A22	2.5	5	10	30	10	40	15	2.5	20	20
2A27	2.5	5	10	30	10	40	15	0	10	10
2A5	2.5	5	10	30	10	35	15	0	20	20
3AA23	2.5	0	5	20	20	40	20	0	40	40
3AA11	2.5	10	10	30	5	20	15	10	15	0
3AA9	2.5	5	10	30	10	30	15	0	15	20
LC44	2.5	2.5	5	20	40	40	20	2.5	20	20
GE2-14	2.5	5	5	30	5	5	5	10	5	0
4AA28	2.5	2.5	2.5	5	5	30	20	2.5	10	10
CECT 967^T	0	2.5	5	20	5	10	5	0	0	0

[a] CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
[b] Units of activity are expressed as nanomoles of substrate hydrolyzed under the assay conditions. Valine arylamidase, cysteine arylamidase, lipase (C_{14}), trypsin, α-quimiotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities were never detected.

ent work could help to discover new traits for distinguishing between *L. lactis* subspecies.

The typing results obtained in this study by PFGE agreed well with those previously obtained by random amplification of polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) techniques (1). In general, cluster analysis of macrorestriction patterns by PFGE showed less similarity between the strains than that obtained by PCR-based typing methods. PFGE is a powerful means of assessing genetic relationships for bacteria due to its larger genome coverage (greater than 90%) than other typing techniques (23). Cluster analysis of the typing results and sequencing of the 16S rRNA genes and housekeeping genes consistently provided two clear-cut clusters—formed separately by the strains of the *lactis* and *cremoris* genotypes—with only low-level similarity to one another. Similar results have been reported with other typing (11, 21, 37, 40) and sequencing (17, 40, 49, 54) techniques. The more robust MLST technique, which gathers together several gene sequences, produced strongly separated *lactis* and *cremoris* genotypes in deeply branched trees, as reported by other authors (39, 40, 50). Two major genomic lineages for the *lactis* and *cremoris* genotypes have also been recently recognized using pangenomic DNA array hybridization, determining the presence or absence of 4,571 gene orthologs (7). Proteomic analysis of the ribosomal proteins by matrix-assisted laser desorption ionization–time of flight mass spectrometry has provided similar results (52). All this would seem to indicate that, irrespective of their phenotype, the *lactis* and *cremoris* lineages are phylogenetically related but that they have long been on separate evolutionary paths. In fact, on the basis of 16S rRNA gene divergence (less than 0.8%), the deviation of the *lactis* and *cremoris* genotypes has been estimated to have occurred some 17 million years ago (9). Though it is difficult to infer divergence times for the different evolutionary steps, recent independent MLST analyses have confirmed an early separation of the *lactis* and *cremoris* genotypes (39, 40).

Despite the similarity of the *lactis* and *cremoris* genotypes shown in the present phenotypic assays, their member strains showed distinguishable carbohydrate utilization and enzyme activity profiles. Surprisingly, the strains with a *cremoris* genotype showed greater fermentation and enzyme activity profiles, even though true *L. lactis* subsp. *cremoris* strains are reported to have extremely reduced fermentation and enzyme activity profiles (20, 25, 45). The ability of *L. lactis* to ferment carbohydrates is thought to be related to the degree of adaptation of the strains to the dairy environment (30), independent of genotype. The genome sequence of two plant-associated *L. lactis* subsp. *lactis* strains shows the largest number of genes in the carbohydrate metabolism and transport category (46); therefore, they ferment more carbohydrates than any of the three sequenced dairy strains (MG 1363, IL 1403, and SK 11). Furthermore, the genome of *L. lactis* subsp. *lactis* MG 1363 (*cremoris* genotype) has been shown to carry more genes in these two categories (57) than that of *L. lactis* subsp. *lactis* IL 1403 (*lactis* genotype) (8); consequently, it can utilize more sugars. Chromosome size has been shown to vary widely among *L. lactis* strains of both subspecies (30, 39). However, the origin of the *L. lactis* strains has been shown to correlate with chromosome size, particularly for the phenotypic *L. lactis* subsp. *cremoris* strains, which have smaller chromosomes than the *L. lactis* subsp. *lactis* strains of both the *lactis* and *cremoris* genotypes (30). Chromosome sizes in this study fell within the normal range for *L. lactis* strains of either the *lactis* and *cremoris* genotype (30, 39). Adaptation of *L. lactis* to grow in milk is

TABLE 5. MICs of 16 antibiotics to wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes

Genotype/strain[a]	MIC (μg/ml)[b]															
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	PG	Va	Vi	Lz	Tm	Ci	Ri
Genotype *lactis*																
1AA59	4	8	32	8	0.50	0.25	0.12	8	0.50	0.25	0.50	1	2	>64	8	16
CAS3	4	16	32	8	0.25	0.25	0.12	2	0.25	0.25	0.50	2	2	>64	4	16
L39	1	8	16	2	0.50	0.25	0.25	4	0.25	0.25	0.50	2	4	>64	4	16
3AA15	2	16	16	4	0.50	0.25	0.12	4	0.25	0.25	0.25	1	2	>64	4	16
2BA36	2	8	32	2	0.50	0.25	0.25	8	0.12	0.25	0.25	2	4	>64	4	16
1A38	4	16	64	8	0.50	0.25	0.25	4	0.25	0.25	0.50	2	2	>64	2	32
2A83	2	8	32	8	0.50	0.50	0.12	4	0.25	0.12	0.50	0.50	2	>64	4	32
GE-1	4	16	32	8	0.50	0.25	0.12	8	0.25	0.25	0.25	1	4	>64	8	16
Q1-6	2	8	16	4	0.50	0.25	0.12	4	0.12	0.12	0.50	1	2	>64	4	16
4AA10	4	32	64	16	0.50	0.25	0.25	8	0.25	0.25	0.50	2	2	>64	4	16
CECT 185^T	16	32	64	32	0.50	0.25	0.12	8	0.50	0.50	0.50	8	4	>64	8	>64
Genotype *cremoris*																
1AA23	1	8	16	16	0.50	0.25	0.25	4	0.25	0.25	0.50	2	2	>64	8	8
2A22	4	16	64	32	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
2A27	8	32	64	32	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
2A5	8	16	64	16	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
3AA23	16	64	128	64	0.50	0.25	0.50	16	0.25	0.25	0.50	2	2	>64	8	8
3AA11	2	8	32	8	1	0.12	0.25	8	0.25	0.25	0.50	2	2	>64	8	32
3AA9	16	64	128	64	0.50	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	8	8
LC44	2	16	32	8	0.50	0.25	0.25	8	0.25	0.50	0.50	2	2	>64	8	32
GE2-14	8	32	64	8	0.50	0.12	0.25	8	0.25	0.25	0.50	2	2	>64	4	32
4AA28	2	16	32	4	0.50	0.25	0.25	8	0.12	0.12	0.50	2	2	>64	8	16
CECT 967^T	0.50	2	2	1	0.50	0.25	0.12	8	0.25	0.25	0.50	1	2	64	4	>64

[a] CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
[b] Antibiotic abbreviations: Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Nm, neomycin; Tc, tetracycline; Em, erythromycin; Cl, clindamycin; Cm, chloramphenicol; Am, ampicillin; PG, penicillin G; Va, vancomycin; Vi, virginiamycin; Lz, linezolid; Tm, trimethoprim; Ci, ciprofloxacin; Ri, rifampin.

thought to have occurred by gene decay and acquisition of key traits (30, 46, 57), most probably under the high selective pressure imposed by cheese- and butter-making technologies. The analysis of the implicated genes and the whole-genome sequences of more strains might give clues about the current phylogenetic position of the *lactis* and *cremoris* genotypes and the evolutionary processes that gave rise to these dairy-adapted starter strains.

In the present work, acid production in milk was variable among the strains of the two genotypes; a few strains even failed to coagulate the provided UHT milk after 24 h of incubation at 22°C. In general, the *cremoris* genotypes usually caused the pH to decrease more than the *lactis* genotypes, as reported elsewhere (37, 54).

Wild and nondairy *L. lactis* strains have occasionally been associated with off-flavor production (3, 54), which correlates with the formation of large amounts of volatile compounds via the degradation of branched-chain amino acids (Leu, Ile, Val). These compounds have a very low taste threshold and have been connected with malty and burnt notes in dairy products (47). However, selected wild strains or combinations of wild and starter strains have been shown to enhance the typical flavors and to increase ripening indices (4, 5). Moreover, the same volatile compounds that caused the off flavors mentioned above seem to be involved in the desired strong flavors of cheeses made from raw milk (12); these flavors are strongly fostered in traditional cheese varieties, particularly those with protected designation of origin (PDO) status. Starter candidates might therefore be selected among wild *L. lactis* strains to ensure the production of intensely flavored cheeses.

In conclusion, the overall phenotypic and genotypic relatedness of the strains belonging to the *lactis* and *cremoris* genotypes suggests that they should be considered members of the same species, as their properties meet the criteria presently used in the species concept for prokaryotes (41). However, despite their similarity, the *lactis* and *cremoris* genotypes consistently cluster separately when investigated with simple molecular genetic techniques, suggesting that they should be considered true subspecies, a possibility contemplated in the species definition referred to above (41) and on the more recent recommendations for the taxonomy of prokaryotes (53). Separate or combined simple matching-based cluster analysis of the phenotypic traits analyzed in the present study (carbohydrate fermentation, enzyme activities, production of volatile compounds) consistently gave the same two well-separated clusters as the molecular genetic techniques (data not shown). Thus, in disagreement with some other authors (16, 40), irrespective of their phenotype, all the *cremoris* genotypes should be considered to belong to the *cremoris* subspecies.

Analysis of more *L. lactis* collections, including representative strains of all subspecies and biovars from different environments, using state-of-the-art high-throughput phenotypic (Biolog, cheese models) and molecular genetic (genome sequencing, microarray hybridization, comparative genomic hybridization) screening techniques, should further help in assessing the diversity of the lactococci. These studies should

TABLE 6. Absolute or relative abundance of volatile compounds produced in UHT milk at 30°C for 48 h by the wild *L. lactis* subsp. *lactis* strains of the *lactis* and *cremoris* genotypes detected by HS/GC/MS[a]

Genotype/strain[b]	Volatile compound concn (μg/ml)					Relative abundance[c]						
	Acetaldehyde	2-Propanone	Ethanol	Diacetyl	Acetic acid	Acetoin	Methanethiol	2-Methyl propanal	2-Methyl propanol	2-Methyl butanal	3-Methyl butanal	2- and 3-Methyl butanol
Genotype *lactis*												
1AA59	6.41	11.47	802.33	7.31	—[d]	0.06	0.88	3.60	—	3.19	15.49	8.10
CAS3	5.35	13.89	863.77	3.71	—	—	0.87	—	—	—	—	—
L39	6.06	13.55	586.84	—	—	—	0.79	6.30	3.35	2.99	32.89	34.71
3AA15	5.60	13.87	865.09	8.35	—	0.06	0.91	4.42	0.88	3.06	15.64	8.71
2BA36	5.88	12.05	650.70	9.27	—	0.08	0.87	0.95	—	0.66	6.34	5.19
1A38	27.57	9.03	140.84	2.55	—	0.19	—	—	—	—	—	—
2A83	6.41	11.47	802.33	7.31	—	0.05	0.96	0.28	—	—	0.98	4.97
GE-1	6.38	13.05	667.91	6.57	—	—	0.75	—	—	—	—	—
Q1-6	1.55	9.93	153.24	4.31	—	0.43	—	—	—	—	—	—
4AA10	20.89	8.63	1,239.09	3.71	—	—	0.87	—	—	—	—	—
CECT 185^T	5.26	1.38	238.9	—	—	—	—	—	0.35	—	—	2.48
Genotype *cremoris*												
1AA23	33.71	15.05	731.74	0.70	—	—	—	—	—	—	—	—
2A22	22.33	12.25	1,146.28	4.80	—	—	0.85	14.33	5.75	3.25	84.50	36.13
2A27	21.89	11.50	1,140.95	4.78	—	—	0.78	14.49	6.42	3.23	88.33	39.87
2A5	23.89	14.58	1,287.38	5.52	—	—	0.81	15.13	5.91	3.53	87.65	37.10
3AA23	17.91	9.75	1,170.95	4.84	—	—	0.46	19.76	9.10	2.79	89.76	53.99
3AA11	26.93	10.83	747.33	3.70	—	0.07	0.25	32.71	6.24	35.82	108.76	70.72
3AA9	79.56	10.76	823.03	2.40	—	—	0.38	17.38	9.76	0.95	58.39	66.17
LC44	25.00	9.31	402.08	—	—	—	0.26	—	—	—	—	—
GE2-14	33.81	12.46	679.72	2.56	—	—	0.24	35.74	6.06	35.09	113.76	83.26
4AA28	25.95	15.31	746.83	—	—	—	0.70	8.27	0.97	2.69	55.39	17.88
CECT 967^T	2.25	0.87	77.50	—	—	—	—	—	—	—	—	—

[a] Average results of duplicate analyses are shown.
[b] CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
[c] Relative abundance compared to an internal control (cyclohexanone, 0.36 mg/ml).
[d] —, not detected.

be aimed at correlating genomic makeup and phenotypic traits with industrial performance, which has ultimately to be assessed by carefully controlled trials using defined mixtures of phenotypic and genotypic strains of both subspecies.

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Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk

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ABSTRACT

Sixty bacterial strains were encountered by random amplification of polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) typing in a series of 306 *Lactococcus lactis* isolates collected during the manufacturing and ripening stages of five traditional, starter-free cheeses made from raw milk. Among the 60 strains, 17 were shown to produce bacteriocin-like compounds in both solid and liquid media. At a genotypic level, 16 of the strains were identified by molecular methods as belonging to *L. lactis* subsp. *lactis* and one to *L. lactis* subsp. *cremoris*. Among the *L. lactis* subsp. *lactis* strains, phenotypic and genetic data determined that eleven produced either nisin A (nine strains) or nisin Z (two strains), and that five produced lactococcin 972. Variable levels of the two bacteriocins were produced by different strains. In addition, nisin was shown to be produced in inexpensive, dairy- and meat-based media, which will allow the practical application of its producing strains in industrial processes. Specific PCR and nucleotide and deduced amino acid sequence analysis identified the inhibitor produced by the single *L. lactis* subsp. *cremoris* isolate as a lactococcin G-like bacteriocin. Beyond the use of bacteriocins as functional ingredients for the biopreservation of foods, the newly identified bacteriocin-producing *L. lactis* strains from traditional cheeses may also be useful for designing starter cultures with protective properties and/or adjunct cultures for accelerating cheese ripening.

1. Introduction

Many microbial groups produce bacteriocins—peptides and proteins with bactericidal activity. Bacteriocins of some bacteria inhibit growth of closely related microbes, while others inhibit a much wider range of microorganisms, including food-borne pathogens and spoilage microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium tyrobutyricum* (Gálvez et al., 2008).

From a biochemical point of view, two types of bacteriocins have been identified in lactic acid bacteria (LAB), those characterized by the presence of dehydrated (dehydroalanine and dehydrobutyrine) and/or thioether amino acids (lanthionine and β-methyllanthionine), usually referred to as lanthibiotics (or class I), and those containing unmodified amino acids (non-lanthibiotics) (Jack et al., 1995). Non-lanthibiotics are divided into classes II through IV depending on their size and the presence of non-protein moieties. Both lanthibiotics and non-lanthibiotics are synthesized via a ribosomal pathway, but the former are later modified enzymatically. In the last 25 years, intensive research into the bacteriocins produced by LAB has been undertaken with the aim of improving the microbial quality and safety of fermented products (de Vuyst and Leroy, 2007).

Lactococcus lactis strains are the majority LAB components of commercial starter cultures used by the dairy industry for the manufacture and ripening of cheese and fermented milk (Limsowtin et al., 1995). Lanthibiotic and non-lanthibiotic bacteriocins produced by *L. lactis* from different sources have been identified and characterized (Venema et al., 1995). The first bacteriocin isolated from *L. lactis* was nisin (Mattick and Hirsch 1947), a 34-amino acid lanthibiotic. This is currently approved and exploited in over 50 countries as a food additive (code E234) (Delves-Broughton et al., 1996). To date, five natural nisin variants (A, Z, Q, U, and F) have been identified (de Kwaadsteniet et al., 2008). Other lanthibiotics produced by *L. lactis* include the single peptide lacticin 481 and the two-component system lacticin 3147 (de Vuyst and Leroy 2007). Non-lanthibiotic bacteriocins from *L. lactis* include pediocin-like bacteriocins (class IIa) such as lactococcin MMFII, two-peptide component bacteriocins (class IIb) such as lactococcin G and M, thiol-activated bacteriocins (class IIc) such as lactococcin B, and heat-labile, lactococcus-specific bacteriocins (class IId) such as

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lactococcin A (diplococcin) and lactococcin 972 (Venema et al., 1995; Oppegård et al., 2007).

The incorporation of bacteriocin-producing lactococci as starter or adjunct cultures in the manufacture of fermented food provides an attractive and economic alternative to the addition of purified bacteriocins (indeed, metabolic compounds produced during fermentation are no longer considered as additives). Bacteriocin-producing *L. lactis* has therefore been experimentally tested in the manufacture of several cheese varieties (Ryan et al., 1996; Martínez-Cuesta et al., 2001; O'Sullivan et al., 2003; Rilla et al., 2003; Garde et al., 2006) and other fermented products (Diop et al., 2009). Following its addition, starter lysis is increased (O'Sullivan et al., 2003) and peptidolytic and transamination activities, key factors in the formation of aroma and taste compounds, may also be enhanced (Martínez-Cuesta et al., 2003; Fernández de Palencia et al., 2004). In addition to its technological applications, bacteriocin-producing *L. lactis* has been assayed for the treatment of mastitis in cows (Ryan et al., 1999; Twomey et al., 2000; Klostermann et al., 2009), and is being evaluated as an antipathogenic agent in human gastrointestinal infections (O'Connor et al., 2006; Millette et al., 2008).

The aim of the present work was to screen for bacteriocin production in a large number of *L. lactis* strains isolated during the manufacturing and ripening stages of different batches of five traditional, Spanish, starter-free cheeses made from raw milk. Efforts were also made to identify these antimicrobial compounds by searching for bacteriocin-encoding genes. Of the 17 bacteriocin producers detected, phenotypic and genetic analyses identified eleven as nisin producers, five as lactococcin 972 producers, and a single producer of lactococcin G.

2. Material and methods

2.1. Strains, media and culture conditions

A series of 306 lactococcus-like isolates collected during the manufacture and ripening of five Spanish traditional, starter-free cheeses made from raw milk were grouped by typing and identified by partial ARDRA, sequencing and sequence comparison. These isolates came from *Casín* (80), *Cabrales* (106), *Genestoso* (63), *Peñamellera* (44), and *Valle del Narcea* (13) cheeses. Representative isolates of the 60 different strains found were tested for the production of antimicrobial compounds against a series of Gram-positive indicator bacteria. The indicator strains included *L. lactis* subsp. *cremoris* MG 1363, *L. lactis* subsp. *lactis* NCDO 497 (nisin producer), *L. lactis* subsp. *lactis* IPLA 972 (lactococcin 972 producer), *Lactobacillus sakei* CECT 906^T, *Lactobacillus plantarum* LL 441 (plantaricin C producer), *Listeria innocua* 86/26 and *S. aureus* CECT 86^T. Cryopreserved cultures of cheese isolates and control strains in glycerol were recovered on M17 agar plates (lactococci), de Man, Rogosa and Sharpe (MRS) agar plates (lactobacilli), or in tryptone soy broth (TSB) (*L. innocua* and *S. aureus*), and incubated at the corresponding optimum temperature for 24 h. *Micrococcus luteus* CECT 245 (=ATCC 10240) was used as the indicator strain for measuring nisin activity. This strain was grown in nutrient broth (NB) with shaking at 37 °C for 24 h.

2.2. Identification and typing of isolates

Total genomic DNA from isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the bands were stained with ethidium bromide (0.5 μg/mL) and photographed under UV light. Isolates were grouped by repetitive extragenic palindromic (REP) fingerprinting employing the polymerase chain reaction (PCR) and the primer BoxA2-R (Table 1), as reported by Koeuth et al. (1995), followed by random amplification of polymorphic DNA (RAPD) typing with the primer M13 (Table 1), as reported by Rossetti and Giraffa (2005). Reproducibility studies of the combined REP and RAPD techniques showed a percentage similarity of over 95%.

Representative isolates of the REP and RAPD groups were identified by partial ARDRA, followed by sequencing of representative amplicons and comparison of the sequences obtained against those in databases. For ARDRA, 16S rRNA genes were almost completely amplified using universal primers 27-F and 1492-R (Table 1). Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich), digested with restriction enzymes *Hae*III and *Hin*fI (Invitrogen Ltd., Paisley, UK), and electrophoresed as above. When required, amplicons were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and to those held by the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

2.3. Antimicrobial activity

Antimicrobial activity was successively examined by an agar spot test and a well-diffusion assay. For the former, overnight cultures of the isolates were spotted (5 μL) on the surface of M17, MRS and TSB agar plates and incubated at 30 °C for 24 h. Spots were then covered with 10 mL of soft agar (0.75%) inoculated at 0.25% with indicator

Table 1
Primers used throughout this study.

Name	Sequence (5′→3′)	Technique/Amplification	Reference/GenBank accession no
BoxA2-R	ACGTGGTTTGAAGAGATTTTCG	REP-PCR typing	Koeuth et al. (1995)
M13	GAGGGTGGCGGTTCT	RADP typing	Rossetti and Giraffa (2005)
27-F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene	S-D-Bact-0008-a-S-20
1492-R	GGTTACCTTGTTACGACTT	16S rRNA gene	S-*-Univ-1492R-b-A-21
Nis-F	CGGCTCTGATTAAATTCTGAAG	Nisin genes	M65089
Nis-R	GGATTAGCTAGTAGTAACTGTTC	Nisin genes	M65089
Lact3147-F	GTCTTTGTGTTGTTTGGAGATG	Lacticin 3147 gene	AE001272
Lact3147-R	CAACTCCCGAAATAAATCATCG	Lacticin 3147 gene	AE001272
Lact481-F	CCAATGTCATTGCATCTGCAC	Lacticin 481 gene	X71410
Lact481-R	GTCCTTATGTTGCTATTCATC	Lacticin 481 gene	X71410
Lcn972-F	TTGTAGCTCCTGCAGAAGGAACATGG	Lactococcin 972 gene	Martínez et al. (1999)
Lcn972-R	GCCTTAGCTTTGAATTCTTACCAAAAG	Lactococcin 972 gene	Martínez et al. (1999)
LactABM-F	GAAGAGGCAATCAGTAGAG	Lactococcin A, B, and M genes	M90969, S38128, van Belkum et al. 1991
LactA-R	GTGTTCTATTTATAGCTAATG	Lactococcin A gene	M90969
LactB-R	CCAGGATTTTCTTTGATTTACTTC	Lactococcin B gene	S38128
LactM-R	GTGTACTGGTCTAGCATAAG	Lactococcin M gene	van Belkum et al. (1991)
LactGQ-F	GAAAGAATTATCAGAAAAAG	Lactococcin G and Q genes	FJ938036, AB182406
LactGQ-R	CCACTTATCTTTATTTCCCTCT	Lactococcin G and Q genes	FJ938036, AB182406

bacteria. These plates were then incubated under the conditions required by the indicator species. Positive cultures were subjected to a well-diffusion assay with neutralized, filter-sterilized supernatants, essentially as reported by Schillinger and Lücke (1989). Briefly, 20 mL of the agar medium at 45 °C was vigorously mixed with 200 μL of an overnight culture of the indicator strain and poured into Petri dishes. Supernatants from overnight cultures of the producing strains were neutralized to pH 6.5–7.0 with NaOH 0.1 M, centrifuged at 14,000 rpm for 5 min, and filter-sterilized through a 0.20 μm pore membrane (Millipore, Bedford, MA, USA). Aliquots of 50 μL of each supernatant were placed in wells excavated into the agar. The inhibition of indicator growth was examined after incubation for 24 h under appropriate culture conditions.

2.4. Search for bacteriocin-encoding genes by PCR

Genes coding for the most common bacteriocins produced by *L. lactis* strains were sought by specific PCR. Based on published sequences and sequences on the databases, primers were designed for genes encoding nisin, lacticin 3147, lacticin 481, lactococcin 972, lactococcin A, lactococcin B, lactococcin G, lactococcin M, and lactococcin Q (Table 1).

Amplifications were all conducted under standard conditions at an annealing temperature of 50 °C. Then, amplicons were purified and sequenced, and their sequences compared as above.

2.5. Quantification of bacteriocin production

Nisin released in MRS broth was quantified and its activity expressed in international standard units per mL (IU/mL) by comparing the activity of the supernatants with that of commercial nisin (Nisaplin®, Danisco, UK) dilutions. Cultures were centrifuged at 12,000×*g* for 10 min and the supernatants adjusted to pH 2.0 with 0.02 N HCl, heated at 80 °C for 5 min, and centrifuged once again under the same conditions. Dilutions of these supernatants were made in 0.02 N HCl and 50 μL deposited in wells made in NB agar plates previously inoculated with approximately 1.0×10^8 colony forming units (CFU)/mL of *M. luteus* CECT 245. The diameter of the inhibition halos was measured and concentrations were determined against a standard curve for commercial nisin dilutions prepared in the same way.

Lactococcin 972 was quantified by a non-competitive enzyme-linked immunoassay (NCI-ELISA) with rabbit polyclonal antibodies raised against the purified bacteriocin, which were supplied by the Immunotechnology External Service of the University of Oviedo (Spain). NCI-ELISA was essentially performed as described by Sánchez et al. (2008). Briefly, flat-bottom polystyrene microtiter wells (Maxisorp; Rochester, NY, USA) were coated with culture supernatants or different concentrations of pure lactococcin 972, washed and incubated with the primary (1:1000) and the secondary (1:40,000) antibody goat anti-rabbit IgG peroxidase conjugates (Sigma). Plates were revealed with 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS; Sigma-Aldrich) as the substrate and the absorbance at 405 nm recorded in a Benchmark Plus microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Production of nisin in dairy- and meat-based media

The production of nisin in industrial media mimicking dairy- and meat-derived products was analyzed in reconstituted skim milk (10% w/v) supplemented with 0.5% whey protein concentrate (RSM-WPC) and in meat-extract medium (8% w/v) supplemented with soy-extract 2.25% (ME-SY), respectively. In both cases, the basal medium was supplemented with NaCl (2%), potassium sorbate (0.05%), and yeast extract (0.025%), and the pH was adjusted to 6.4. The release of nisin in RSM-WPC and ME-SY media was quantified as above, using control commercial nisin dilutions and the bacteriocin produced in MRS.

2.7. Analysis of plasmid content

Plasmid DNA from *L. lactis* was extracted and purified following the procedure of O'Sullivan and Klaenhammer (1993). Plasmid preparations were electrophoresed in 0.75% agarose gels, stained with ethidium bromide (0.5 μg/mL) and photographed.

3. Results and discussion

3.1. Identification and typing of L. lactis isolates

Typing analysis of the 306 isolates by the combined REP and RAPD techniques gave 60 different fingerprinting patterns with lower percentage similarities than those recorded in a reproducibility study (Supplemented material 1). Consequently, these 60 profiles were considered as different strains and thus subjected to identification by partial ARDRA, sequencing and comparison of the sequences. A single ARDRA profile was obtained with either *Hae*III or *Hin*fI, indicating that they all belonged to a single species. Sequencing of 21 16S rRNA amplicons representative of all strains showing a Spearman's coefficient of similarity in their REP/RAPD profiles of over 0.52% (Supplementary material 1) indicated that they all could be assigned to the *L. lactis* species. The sequences of six amplicons, corresponding to ten strains (Supplementary material 1, codes 14, 15, 16, 44, 46, 47, 49, 50, 54 and 58), were shown to match the 16S rRNA sequence of *L. lactis* subsp. *cremoris*; all others were shown to be identical to those of *L. lactis* subsp. *lactis*. Sequencing of all 10 isolates of the supposed *cremoris* subspecies and 20 more amplicons at random from the *lactis* subspecies further confirmed the identity and number of strains at the subspecies level. As reported for many other traditional cheeses (Callon et al., 2004; Delgado and Mayo 2004; Psoni et al., 2007; Nieto-Arribas et al., 2009), the genetic diversity found among the *L. lactis* isolates from the five raw milk cheeses was rather high. However, the presence of (genetic) *L. lactis* subsp. *cremoris* strains in such cheeses has only rarely been reported (Gaya et al., 1999; Delgado and Mayo 2004; Nieto-Arribas et al., 2009).

3.2. Antimicrobial activity of L. lactis strains

The production of inhibitory compounds by representative isolates of the different strains against a group of indicator bacteria including well recognized food-borne pathogens was first analyzed by an agar spot test. A variable number of the 60 strains inhibited the different indicator organisms. *L. sakei* CECT 906^T, a strain reported to be very susceptible to bacteriocins and other antimicrobials (González et al., 1994), was inhibited by 37 strains (61.66%). In contrast, *S. aureus* CECT 86^T was inhibited by only 11 (18.33%); additionally, in most cases only faint halos were seen. *L. lactis* subsp. *cremoris* MG 1363, *L. innocua* 86/26, *L. plantarum* LL 441 and *L. lactis* subsp. *lactis* NCDO 497 were inhibited by 22, 18, 14 and 13 strains, respectively. Strains with antibacterial activity against any of the indicators were subsequently subjected to the well-diffusion assay. Under the conditions of this test (which requires neutralized, filter-sterilized supernatants), the number of positive strains was severely reduced, as only 17 strains showed clear inhibitory effects (Table 2). These results were not surprising; many authors have reported that confirmation in liquid media of the inhibition detected by the agar spot test is not always obtained (Schillinger and Lücke 1989; Larsen et al., 1993; Martínez et al., 1995; Hernández et al., 2005). Several colony-associated antimicrobial compounds, including fatty acids and H_2O_2, have been considered to be responsible for the inhibitory effects observed in solid media (de Vuyst and Leroy 2007). Strains inhibiting the indicators used in this study were as follows: *L. sakei* CECT 906^T-17 strains, *L. lactis* subsp. *cremoris* MG 1363-17 strains, *L. innocua* 86/26-10 strains, *L. plantarum* LL 441-9 strains, *S. aureus* CECT 86^T (weak inhibition)-9 strains, and *L. lactis* subsp. *lactis* NCDO 497-7 strains. In the present work, the inhibitory strains were all shown to belong to *L. lactis* subsp.

Table 2
Antimicrobial activity of *L. lactis* strains from traditional cheeses against of a series of indicator strains assayed with neutralized supernatants by a well-diffusion assay. Also included, representative genotype as determined by specific PCR and bacteriocin activity or bacteriocin production.

Indicator strain/genes/ bacteriocin production	*L. lactis*[a] strain																
	1A6	1A8	1A16	1A38	1AA16	1AA17	1AA48	2BB9	3AA28	L30	P83A	2A27	Q1-2	Q1-6	Q1-8	T2-26	T2-43
L. lactis subsp. *cremoris* MG 1363	++	++	++	++	++[b]	++	+	++	++	+	++	+	++	++	++	++	++
L. lactis subsp. *lactis* NCDO 497	−	−	−	−	−	(+)	−	−	−	−	−	++	++	++	++	++	++
L. lactis subsp. *lactis* IPLA 972	++	++	++	++	++	++	−	++	++	+	++	++	−	−	−	−	−
Lactobacillus plantarum LL 441	++	++	++	++	++	++	−	++	++	−	+	−	−	−	−	−	−
Lactobacillus sakei CECT 906^T	++	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	++	++	+	++	++
Listeria innocua 86/26	++	++	+	++	++	++	−	++	++	+	++	−	−	−	−	−	−
Staphylococcus aureus CECT 86^T	+	(+)	+	(+)	(+)	+	−	+	+	−	+	−	−	−	−	−	−
Presence of *nisA*	+	+	+	+	+	−	+	−	+	+	+	−	−	−	−	−	−
Presence of *nisZ*	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−
Presence of *lcn972*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
Presence of *lcnG*	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Bacteriocin production	45[c]	88[c]	75[c]	85[c]	50[c]	60[c]	<20[c]	125[c]	96[c]	70[c]	64[c]	Nd[d]	12.4[e]	5.6[e]	5.6[e]	8.1[e]	11.8[e]

[a] Genetically, all strains are *L. lactis* subsp. *lactis* except that of 2A27 which is a *L. lactis* subsp. *cremoris* strain.
[b] The number of crosses in the test is related to the diameter of the inhibition halo; in parenthesis, weak inhibition.
[c] Nisin activity is expressed as IU/mL of culture medium (MRS). Under the same experimental conditions, nisin production by *L. lactis* subsp. *lactis* NCDO 497 was shown to be 85 IU/mL.
[d] Nd, not determined.
[e] Production of lactococcin 972 was measured as μg of protein per ml of culture medium (M17). The original producer, *L. lactis* subsp. *lactis* IPLA 972, produces 4.9 μg/mL.

lactis, except for 2A27 which proved to be a *L. lactis* subsp. *cremoris* strain. All these 17 strains showed distinct typing profiles, as depicted in Fig. 1 in which the REP patterns obtained with primer BoxA2-R are summarized.

Careful inspection of Table 2 shows that 11 strains did not inhibit the nisin producer indicator NCDO 497 (except for a small inhibition by strain 1AA17), suggesting that some strains might be nisin producers. In fact, the nisin production phenotype has been widely found among *L. lactis* strains from many ecosystems (Martínez et al., 1995; Rodríguez et al., 1995; Ayad et al., 2002; Park et al., 2003; Beasley and Saris 2004; Millette et al., 2007; Dal Bello et al., 2010). At the same time, the five strains on the right of the table produced bacteriocin-like substances that inhibited only the *L. sakei* strain and two *L. lactis* indicators (strains MG 1363 and NCDO 497). The availability of *L. lactis* subsp. *lactis* IPLA 972, a lactococcin 972 producer (Martínez et al., 1995; Martínez et al., 1999), allowed all antimicrobial producers to be assayed using this strain as indicator. Table 2 shows that IPLA 972 was inhibited by most strains, including *L. lactis* subsp. *cremoris* 2A27, but not by these five *L. lactis* subsp. *lactis* strains. Therefore, these strains might produce lactococcin 972, a phenotype that has only been reported for strain IPLA 972 (Martínez et al., 1995).

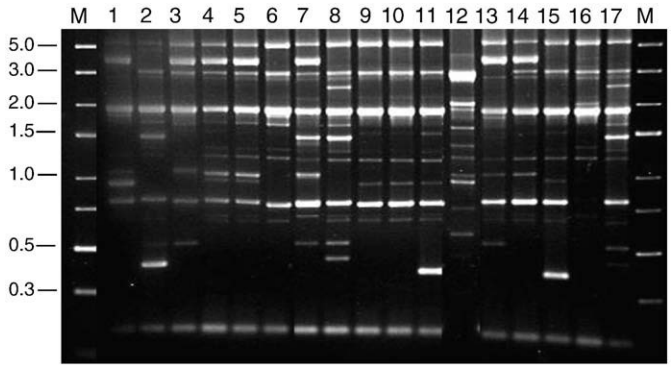

Fig. 1. REP-PCR typing of the seventeen *L. lactis* subsp. *lactis* strains bacteriocin producers with the primer BoxA2R. Order, lines 1 through 11 nisin producer strains 1A6, 1A8, A16, 1A38, 1AA16, 1AA17, 1AA48, 2BB9, 3AA28, L30, and P83A; line 12, *L. lactis* subsp. *cremoris* 2A27; lines 13 through 17 lactococcin 972 producers Q1-2, Q1-6, Q1-8, T2-26, and T2-43. M, Molecular weight marker (Gene Ruler Express™ DNA ladder, Fermentas Gmbh, Germany); molecular weight (kbp) of key bands is indicated.

3.3. Targeting the bacteriocin-encoding genes by PCR

PCR analyses were undertaken using specific primers for genes of the most common lactococcal bacteriocins, i.e., nisin, lacticin 3147, lacticin 481, lactococcins A, B, G, and M, as well as specific primers for lactococcin 972. Amplicons of the expected size for lacticin 3147, lacticin 481, and lactococcins A, B, and M, were never obtained. Sequencing of eventually-produced amplicons showed non-specific amplification of *L. lactis* genes. In contrast, 11 of the 17 strains produced an amplicon of the expected size for nisin (lines 1 through 11 in Fig. 2A) as did five for lactococcin 972 (lines 13 to 17 in Fig. 2B). Amplicons were all sequenced to prove unequivocally that they corresponded to their respective bacteriocin-encoding gene. A nucleotide difference was observed in the sequences of the nisin

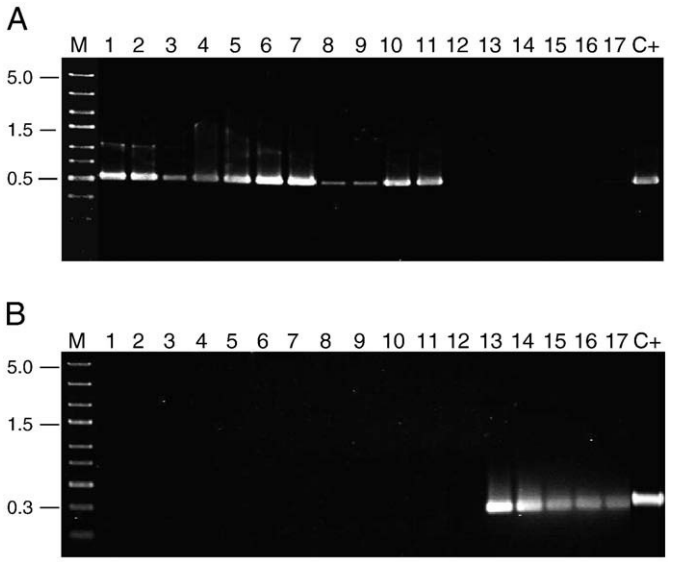

Fig. 2. Specific PCR amplification of the nisin structural gene (A) and that of lactococcin 972 (B) using total DNA of the wild *L. lactis* subsp. *lactis* strains producing inhibitory substances as a template. Order, lines 1 through 11 nisin producer strains 1A6, 1A8, A16, 1A38, 1AA16, 1AA17, 1AA48, 2BB9, 3AA28, L30, and P83A; line 12, *L. lactis* subsp. *cremoris* 2A27; lines 13 through 17 lactococcin 972 producers Q1-2, Q1-6, Q1-8, T2-26, and T2-43; line C+, positive reaction using as a template total DNA from *L. lactis* subsp. *lactis* NCDO 497 and *L. lactis* subsp. *lactis* IPLA 972, respectively; line M, molecular weight marker, indicating molecular weight of key bands in kbp.

structural gene in two strains (1AA17 and 2BB9) with respect to the nisin A structural gene of the other nine strains. This nucleotide change corresponded to the sequence of the structural gene of nisin Z (Table 2) (Mulders et al., 1991).

The sequences obtained for the lactococcin 972 gene were shown to be identical to one another as well as to the sequence from *L. lactis* subsp. *lactis* IPLA 972 (Martínez et al., 1999). Positive amplification with the *L. lactis* subsp. *cremoris* 2A27 strain was only obtained when using specific primers for the genes encoding the two-peptide, related bacteriocins lactococcin G and lactococcin Q. Analysis of nucleotide and amino acid deduced sequences indicated that this strain produced a bacteriocin almost identical to lactococcin G, although small changes at the nucleotide level leading to a few amino acid changes in both α and β peptides were noted (Supplementary material 4).

The slight inhibition of *L. lactis* subsp. *lactis* NCDO 497 by 1AA17 strain is intriguing, since they both are nisin producers. The latter strain might co-produce a second, undetected bacteriocin, as has been reported recently for other *L. lactis* strains (Topisirovic et al., 2006; Bravo et al., 2009; Dal Bello et al., 2010). All five lactococcin 972 producers have recently been isolated during the microbial characterization of *Casín* cheese (Alegría et al., 2009). Since the lactococcin 972 structural gene has been found in plasmid pBL1 (11 kbp) (Martínez et al., 1999), the plasmid content of the lactococcin-producing strains was analyzed. The plasmid profiles of the different lactococcin producers varied (Supplemented material 3), and none of the bands was shared by all strains. This further strengthens the view of the typing results, and suggests that these isolates are indeed different strains and that the lactococcin operon may be located in plasmids of variable size.

3.4. Bacteriocin production in laboratory and industrial media

The activity of nisin released into the culture medium by the different producers was measured by comparing the inhibition halos against a standard curve for commercial nisin (Supplementary Material 2), using *M. luteus* CECT 245 as indicator. Nisin activity ranged from <20 to about 125 IU/mL (Table 2). Activity of the major producers was comparable to or higher than that of *L. lactis* subsp. *lactis* NCDO 497 (85 IU/mL), and those reported on the literature for wild *L. lactis* isolates (Ayad et al., 2002). Nisin activity was further assayed and quantified in industrial media simulating dairy (RSM-WPC) and meat products (MS-YS). The quantification of nisin in these two media showed a general decrease of around 10% in bacteriocin production in RSM-WPC (average 67.3 IU/mL; range 16.7–118 IU/mL). On the contrary, production of nisin in MS-YS was shown to be greatly enhanced in all strains. As compared to that in MRS, nisin activity in this latter medium showed, depending on the strain, a 2–4 fold increase (average 196 IU/mL; range 97–346 IU/mL). Nisin production shows primary metabolite kinetics and is only produced during the exponential growth phase (de Vuyst and Vandamme, 1992). Accordingly, strains 2BB9 and 3AA28 were shown to reach the highest cell density and were the best nisin producers in all media and under all conditions assayed. The production of nisin in low-cost media would facilitate the practical application of the producers for the industrial manufacture of nisin as food preservative, but also their inclusion as starters or adjunct cultures for the preservation of dairy and meat fermented products.

Variable amounts of lactococcin 972 were also measured in the supernatant of the producing strains by an immunoassay (Table 2). Two strains, Q1-6 and T2-43, were shown to produce two-fold bacteriocin as compared to the original producer. *L. lactis* resistant strains to lactococcin 972 have never been reported, except for the immunity of producers (Martínez et al., 1995, 1999). This fact would allow the use of producing-strains as the components of adjunct cultures, which may contribute to accelerate cheese ripening by increasing lysis of starter cells, as it has been proposed for producers of other bacteriocins (Martínez-Cuesta et al., 2001; Fernández de Palencia et al., 2004). In addition to their technological value, these strains could also serve as a suitable source of lactococcin 972 for molecular studies aimed to unravel its atypical mode of action (Martínez et al., 2008).

4. Conclusions

In conclusion, 17 bacteriocin producers were identified in a collection of 60 lactococcal strains from traditional cheeses made from starter-free raw milk, indicating that this phenotype is well spread among wild dairy *L. lactis* strains. Besides the discovering of new bacteriocins, it is also important to identify strains producing higher amounts of the antimicrobials (particularly those with broad inhibitory spectrum such as nisin), which would lead to their commercial application. As the bacteriocin production trait is widely spread among *L. lactis* from artisanal, traditional cheeses made of raw milk, these products could be a good source of strains displaying enhanced outputs. The structural gene of nisin was identified by PCR in 11 strains, which produced nisin at variable concentrations. A remaining set of five strains harboured the lactococcin 972 structural gene and variable amounts of this inhibitory peptide were measured in the culture medium. Finally, specific PCR and analysis of the amplicons strongly suggested that the *L. lactis* subsp. *cremoris* 2A27 produces a two-peptide, lactococcin G-like bacteriocin. Because of their broad inhibitory activity, nisin-producing strains might be of interest in the development of protective starter cultures for cheese and other fermented products. The inhibitory activity of lactococcin 972 and lactococcin G against lactococci alone renders them of interest in the design of adjunct cultures aimed at improving and accelerating cheese ripening. Autochthonous starters and adjunct cultures composed by bacteriocin-producing strains may further help to reinforce typicity and originality of traditional cheeses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2010.07.029.

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Identification, typing and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses

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Abstract

Selected *Leuconostoc* strains are required as aroma producers in dairy starters. In this work, 42 *Leuconostoc* isolates from different raw milk traditional cheeses made without the addition of commercial starters were identified by molecular methods. These isolates were assigned to the species *Leuconostoc citreum* (24), *Leuconostoc mesenteroides* (13) and *Leuconostoc lactis* (5). Typing the isolates by repetitive extragenic profiling (Rep-PCR) identified 22 strains. Of these, 14 were selected for further characterization based on their behaviour in milk. The biochemical, technological and food safety variables recorded were examined to select appropriate candidates for use as adjunct cultures. In agreement with the wide genetic diversity recorded by Rep-PCR, wide phenotypic biodiversity was seen among the species and strains in terms of enzyme profile and carbohydrate fermentation, acidification rate, the production of volatile compounds in milk, and growth under different pH, salt and temperature conditions. No production of biogenic amines was recorded, even when suitable amino acid precursors were present in the growth medium. All 14 strains proved to be susceptible or only intrinsically resistant to a set of 16 antibiotics, except for a single *Leuc. citreum* strain that showed resistance to ciprofloxacin. The remaining 13 strains could be confidently used as components of adjunct cultures in the dairy industry.

1. Introduction

Leuconostoc species are heterofermentative lactic acid bacteria (LAB) widely used in mesophilic cultures to produce aromas during milk fermentation. Presently, the genus *Leuconostoc* includes 13 species, *Leuc. carnosum, Leuc. citreum, Leuc. fallax, Leuc. gasicomitatum, Leuc. gelidum, Leuc. holzapfelii, Leuc. inhae, Leuc. kimchi, Leuc. lactis, Leuc. mesenteroides* (with four subspecies, *cremoris, dextranicum, mesenteroides* and *suionicum*), *Leuc. miyukkimchii, Leuc. palmae,* and *Leuc. pseudomesenteroides* (Ogier et al., 2008; www.dsmz.de). Of these, *Leuc. mesenteroides* subsp. *cremoris* and *Leuc. lactis* are commonly included as components of commercial starters (Hemme and Focaud-Scheunemann, 2004).

Leuconostocs lack a functional extracellular caseinolytic proteinase (Liu et al., 2010), which impedes their reaching high cell densities in milk when growing alone. They are therefore usually employed as adjunct cultures in combination with fast-acid-producing lactococci. *Leuconostoc* species metabolise the citrate of milk to produce

diacetyl (Cogan and Jordan, 1994), a key odour compound in butter, buttermilk and certain cheese varieties (Rincon-Delgadillo et al., 2012). They also participate in the formation of other aroma and flavour compounds, such as lactic, acetic acid, and ethanol (Cogan and Jordan, 1994; Hemme and Focaud-Scheunemann, 2004). These and other metabolic end products contribute not only to the flavour profile of fermented products but, via their antimicrobial action, to their preservation (Hemme and Focaud-Scheunemann, 2004). The production of pediocin-like bacteriocins belonging to subclass IIa by dairy leuconostocs (Stiles, 1994) may further contribute to food safety while reducing food spoilage (Chang and Chang, 2010). As obligate heterofermenters, *Leuconostoc* spp. are gas-formers (CO_2) (Hemme and Focaud-Scheunemann, 2004), and therefore favour the opening of the curd so important in the manufacture of blue cheese.

Though their numbers may vary widely, *Leuconostoc* species have been reported present in many traditional dairy products made form raw milk with or without starters (Alegría et al., 2009; Nieto-Arribas et al., 2010; Sánchez et al., 2005; Cibik et al., 2000; Server-Busson et al., 1999; Joansen and Kibenich, 1992). Together with the mesophilic lactobacilli, *Leuconostoc* spp. form part of the non-starter lactic acid bacteria (NSLAB) populations of dairy environments. As NSLAB members, they are deemed to play a pivotal role in maintaining the flavour patterns and typicity of traditional dairy products (Nieto-Arribas et al., 2010; Sánchez et al., 2005; Flórez et al., 2006). The latter therefore provide a reservoir of phenotypic and genetic biodiversity, from which new strains with novel properties might be selected for improving adjunct cultures.

In this work, *Leuconostoc* isolates from traditional cheeses made from raw milk without commercial starters were identified by molecular methods and genotyped. They were shown to belong to three *Leuconostoc* species, namely *Leuc. mesenteroides, Leuc. citreum* and *Leuc. lactis*. Representative strains of these species were subjected to complete biochemical, genetic and technological characterization in order to identify appropriate candidates for use as adjunct cultures in the dairy industry.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Leuconostoc isolates (n=42) were obtained over the manufacturing and ripening of different batches of five traditional cheeses. Isolates were kept frozen at -80ºC in de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstad, Germany) with 15% glycerol. Unless otherwise stated, cultures were grown aerobically and statically in MRS broth at 25ºC for 18-24 h.

2.2. Isolation and purification of total DNA

Total genomic DNA from the isolates was purified from overnight cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommenddations.

2.3. Identification by ARDRA and 16S rDNA sequencing

Total DNA was used as a template to amplify a segment of the 16S rRNA gene by PCR using the universal prokaryotic primers S-D-Bact0008-a-S-20 (27F) (5'-AGAGTT TGATCCTGGCTCAG-3') and S-*-Univ 1492R-b-A-21 (1492R) (5'-GGTTACCTT GTTACGACTT-3'). Amplicons were purified through GenElute PCR Clean-Up columns (Sigma–Aldrich), digested with HaeIII and HhaI restriction enzymes (Invitrogen, Pasley, UK), and electrophoresed in 1.5% agarose gels. The gels were then stained with ethidium bromide (0.5 mg mL^{-1}) and photographed under UV light.

Isolates were grouped according to their ARDRA profile. Representative amplicons of each group were then sequenced using the 27F primer, employing a ABI 373 DNA sequencer (Applied Biosystems, Foster City, Ca., USA). On average, more than 800 bp were obtained. These sequences were compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and with those in the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Sequences with a percentage similarity of at least 97% to those in the databases were allocated to the same species (Stackebrandt and Goebel, 1994; Palys et al., 1997).

2.4. Strain typing by Rep-PCR

The intraspecies diversity of the isolates was assessed by repetitive extragenic profiling (Rep-PCR) using primer BoxA2R (5'-ACGTGGTTTGAAGAGATTTTCG-3'), as reported by Koeuth et al. (1995). Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA), employing GeneTools software (Syngene, San Diego, CA, USA). The similarity of the patterns was expressed by the Sørensen-Dice coefficient.

2.5. Acidification of milk

Single colonies of the strains cultivated on MRS agar plates at 30ºC for 48 h were grown in UHT milk (CAPSA, Siero, Spain). Overnight milk cultures were used to inoculate 5 mL of UHT milk at 1% (v/v), which were then further incubated at 30ºC for up to 5 days. Microbial counts, pH measurements of milk samples, and visual inspection of clotting were performed at 24 h, 48 h and 5 days. Assays were performed in triplicate. Strains were also grown in UHT-treated milk supplemented with 5% (w/v) sucrose (Merck) and 0.005% bromocresol purple as a pH indicator.

2.5. Metabolic activities

2.5.1. Carbohydrate fermentation

The carbohydrate fermentation profiles of the isolates were determined using the commercial API50 CHL system (bioMérieux, Montalieu-Vercieu, France), following the supplier's recommendations.

2.5.2. Enzyme activities

Enzyme activities were measured using the commercial, semiquantitative API-ZYM system (bioMérieux), following the manufacturer's recommendations.

2.6. Technological characterisation

2.6.1. Proteolytic activity

The proteolytic activity of the strains was investigated by the *o*-phthaldialdehyde (OPA) method (Church et al., 1983). Briefly, after incubation of the strains in milk at 30ºC for 48 h, proteins were precipitated by the addition of 2 mL of 0.75 N trichloroacetic acid (TCA) and 0.2 mL of water to 1 mL incubated milk samples. The mixtures were vortexed for 2 min and then filtered using Whatman nº2 filter paper (Whatman Ltd., Maidstone, UK). The OPA reagent (Sigma-Aldrich) was added to the filtrates and the absorbance of the reaction measured at 340 nm using a Benchmark PlusMicroplate Spectrophotometer (BioRad, Hercules, CA, USA). Results were expressed as mM glycine L^{-1} using an appropriate calibration curve (concentration range 0.1-10 mM).

2.6.2. Production of volatile compounds

Volatile compound analysis was performed after growth of the strains at 30ºC in UHT milk for 24 and 48 h and 5 days. A solution of cyclohexanone (0.36 mg mL-1) was used as an internal standard. The separation and quantification of the volatile compounds was performed by head space/gas chromatography/mass spectro metry (HS/GC/MS), using an Agilent

apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with a capillary column DB-WAXetr 60 m x 0.25 mm x 0.25μm (Agilent). Sample preparation and gas chromatographic separation were performed as previously described (Salazar et al., 2008). Peaks were quantified as the relative total ion count with respect to the internal standard.

2.6.3. Production of antimicrobial substances

Antimicrobial activity was first examined by an agar spot test, followed by a well-diffusion assay using neutralised, filter-sterilised supernatants (Alegría et al., 2010). *Lactococcus lactis* subsp. *cremoris* MG 1363, *Lactobacillus sakei* CECT 906^T, *Listeria innocua* 86/26 and *Staphylococcus aureus* CECT 86^T were used as indicators.

2.6.4. Resistance to NaCl and low pH, and growth at different temperatures

Resistance to NaCl was assessed by inoculation of the strains (at 1% v/v) into a series of 100 μL MRS broths containing concentrations of NaCl ranging from 4 to 7% (w/v) with 0.5% intervals. To test the resistance of the strains to acid, they were inoculated (at 1% v/v) into 100 μL MRS broth adjusted with acetic acid to pH values between 3.5 and 6.5 with 0.5 unit intervals. As a control for both tests, a culture in standard MRS (pH 6.8, no extra NaCl) was used. Finally, strains were inoculated (1% v/v) into MRS broth and incubated at 10, 25, 32, and 45ºC to determine their growth at different temperatures. In all cases, optical density (OD) was measured spectrophotometrically at 595 nm. All assays were performed in triplicate. The results were expressed as the difference between the OD_{595} recorded for standard MRS and that associated with the corresponding NaCl, pH or temperature condition.

2.7. Safety of *Leuconostoc* strains

2.7.1. Antibiotic resistance

The minimum inhibitory concentration (MIC) of several antibiotics of biological and clinical significance were determined by the microdilution method, using dedicated plates containing serial two-fold dilutions of 16 antibiotics (VetMIC™, National Veterinary Institute of Sweden, Uppsala, Sweden) (Flórez et al., 2007). Briefly, individual colonies on Mueller-Hinton agar plates (Oxoid, Basingstoke, Hampshire, UK) were suspended in 2 mL of sterile saline solution (Oxoid) until a density corresponding to McFarland standard 1 or its spectrophotometric equivalent (around $3x10^8$ cfu mL-1) was obtained. This suspension was then diluted 1:1000 with Mueller-Hinton broth to a final concentration $3x10^5$ cfu mL^{-1}. An aliquot of 100 mL of this inoculum was then added to each well of the VetMIC™ plates, which were then incubated at 30ºC for 48 h. The MIC was defined as the lowest antibiotic concentration at which no visual growth was observed in the wells.

2.7.2. Production of biogenic amines

The production of the toxic amines tyramine and histamine from their respective precursor amino acids (tyrosine and histidine) was investigated using the plate assay described by Joosten and Northolt (1989).

3. Results and Discussion

3.1. Identification and typing of *Leuconostoc*

The restriction enzyme HaeIII yielded two ARDRA profiles for the 42 isolates - profile a (24 isolates) and profile b (18 isolates) (Fig. 1A). HhaI, in contrast, yielded three different profiles: profile 1 (24 isolates), profile 2 (13 isolates), and profile 3 (5 isolates) (Fig. 1B). After the sequencing and comparison of representative amplicons the profiles were assigned to *Leuc. citreum* (profile a), *Leuc. lactis* or *Leuc. mensenteroides* (profile b),

Leuc. lactis (profile 1), *Leuc. mensenteroides* (profile 2), and *Leuc. citreum* (profile 3). The intraspecies genetic diversity of the isolates belonging to the different species was assessed by Rep-PCR. Sixteen profiles were returned by the 24 *Leuc. citreum* isolates, nine for the 13 *Leuc. mesenteroides* isolates, and three for the five *Leuc. lactis* isolates (Fig. 2). Reproducibility studies of the Rep-PCR technique using different isolates and independently purified DNA showed a percentage similarity of >85% (data not shown). Using this cut-off, 15 strains were contemplated among the 24 *Leuc. citreum* isolates, five among the 13 *Leuc. mesenteroides*, and two among the 5 *Leuc. lactis* (Fig. 2). To select the best strains for use in dairy systems, all 22 strains were cultured in UHT milk. Eight neither grew nor acidified the milk; they were therefore discarded from further analysis.

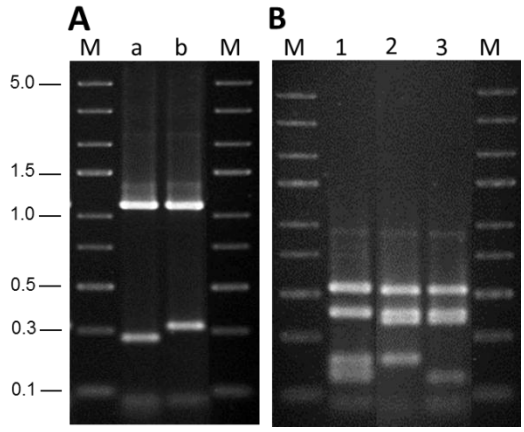

Figure 1.- ARDRA profiles obtained after amplification of the 16S rRNA genes of the Leuconostoc strains with the universal primers 27F and 1492R and digestion of the amplicons with the restriction ennzymes HaeIII (Panel A) and HhaI (Panel B). M, Gene Ruler ExpressTM DNA ladder (Fermentas, Vilnius, Lithuania); the molecular weight (kbp) of some bands is indicated on the left.

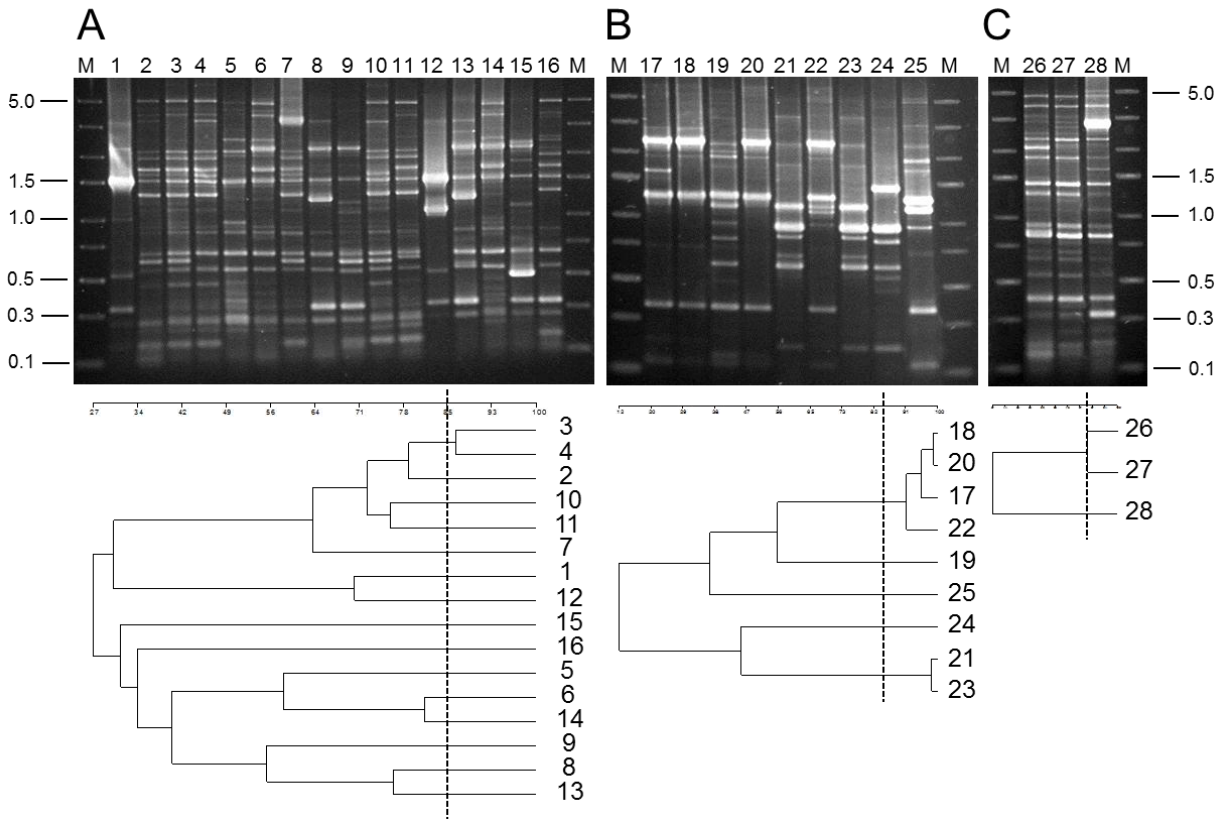

Figure 2.- REP-PCR profiles obtained with primer BOXA2R corresponding to 16 isolates of *Leuc. citreum* (Panel A), 9 isolates of *Leuc. mesenteroides* (Panel B), and 3 isolates of *Leuc. lactis* (Panel C). M, Gene Ruler Express ™ DNA ladder (Fermentas). The dendrograms of similarity of the different profiles are shown below the panels. The broken line indicates the position of 85% of similarity which, as suggested by reproducibility studies, separated different strains.

The phenotypic and metabolic activity of the selected 14 strains was analysed using the API 50 CHL and API-ZYM systems. Tables 1 and 2 show the results obtained. In agreement with the high genetic diversity detected by Rep-PCR, high phenotypic diversity was encountered. Most strains showed single patterns in both phenotypic assays. Ten different profiles were obtained for the 14 strains in both tests. All the *Leuconostoc* strains fermented D-glucose, D-fructose, N-acetyl-glucosamine, D-lactose, D-maltose, D-mannose and D-sucrose.

However, diversity in the utilization of another 17 carbohydrates was detected (Table 1). Twelve of the 19 enzymatic activities tested for were detected among the analysed strains. Of these, some activities (such as those of as leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase) were shown by all or most strains. In contrast, other activities (such as those of esterase lipase C8, cystine arylamidase, α-chymotrypsin and α-galactosidase) were only rarely detected (Table 2).

3.2. Technological characterization of *Leuconostoc* strains

Table 3 shows the behavior of the 14 selected strains in UHT milk. All strains attained a similar maximum cell density after 24 h of incubation; the number of live cells declined thereafter. Acidification and milk clotting progressed slowly. By day 5, pH 5.0 or lower was reached with most *Leuc. citreum* strains, and a stable coagulum had been produced. However, the final pH of the milk fermented by the *Leuc. mesenteroides* and *Leuc. lactis* strains was higher, and the viscosity of the coagulum much lower. In some cases, small- and medium-sized gas bubbles were clearly observed within the coagulum, indicating the active production of CO_2. Gas production by *Leuc. citreum* 7A7b was so strong that the curd broke, allowing for some whey drainage (Table 3). In the sucrose-supplemented milk, the *Leuc. citreum* strains generally grew faster than those of *Leuc. mesenteroides* and *Leuc. lactis*. All but two strains (*Leuc. mesenteroides* 3AC2 and 3AC16) developed well and turned the pH indicator yellow. However, the glucans and dextrans that increase the viscosity of milk were not produced under these culture conditions, as determined by the Posthumus funnel method (data not shown).

Table 4 shows the production of volatile compounds in milk by the strains. At least eight volatile compounds were detected by GC/MS. Though differences among strains were found, ethanol was the major volatile compound in the profiles and was produced by all strains. Acetic acid was also produced by all strains, except for *Leuc. lactis* 4AB2. Small amounts of 2-propanone and ethyl-acetate were also produced by most strains. Compounds such as 2-butanone, diacetyl, 2-heptanone and butanoic were rarely produced.

The pH and NaCl content of the medium, and the incubation temperature, had great influence on the final cell density attained by the *Leuconostoc* cultures. Figure 3 shows the results of growth under different conditions. For reasons of clarity, only key results are shown in the figure panels.

Figure 3A shows the strain to strain variation in growth at the different temperatures assayed. In general, the same OD was eventually obtained for all strains at 10, 25 and 30ºC. However, at 40ºC none of the strains grew, except for the two *Leuc. lactis* strains (4AB2 and 7G3a), which grew to 17% and 29% of that seen at 25ºC (as measured by OD).

The growth of all strains was inversely related to the NaCl content of the growth medium (Fig. 3B). *Leuc. citreum* strains 4AC15, 7A7b and 7G2c reached higher cell densities than all the other strains at all salt concentrations up to 6.5%. The strain *Leuc. lactis* 7G3a also grew well up to an NaCl concentration of 5.5%.

Compared to growth in unadjusted MRS (pH 6.8), all strains showed 20% reductions in OD at pH 5.5, and more than 80% at pH 5. None of the strains grew at pH 4.5 or lower (Fig. 3C). Small, strain-specific differences were observed at all pHs, although at pH 5 *Leuc. mesenteroides* 3AC2 and 3AC16 doubled the optical density reached by all other strains.

The OPA assay detected significant differences in proteolytic activity between the isolates (Table 5). *Leuc. lactis* 7G3a was associated with the strongest release of amino acids from the milk proteins, followed by *Leuc. mesenteroides* 3AC16, to equivalents of 8.09±0.01 and 13.66±0.02 mM L^{-1} of glycine respectively. In contrast, one strain each of these two species showed no detectable proteolytic activity in the OPA test. Most strains produced antimicrobial substances against one or more indicators in the agar spot test. However, in the well-diffusion assay, only *Leuc. citreum* 4AC4 show-ed a consistent and specific inhibitory effect against the *L. innocua* indicator.

3.3. Safety of *Leuconostoc*

Table 6 shows the MIC values for the assayed antibiotics. Where defined, MICs were compared to the microbiological breakpoints stated for the purpose of distinguishing resistant from susceptible bacteria by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (EFSA, 2012). For most of the antibiotics, the MIC values were lower than the FEEDAP cut-offs. The MICs of kanamycin and chloramphenicol for some strains were one dilution higher than their corresponding cut-offs. The MIC values of all the antibiotics for the different strains

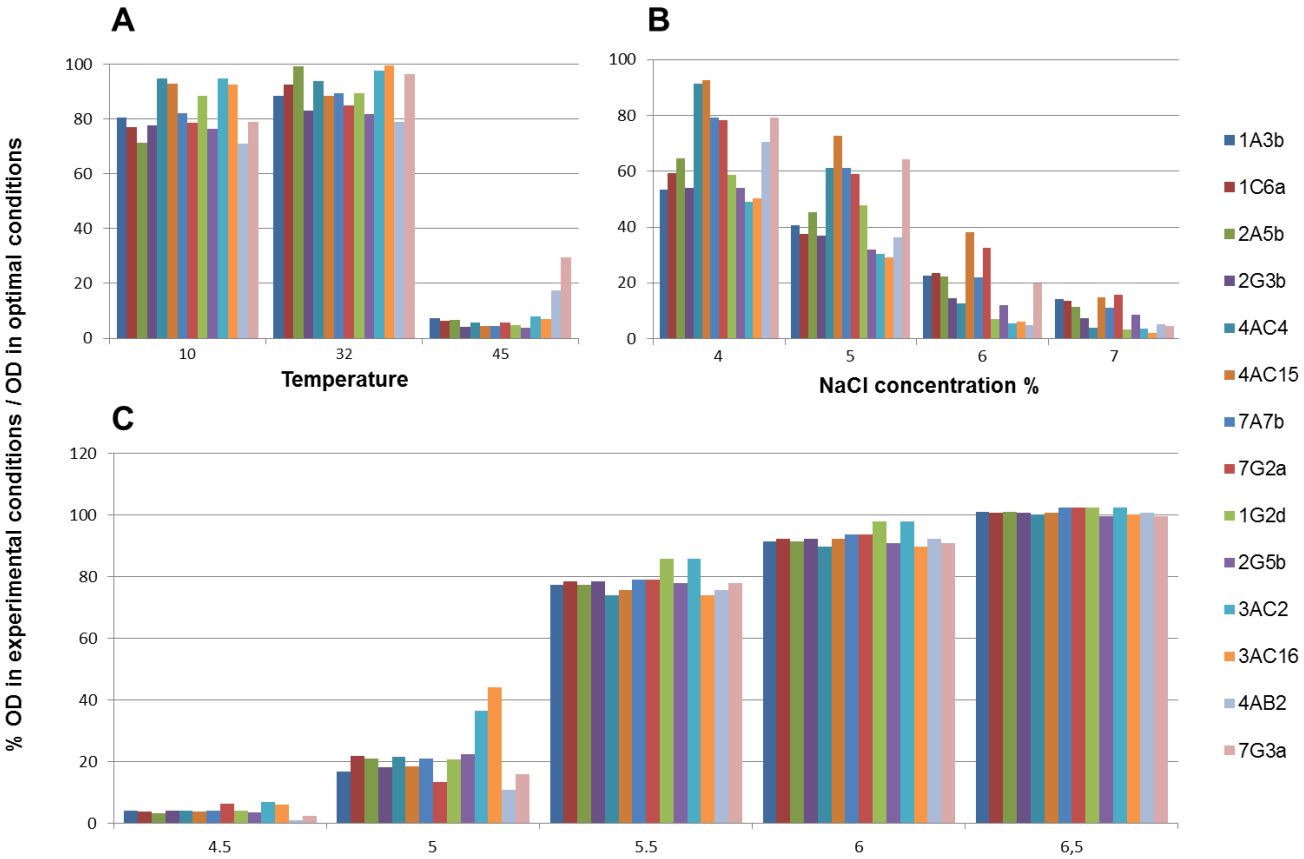

Figure 3.- Effect of acid (pH) on the growth of *Leuconostoc* strains. Results are expressed as the percentage of OD_{600} in experimental conditions with respect to OD_{600} optimal conditions. Optimal conditions correspond to 25 C in the temperature assay (A), MRS without added NaCl in the salt resistance assay (B) and pH 6,8 in the acid resistant assay (C). Results are the average of three independent cultures with a standard deviation always lower than 5%.

suggested that all but one were either susceptible or only intrinsically resistant. The exception was *Leuc. citreum* 7A7b, for which the MIC of ciprofloxacin was 64 μg mL^{-1}; a value eight times that associated with most other strains.

None of the strains produced tyramine or histamine on plates containing their precursor amino acids tyrosine or histidine. Production of these and other biogenic amines has been further excluded by ultra-high pressure liquid chromatography (UPLC) analysis (Ladero et al., unpublished).

4. Discussion

Traditional cheeses are a good source of new dairy LAB strains, including *Leuconostoc* spp. (Server-Bussson et al., 1999; Cibik et al., 2000; Sánchez et al., 2005; Nieto-Arribas et al., 2010; Cardamone et al., 2011). In this work, 42 *Leuconostoc* isolates were identified and typed by molecular methods. Rep-PCR identified 22 different strains, 14 of which were selected for further study based on their acidification and growth behaviour in milk. These were subjected to phenotypic characterization, including for different technological and safety traits. The genetic diversity revealed by Rep-PCR correlated well with the biochemical diversity seen in the different phenotypic assays. A similar level of phenoltypic and genetic diversity has been reported elsewhere for other mesophilic LAB species isolated from traditional, starter-free, raw-milk cheeses (Delgado and Mayo, 2004; Alegría et al., 2009).

Qualitative and quantitative variations between the strains in terms of their volatile compound profiles in milk were recorded. Surprisingly, diacetyl was only identified as a minority component in the profiles of two strains, *Leuc. mesenteroides* 2G5b and *Leuc. lactis* 4AB2. In *Lactococcus lactis* it is well known that the transport of citrate only occurs after acidification of the medium during cell growth (García-Quintans et al., 1998). Similar pH-mediated induction of the components required for citrate utilization in *Leuconostoc* may hamper the formation of diacetyl at the high pH recorded for all cultures at 48 h (Table 3). However, *Leuconostoc* does not grow well in milk when on its own. Thus, diacetyl production, and perhaps that of other volatile compounds, might be enhanced when strains are cultivated with starter lactococci. Such production might reflect greater growth or the complementation of metabolic routes (Ayad et al., 2001).

Proteolysis is the most critical process for the formation of volatile compounds during the ripening of dairy products (Smit el al., 2005). Differences in the proteolytic ability of the strains, as judged by the OPA test, were noted. Though proteolysis of casein is essential for full growth in milk, the activity recorded did not correlate with the acidification and clotting of the milk (Table 3). Proteolytic levels higher than those reported in the literature (Nieto-Arribas et al., 2010) were scored for two strains. Since no extracellular proteinases have ever been described for *Leuconostoc* species (Liu et al., 2010), differential lysis during growth in milk and the release of intracellular proteolytic enzymes might explain such differences.

The antimicrobial activity of *Leuconostoc* in solid media has been attributed to metabolic end products such as lactic and acetic acids and diacetyl (Hemme and Foucaud-Sheunemann, 2004), as well as colony-associated compounds such as fatty acids and H_20_2 (Hemme and Foucaud-Sheunemann, 2004; de Vuyst and Leroy, 2007). Consequently, the inhibitory effects detected by the agar spot test are not always confirmed in liquid (Alegría et al., 2010; Dal Bello et al., 2010). In the present work, a single strain (*Leuc. citreum* 4AC4) showed inhibitory activity when neutralised supernatants were used. The production of bacteriocin-like substances by different strains has frequently been reported

(Héchard et al., 1992; Stiles, 1994; Trias et al., 2008). The production of a bacteriocin by 4AC4 is suspected, but the nature of the inhibitory compound was not further examined. The inclusion of bacteriocin-producing strains in starter and adjunct cultures could further help improve the quality and safety of fermented dairy products.

Whenever possible, the use of starter and adjunct cultures carrying antibiotic resistance should be avoided in food systems to help prevent the spread of antibiotic resistance determinants through the food chain (EFSA, 2012). The MICs of antibiotics for all assayed strains were lower than the microbiological breakpoints defined by the European Food Safety Authority, or just a dilution higher. Increased MICs might be the result of methodological limitations, such as a lack of standardised methods for use with *Leuconostoc* species, the absence of appropriate control strains of this genus, or the use of different antibiotic-testing media (Katla et al., 2001; Flórez et al., 2005). Analysis of the MIC distributions for all antibiotics, and comparison with previous results (Katla et al., 2001; Flórez et al., 2005), strongly suggests that the strains of this study are susceptible to all the antibiotics assayed - with the exception of *Leuc. citreum* 7A7b, which showed strong resistance to ciprofloxacin. The mechanism of resistance to this antibiotic deserves further investigation.

The production of biogenic amines by *Leuconostoc* species from wines is well known (Lonvaud-Funel and Joyeux, 1994; Moreno-Arribas et al., 2003), but this activity has never been seen in cheese isolates (Bober-Cid and Holzapfel, 1999; Sánchez et al., 2005; Nieto-Arribas et al., 2010). As would seem to be the case for *Lact. lactis* (Ladero et al., 2011), the formation of biogenic amines by *Leuconostoc* spp. may have been selected against during adaptation to the milk environment.

Leuconostoc spp. have been described as moderately susceptible to bacteriophage attack (Dessart and Steenson, 1995). This may result from their low growth rates compared to those of lactococci. Thus, *Leuconostoc* species usually reach their maximum numbers during cheese ripening, when the matrix is in a semi-solid state, which might hinder the spread of phages. In the present work, phage resistance - a desirable characteristic for starter and adjunct cultures - was not examined due to the lack of a suitable collection of phages that infect species of this genus.

In conclusion, several *Leuconostoc* strains from traditional cheeses were fully characterised. The genetic and phenotypic diversity detected among the strains of the different species, including their different proteolytic activities, should allow for the rational selection of cultures that would meet different technological demands. Those characterised here might complement currently available strains for the industrial manufacture of dairy products. They could also be used in the design of specific starter and adjunct cultures based on native strains, which might contribute towards the maintenance of typicity in traditional cheeses. The testing of individual strains and mixtures as adjunct cultures, and their evaluation in cheese trials together with starter strains of *Lact. Lactis*, is currently in progress.

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Table 1.- Variability in the carbohydrate fermentation profiles of *Leuconostoc* strains isolated from traditional, starter-free cheeses made of raw milk.

Species/Strain	Fermentation of carbohydrate[a]																
	LARA	RIB	DXYL	GAL	MDG	ARB	ESC	SAL	CEL	MEL	TRE	RAF	GEN	DTUR	GNT	2KGN	5KGN
Leuc. citreum																	
1A3b	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-
1C6a	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-
2A5b	+	-	-	+	+	-	-	-	-	-	+	-	-	+	+	-	-
2G3b	-	-	-	+	+	+	+	+	-	-	+	-	-	+	+	-	-
4AC4	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-
4AC15	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-
7A7b	-	-	-	+	+	+	+	+	-	-	+	-	-	+	+	-	-
7G2a	-	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+	-
Leuc. mesenteroides																	
1G2d	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-
2G5b	-	-	-	-	-	+	+	+	-	-	+	-	-	+	+	-	-
3AC2	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
3AC16	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Leuc. lactis																	
4AB2	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-
7G3a	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Key of carbohydrates: LARA, L-arabinose; RIB, D-ribose; DXYL, D-xylose; GAL, D-galactose; MDG, α-methyl-D-glucoside; ARB, arbutin; ESC, Esculin; SAL, salicin; CEL, D-cellobiose; MEL, D-melibiose; TRE, D-trehalose; RAF, D-raffinose; GEN, gentiobiose; DTUR, D-turanose; GNT, gluconate; 2KGN, 2-keto-gluconate; and 5KGN, 5-keto-gluconate. All strains fermented D-glucose, D-fructose, D-mannose, D-maltose, N-acetyl-glucosamine, D-lactose, and D-sucrose, while proved to be negative for glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-manitol, D-sorbitol, α-methyl-D-mannoside, amygdalin, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, and L-arabitol.

Table 2.- Diversity of enzymatic activities of *Leuconostoc* strains from traditional, starter-free cheeses made of raw milk.

Species/strain	Enzymatic activity[a]											
	Alkaline phosphatase	Esterase C4	Esterase Lipase C8	Leucine arylamidase	Valine arylamidase	Cistine arylamidase	α-chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	α-glucosidase
Leuc. citreum												
1A3b	5	5	0	5	5	0	0	10	5	0	40	10
1C6a	5	0	0	5	5	0	0	10	5	0	40	10
2A5b	5	0	0	5	5	0	0	10	5	0	40	10
2G3b	5	5	0	5	5	0	0	10	5	0	40	10
4AC4	0	10	5	5	5	0	0	5	5	5	40	10
4AC15	0	10	5	5	5	0	0	5	5	0	40	20
7A7b	5	0	0	5	5	5	0	20	5	0	40	20
7G2a	0	5	0	5	5	0	0	5	5	0	40	10
Leuc. mesenteroides												
1G2d	5	0	0	10	0	5	0	5	5	0	40	10
2G5b	5	5	0	5	5	0	0	5	5	0	40	10
3AC2	0	20	0	5	5	0	0	5	5	0	40	5
3AC16	0	20	0	5	5	0	0	5	5	0	40	5
Leuc. lactis												
4AB2	5	0	0	10	5	0	10	5	0	5	0	0
7G3a	5	0	0	10	5	0	0	10	5	0	20	0

[a]Units of activity are expressed as nanomoles of substrate hydrolyzed under the assay conditions.

Lipase C14, trypsin, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities were never recorded.

Table 3.- Growth and survival of *Leuconostoc* strains isolated from traditional, starter-free cheeses in UHT-milk.

Species/strain	Incubation in milk for[a]								
	24 h			48 h			5 day		
	pH[b]	Log_{10} cfu/ml	Clotting of milk	pH	Log_{10} cfu/ml	Clotting of milk	pH	Log_{10} cfu/ml	Clotting of milk
Leuc. citreum									
1A3b	6.07	8.81	+/-	5.45	8.46	+/-	5.00	8.02	+++ (gb)[c]
1C6a	6.03	8.35	+/-	5.50	8.59	+	5.02	7.81	+++
2A5b	6.21	8.30	+/-	5.55	8.35	++	5.11	7.78	+++ (gb)
2G3b	6.28	8.19	-	5.93	8.05	+	5.21	8.61	+
4AC4	6.05	8.38	+/-	5.53	7.32	+	4.80	7.16	+++
4AC15	6.06	8.54	+/-	5.50	8.26	++	5.01	6.30	+++ (gb)
7A7b	6.32	8.52	-	6.08	7.79	+	5.70	6.89	+++ (w)[d]
7G2a	6.03	8.61	+/-	5.58	8.25	+	4.97	6.83	+++
Leuc. mesenteroides									
1G2d	6.27	8.21	-	5.75	8.48	+/-	5.66	6.42	+/- (gb)
2G5b	5.99	8.74	-	5.58	8.12	+	5.62	6.48	++
3AC2	6.22	8.30	-	5.80	7.99	+/-	5.39	7.89	+/-
3AC16	6.28	8.23	-	5.81	8.03	+/-	5.39	7.71	+ (gb)
Leuc. lactis									
4AB2	6.24	8.38	-	5.78	8.26	+/-	5.27	7.11	+
7G3a	6.25	8.24	-	6.00	7.80	+/-	5.72	7.96	+/-

[a]Inoculated with 10^6 cfu from overnight cultures in UHT milk and incubated at 30ºC.
[b]pH of uninoculated milk 6.66
[c](gb), visible gas bubbles in the curd.
[d](w), broken curd and whey drainage.

Table 4.- Relative abundance of volatile compounds detected by Head Space, Gas Chromatography, Mass Spectrometry (HS/GS/MS) after growth of the strains at 32ºC for 48 h in UHT-treated milk.

Species/Strain	Volatile compound[a]							
	2-propanone	Ethyl-acetate	2-butanone	Ethanol	Diacetyl	2-heptanone	Acetic acid	Butanoic acid
Leuc. citreum								
1A3b	4.67	-	-	15,576.69	-	-	383.87	-
1C6a	3.31	-	-	14,498.04	-	-	382.99	-
2A5b	5.26	8.44	-	16,817.62	-	-	341.06	-
2G3b	4.49	5.33	-	12,527.85	-	-	261.55	-
4AC4	3.16	8.47	-	18,265.82	-	-	394.64	-
4AC15	4.56	8.75	-	20,265.67	-	-	492.00	8.45
7A7b	7.56	-	-	8,914.78	-	-	19.77	-
7G2a	4.30	6.26	1.08	16,991.22	-	-	407.07	2.67
Leuc. mesenteroides								
1G2d	2.49	-	-	11,349.17	-	-	227.62	-
2G5b	0.28	2.47	1.41	8,208.76	3.12	4.07	117.07	-
3AC2	-	4.75	1.33	10,798.79	-	-	20.04	-
3AC16	-	4.72	-	12,711.59	-	-	30.80	-
Leuc. lactis								
4AB2	0.75	3.67	1.09	7,833.15	2.17	3.65	-	3.48
7G3A	2.65	-	-	6,688.42	-	4.41	92.89	4.76

[a]Results are expressed as the normalized value of chromatogram peak areas using cyclohexanone (3.6 µg of per gram of sample) as an internal standard, which was given a value of 100. Results were calculated as the difference between values obtained for the sample and that of a non-inoculated UHT-milk sample incubated under the same conditions.
-, not detected.

Table 5.- Protoelytic activity of *Leuconostoc* strains isolated from traditional, starter-free cheeses as determined by the OPA assay.

Species/strain	Proteolytic activity[a]	
	Average[b]	SD[c]
Leuc. citreum		
1A3b	0.87	0.02
1C6a	0.93	0.02
2A5b	0.46	0.00
2G3b	1.26	0.02
4AC4	1.05	0.46
4AC15	1.13	0.01
7A7b	0.00	0.15
7G2a	0.61	0.01
Leuc. mesenteroides		
1G2d	0.18	0.01
2G5b	1.40	0.01
3AC2	0.00	0.00
3AC16	8.09	0.01
Leuc. lactis		
4AB2	0.00	0.00
7G3a	13.66	0.02

[a]Determined as the mmol of glycine released after incubation in milk under the conditions of the assay (30ºC for 48 h), using a glycine calibration curve (Supplementary Figure 1).
[b]Average results of three independent experiments.
[c]SD, standard deviation.

Table 6.- Microdilution MICs of *Leuconostoc* strains from traditional, starter-free cheeses made of raw milk to 16 antibiotics.

Species/Strain	Antibiotic[a]															
	GM	KM	SM	NM	TC	EM	CL	CM	AM	PC	VA	VI	LZ	TM	CI	RI
Leuc. citreum																
1A3b	2	32	16	2	2	0.25	≤0.03	8	0.5	0.12	>128	0.5	2	>64	8	4
1C6a	1	16	8	2	2	0.12	≤0.03	4	0.5	0.12	>128	0.5	4	>64	16	8
2A5b	1	16	8	2	2	0.12	0.06	4	0.5	0.12	>128	0.5	2	>64	8	2
2G3b	2	32	16	4	2	0.25	0.06	4	0.5	0.25	>128	0.5	2	>64	8	2
4AC4	1	32	16	2	2	0.25	≤0.03	8	0.5	0.12	>128	0.5	2	>64	8	2
4AC15	1	32	16	2	2	0.25	≤0.03	8	0.5	0.12	>128	1	2	>64	8	2
7A7b	1	32	16	4	2	0.25	≤0.03	8	0.5	0.12	>128	1	2	>64	64	2
7G2a	1	32	16	2	2	0.25	0.06	8	0.5	0.25	>128	1	2	>64	4	2
Leuc. mesenteroides																
1G2d	≤0.5	16	16	≤0.5	4	0.12	0.06	8	0.5	0.25	>128	0.5	2	>64	4	1
2G5b	2	32	16	8	2	0.25	0.06	8	0.5	0.25	>128	0.5	2	>64	4	2
3AC2	2	8	16	2	4	0.12	0.06	4	0.5	0.25	>128	1	2	>64	8	1
3AC16	0.5	8	16	0.5	2	0.12	0.06	4	0.5	0.25	>128	0.5	2	>64	8	1
Leuc. lactis																
4AB2	1	32	16	2	2	0.12	≤0.03	8	0.5	0.25	>128	1	2	>64	4	2
7G3a	2	16	16	1	2	0.25	0.06	8	0.5	0.25	>128	1	2	>64	4	4
FEEDAP breakpoints[b]	**16**	**16**	**64**	**-[c]**	**8**	**1**	**1**	**4**	**2**	**-**	**nr**	**-**	**-**	**-**	**-**	**-**

Key of antibiotics: GM, gentamicin; KM, kanamycin; SM, streptomycin; NM, neomycin; TC, tetracycline; EM, erythromycin; CL, clindamycin; CM, chloramphenicol; AM, ampicillin; PC, penicillin G; VA, vancomycin; VI, virginiamycin; LZ, linezolid; TM, trimethoprim; CI, ciprofloxacin; RI, rifampicin.

[a]MIC values in μg mL^{-1}.

[b]Microbiological cut-offs in μg mL^{-1} as defined by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for *Leuconostoc* species (European Commission, 2012).

[c]Not defined (-) or not required (nr).

Cuarta parte

CUARTA PARTE: DISEÑO Y ENSAYO EXPERIMENTAL DE FERMENTOS

• Artículo VIII

Technological characterization of mixtures of mesophilic lactic acid bacteria strains isolated from traditional cheeses made of raw milk and their experimental evaluation as cheese starters.
(En preparación para su publicación)

En la última fase de este trabajo se combinaron distintos grupos de bacterias lácticas previamente caracterizadas para crear nueve mezclas iniciadoras diferentes. Además de una caracterización exhaustiva de los integrantes de los fermentos, en nuestra opinión, ha de realizarse también una caracterización de las mezclas y, por supuesto, su ensayo experimental en las condiciones reales de elaboración. Solo así se podrán proponer mezclas con las garantías suficientes para su utilización en el exigente entorno industrial. Las mezclas se compusieron principalmente de distintas cepas de la especie *Lactococcus lactis,* combinadas en ocasiones con cepas de los géneros *Lactobacillus* y *Leuconostoc*. Entre las cepas de *Lc. lactis* se incluían también cepas de las subespecies *lactis* y *cremoris* y cepas de la biovariedad *diacetylactis*. Ensayamos también dos mezclas productoras de bacteriocinas. En una de ellas se incluyeron las cepas productoras de nisina y en la otra las productoras de lactococina 972. Estas nueve mezclas se sometieron a diversos análisis con el objetivo de evaluar

su crecimiento en leche, su poder de acidificación, la producción de ácidos orgánicos, compuestos volátiles, aminoácidos libres, etc.

Posteriormente las cuatro mezclas que mostraron mejores aptitudes como fermentos lácticos se ensayaron en sendas elaboraciones experimentales de queso. En éstos se analizaron las características físico-químicas básicas, así como la evolución de diversas poblaciones microbianas a lo largo de la elaboración y maduración. Finalmente se evaluaron las características organolépticas de los quesos maduros por el Panel de Cata del IPLA. Como conclusión, dos de las mezclas produjeron quesos con buenas cualidades aromáticas y de sabor, por lo que, por sí mismas, constituyen buenos cultivos iniciadores. Los resultados obtenidos con las otras dos mezclas, sin embargo, no fueron concluyentes, debido a un problema con la leche de elaboración y deberán, por tanto, ser evaluadas de nuevo.

Technological characterisation of mixtures of mesophilic lactic acid bacteria strains isolated from traditional raw milk cheeses and their experimental evaluation as cheese starters

Abstract

The technological behavior in milk of nine mixtures of strains of *Lactococcus lactis*, alone or in combination with strains of *Leuconostoc* spp. and *Lactobacillus* spp. (all isolated from traditional raw milk cheeses made without the addition of commercial starter), was investigated. The strains of *L. lactis* examined belonged to *L. lactis* subsp. *lactis* (including producers of the bacteriocins nisin and lactococcin 972), *L. lactis* subsp. *lactis* biovar *diacetylactis*, and *L. lactis* subsp. *cremoris*. The mixtures were analysed for bacterial growth, milk acidification and the production of organic acids, volatile compounds, free amino acids and biogenic amines during milk fermentation. The acidification curves for all mixtures were similar, except for the mixture containing the nisin-producing *L. lactis* strains. Mixture-specific differences were recorded for the other variables measured. Four mixtures appropriate for use as dairy starters were tested in trials, recording microbial and chemical variables during cheese manufacture and ripening. The sensorial properties of the final cheeses were rated by a trained panel. Two mixtures produced cheeses of good flavour and taste; these could be confidently used as cheese starters. Due to a problem in the supply of milk, inconsistent results were recorded for the other two mixtures; these should be re-examined for clear-cut conclusions to be drawn.

1. Introduction

Strains of *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* are important components of mesophilic starter cultures used in the manufacture of many fermented dairy products (Mills et al., 2010). The main function of starter bacteria during fermentation is the production of lactic acid at an acceptable rate (Mills et al., 2010). However, during ripening they also contribute to the development of flavour via their proteolytic action (Smit et al., 2005), and improve food safety via the synthesis of substances with antimicrobial activity, such organic acids, H_2O_2 and bacteriocins (Topisirovic et al., 2006). *L. lactis* is commonly combined with strains of *Leuconostoc* spp. and other species of lactic acid bacteria (LAB) (mainly mesophilic lactobacilli); acting as adjunct cultures these contribute to the production of key flavour compounds (Hemme and Foucaud-Scheunemann, 2004; Settanni and Moschetti, 2010).

Traditional dairy products made from raw milk without the addition of commercial cultures provide a reservoir of biodiversity from which new LAB strains might be selected to improve starter and adjunct

cultures (Kelli and Ward, 2002; Rademaker et al., 2006; Brandsma et al., 2008). Much effort has already been invested in the identification, typing and characterisation of candidate strains (Ayad et al., 2000; Flórez et al., 2006; Alegría et al., 2010). In industrial scale milk fermentations, however, single-strain starters are of little use since they can easily be rendered unserviceable by bacteriophages, the activity of which lead to flavour, texture and food safety problems (Garneau and Moineau, 2011). Closed fermentation vats, the propagation of starters in antiphage, calcium-depleted media, and the selection of phage-resistant strains have all been explored as ways of reducing the economic losses caused (Everson, 1991). However, starters composed of phage-unrelated strains, and the rotation of starter mixtures, continue to be the simplest and most cost-effective methods of protecting against phage attack (Everson, 1991; Garneau and Moineau, 2011). New LAB strains that can be part of such mixtures and rotations might, therefore, also be sought in such reservoirs (van Hylckama Vlieg et al., 2006; Mills et al., 2010).

In this work, starter (lactococci) and non-starter (leuconostocs and lactobacilli) LAB strains isolated from traditional, raw milk cheeses, made without added starters and characterised in previous work, were used in the design of nine culture mixtures. Their growth in milk was then examined, as were their associated acidification rates, the degree of milk acidification reached, and their production of organic acids, volatile compounds and free amino acids. The suitability of the most promising mixtures for the manufacture of cheese was then tested in cheese manufacturing trails.

2. Materials and methods

2.1. Strain propagation and design of starter mixtures

Lactococcus lactis strains were chosen as the base material for the design of nine experimental starter mixtures. These mixtures involved combinations of strains of the subspecies *lactis* and *cremoris* and the *lactis* biovar *diacetylactis* (mixtures S1, S2, S3, S4 and S5), plus strains of the non-starter LAB species *Leuconostoc* spp. (mixture S6) or strains of both *Leuconostoc* spp. and *Lactobacillus* spp. (mixture S7). Mixtures S8 and S9 were composed exclusively of *Lactococcus lactis* subsp. *lactis* strains that produce the bacteriocins nisin and lactococcin 972 respectively (Table 1). The undefined commercial starter Flora Danica (Chr. Hansen, Denmark) was used as a control mixture.

Stock mixtures were produced by pooling identical volumes of individual overnight cultures raised in M17 (Oxoid, Basingstoke Hampshire, UK) (lactococci), Brain Heart Infusion (BHI; Merck, Darmstad, Germany) (leuconostocs) or de Man, Rogosa and Sharpe (MRS; Merck) (lactobacilli) broth at 30, 25 and 37ºC respectively. These mixtures were then centrifuged, washed with sterile saline (NaCl 0.9%), and the pellets suspended (at one tenth of the original fluid volume) in UHT semi-skimmed milk (CAPSA, Siero, Spain). These samples were then frozen at -80ºC for 24 h before lyophilisation in a Virtis Freezemobile 12EL lyophilser (Sp. Scientific, Gardiner, NY, USA), and stored at -80ºC until use.

2.2. Technological characterisation of the mixtures

2.2.1. Growth in milk

To analyse the acidification capacity of the mixtures, 1 g of the different lyophilised mixtures was suspended in 100 mL of UHT semi-skimmed milk and incubated overnight at 30ºC. Overnight cultures were used to inoculate new milk aliquots at an approximate concentration of 10^6 cfu mL^{-1}, which were once again incubated at 30ºC. The pH was measured at regular intervals over the first 11 h and after 24 and 48 h using a pH meter (Crison Instruments S.A., Barcelona, Spain).

2.2.2. Microbial counts

The number of aerobic mesophilic bacteria in the mixtures was determined on Plate Count Milk Agar (PCMA; Merck) after 72 h of incubation at 30ºC. Total lactococci were determined on M17 agar after 48 h incubation at 30ºC. *L. lactis* subsp. *lactis* biovar *diacetylactis* were enumerated on Kempler and McKay agar plates (KMA; Kempler and McKay, 1980) after incubation at 30ºC for 48 h. In this medium, citrate-fermenting *L. lactis* colonies (which are blue in colour) are easily distinguished from non-fermenting colonies (white). *Leuconostoc* spp. were counted on Mayeux, Sandine and Elliker agar (MSEA) (Biokar Diagnostics, Beauvais, France) containing 30 μg mL^{-1} vancomycin (Sigma-Aldrich, St. Louis, MO, USA) after incubation at 25ºC for five days. Lactobacilli were grown on MRS agar adjusted to pH 5.4 and enumerated after incubation at 32ºC for 72 h.

2.2.3. Detection and quantification of sugars and organic acids in milk

The detection of sugars and organic acids in milk was performed by HPLC after growing the mixtures in UHT milk at 30ºC for 48 h. After incubation, 5 g samples were diluted in 25 mL of 5.4 mM H_2SO_4, and the suspension shaken for 1 h at 37ºC before centrifuging at 4500 g for 10 min. The supernatant was filtered through a 0.45 nm sterile membrane and frozen at -20ºC until analysis. The separation of sugars and organic acids was performed using an ICSep ICE-ION-300 ion-exchange column (mobile phase 0.0085 N H_2SO_4, operating temperature 65ºC, flow rate 0.4 ml min^{-1}). Two detectors were connected in series to a Waters liquid chromatograph controlled by Millenium 32 Software (Waters, Milford, MA, USA): a 996 Photodiode Array Detector (Waters) for the determination of organic acids (detection wavelength 210 nm), and a Waters 410 differential refractometer for sugar determination (detection wavelength 280 nm). Quantification was performed using calibration curves for the identified sugars and organic acids. The results were recorded as milligrams of acid or sugar per 100 mL of sample.

2.2.4. Detection of free amino acids

For the detection of free amino acids, the mixtures were grown in UHT milk as described above. A 1 g sample of each was then homogenised with 10 ml of 0.2% thiodipropionic acid (TDPA) (Fluka, Madrid, Spain) and 250 mM of internal standard (norvaline; Sigma-Aldrich) using an Ultra-Turrax homogeniser (OMNI International, Watersburry, USA) at 20,000 rpm for 2 min. This was then placed in an ultrasonic bath for 30 min and centrifuged at 5000 x g for 20 min. After the removal of the fat layer, the supernatant was filtered through a 0.45 μm membrane and 3 ml of the filtrate deproteinised by passing it through an AmiconUltra 0.5 filter (Millipore, Bedford, CA, USA) by centrifugation at 3000 g for 1 h. Twenty millilitres of the sample were derivatised following the protocol of Krause et al. (1995). The separation of amino acids was performed by reverse-phase (RP)-HPLC using an Alltima HP C18 Hi-Load column (6 mm) (Waters). The gradient and detection conditions were similar to those described by the above authors. Quantification was performed using dedicated calibration curves for all amino acids and derived compounds. Results were recorded as micrograms of amino acid per millilitre of sample.

2.2.5. Detection of volatile compounds

Volatile compounds were analysed by headspace/gas chromatography/mass spectrometry (HS/GC/MS) using an Agilent apparatus with G 1888 HS, 6890 GC and 5975BMSD components (Agilent Technologies, Wilmington, DE, USA), equipped with a 60 m/0.25 mm HP-Innovax column (Agilent). Sample preparation and gas chromatographic separation were performed as described by Salazar et al. (2008). Briefly, starter mixtures were incubated in milk as above before adding 100 μL of internal

standard (cyclohexanone, 0.36 mg mL), and then stored at -80ºC until analysis. Peaks were quantified as the relative total ionic count with respect to the internal standard.

2.3. Cheesemaking

Figure 1 shows the cheesemaking process followed. Milk was pasteurised at 63ºC for 30 min and then cooled to 32ºC. It was then inoculated with a freshly-prepared starter aliquot (via overnight incubation of the lyophilised mixture in UHT milk at 30ºC) at 1% v/v. Calcium chloride was also added to a final concentration of 0.02% (w/v). Rennet with a calculated strength of 1:6000 was added to the milk at a concentration of 0.017% (v/v) when the pH reached 6.65. The curd was cut to pea-sized grains and heated to 36ºC before draining and moulding. The curd was then subjected to hydraulic pressure at 1.5 bar for 2 h. When a pH of 5.5 was reached, the cheeses were removed from their moulds and immersed in saturated brine (20º Baumé) at 4ºC for 25 min.

2.4. Milk, curd and cheese analysis

2.4.1. Microbial counts

Ten gram samples of milk, curd and cheese were homogenised with 90 ml of a 2% (w/v) sodium citrate solution at 45ºC in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3×1 min). Ten-fold dilutions were then made in maximum recovery diluent (Scharlau, Barcelona, Spain) and plated in duplicate on general and selective media. Total aerobic mesophilic bacteria, lactococci, lactobacilli and leuconostocs were counted as described above. Enterococci were counted on Slanetz and Bartley agar (SBA) (Merck) after incubation at 44ºC for 24 h. Enterobacteria and coliforms were grown on violet red bile glucose agar (VRBGA) and violet red bile lactose agar (VRBLA) (both from Merck) respectively, using the overlay pour-plate technique. For this, dilutions were mixed with 15 ml of temperate agar (38ºC) and poured into Petri dishes. After solidification, a second agar layer of 10 ml was added. Bacteria were enumerated after 48 h of incubation at 30ºC. Staphylococci were grown on Baird-Parker agar (BPA) (Merck) supplemented with egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearance halos were recorded after 24 h of incubation at 37ºC. Yeasts and moulds were enumerated on yeast extract glucose chloramphenicol agar (YGCA) (Merck) after incubation at 25ºC for 3-5 days.

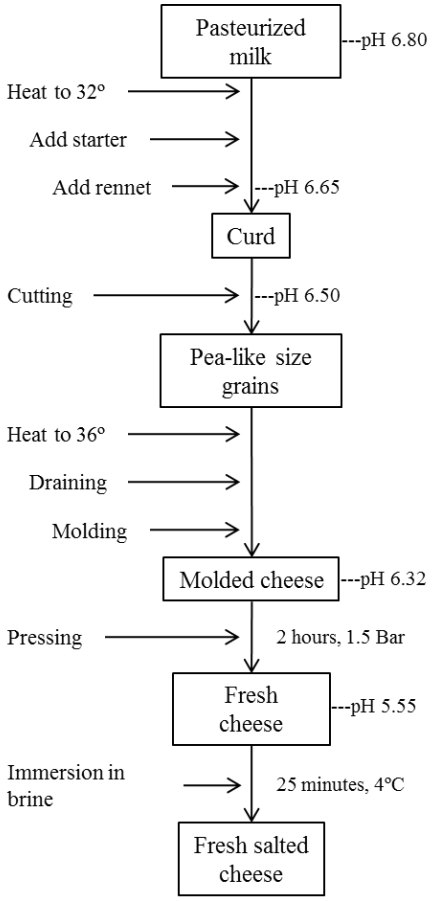

Figure 1.- Flow scheme of the manufacturing process of experimental cheeses. Approximate duration of manufacturing steps and temperature changes through the process is indicated.

2.4.2. Biochemical analysis

Standard International Dairy Federation procedures were followed to determine basic chemical properties (protein, fats and total solid contents). Total N (TN) and other nitrogen fractions were determined by the

Kjeldahl method (International Dairy Federation, 1993) using a Kjeldatherm KT 20S digestion apparatus and a Vapodest 50 titramatic distillation system (Gerhardt GmbH & Co KG, Bonn, Germany). Total protein was calculated as 6.3 times the TN content (the usual conversion coefficient for dairy proteins). A multiplication factor of 6.29 was used to calculate the non-protein nitrogen fraction. The percentage fat content was determined using a butyrometer, following the methods of Gerber (International Dairy Federation, 1981) and Van Gulik (International Dairy Federation, 2008) respectively. Total solids for cheese/curd and milk were calculated by drying samples at 102±2ºC until a constant weight was reached (International Dairy Federation, 1987 and 1982 respectively). Sodium chloride (NaCl) was determined using a Corning 926 Chloride Analyzer (Corning Medical and Scientific, Halstead, UK) following the manufacturer's recommendations. For this, samples were first homogenised in a 2% sodium citrate solution.

2.4.3. Sensory analysis

A trained panel of 20 experts (10 men and 10 women aged between 24 and 65 years) was recruited from among the IPLA staff. The terms describing the attributes of the cheeses were grouped into five categories (main descriptors): appearance (shape, size, rind colour, rind thickness, rind rugosity, inner colour, inner homogeneity and presence of eyes), odour (strength, cleanliness), taste (balance, spiciness, acidity, saltiness, bitterness, sourness, rancidness, and the presence of strange flavours), texture (firmness, creaminess, grain presence, and adherence), and overall impression. All these descriptors were rated on a hedonistic scale from 1 (very unsatisfactory) to 5 (very satisfactory).

3. Results and Discussion

All the starter mixtures coagulated the milk after 24 h of incubation at 30ºC. The final pH obtained ranged from 4.12 (mixtures S2 and S4) to 4.37 (mixture S9) (Table 2). In all cases, the pH fell a little further by 48 h of incubation.

Acidification curves for the mixtures were produced over 24 h (Fig. 2). The curves for all the mixtures were similar to one another and to that of the Flora Danica commercial starter, except for mixture S9. The latter took much longer to acidify the milk and the final pH was higher.

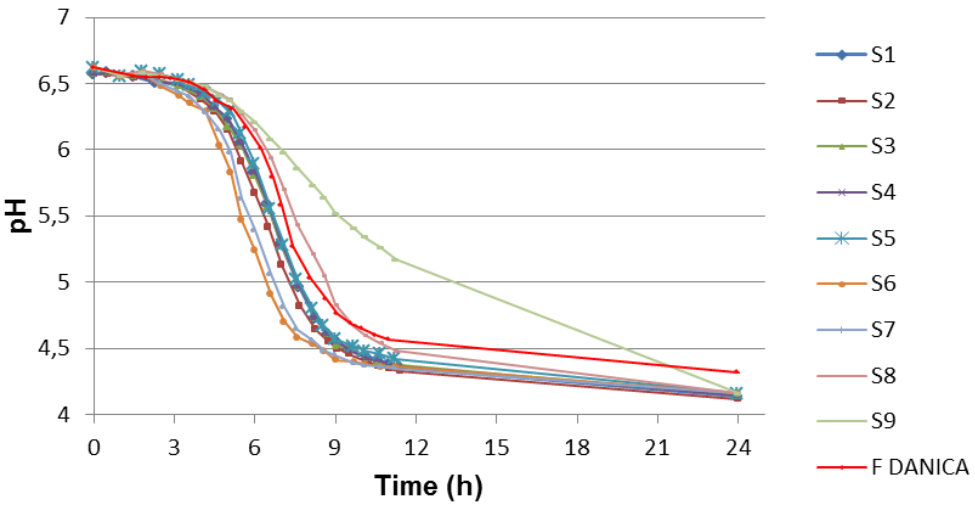

Figure 2.- Flow scheme of the manufacturing process of experimental cheeses. Approximate duration of manufacturing steps and temperature changes through the process is indicated.

The total counts for the different starter mixtures after 24 h of incubation in milk reached values surpassing 9 $\log_{10}$ cfu ml^{-1} except for mixtures S5 and S9 (8.88 and 8.87 $\log_{10}$ cfu ml^{-1} respectively). Prolongation of the incubation period to 48 h under the same conditions greatly reduced the viability of the cells of all the mixtures between one and almost three $\log_{10}$ cfu ml^{-1}. All four mixtures containing *L. lactis* subsp. *lactis* biovar *diacetylactis* strains achieved one $\log_{10}$ cfu ml^{-1} less than those containing non-citrate fermenting lactococci.

Leuconostoc and *Lactobacillus* were only scored for the mixtures that contained these bacterial types. After 24 h of incubation, strains of these two genera reached a cell density approximately half that of the lactococci. While the leuconostocs seemed to fall in number between 24 and 48 h, the lactobacilli increased from 3.60 to 5.21 $\log_{10}$ cfu per ml^{-1}. Cell numbers and growth values similar to those observed in this work have recently been reported in the development of starters from wild strains isolated from traditional dairy products (Ayad et al., 2000; Centeno et al., 2001; Randazzo et al., 2007; Franciosi et al., 2009; Nieto-Arribas et al., 2009).

Table 3 shows the production or consumption of organic acid in milk by the different mixtures. The table also shows the lactose, glucose and galactose contents of the milk samples after fermentation, plus the results obtained with the Flora Danica complex commercial starter. All the mixtures produced large quantities of lactic acid, and small amounts of pyruvic, formic, uric, acetic and butyric acids, while they all consumed hippuric, orotic and citric acids. Surprisingly, this last organic acid was not differentially used by mixtures containing strains of *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. The utilisation of milk citrate is linked to the production of diacetyl, a key odour compound in many fermented dairy products. The consumption of all the citric acid in the milk (around 30 mg 100 ml^{-1}) and the production of more acetic acid (8.52 mg 100 ml^{-1}) were the main differences observed with respect to the Flora Danica starter. Lactose and galactose were the only sugars detected in milk after 48 h of incubation. With small mixture-specific differences, all strains consumed lactose, releasing some galactose.

The consumption and production activities determined the final organic acid profile of the milks fermented with the different mixtures. Lactic acid is the main end product of the metabolism of LAB, although they are known to produce small quantities of other organic acids such as acetic, formic and butyric acids (Mayo et al., 2010). Orotic acid is an intermediary in the synthesis of nucleotides (Østile et al., 2003); it may also act as a growth promoter for some LAB species. Pyruvic acid is a key intermediate in the metabolism of LAB (Mayo et al., 210); its presence after fermentation may result from the (partial) lysis of cells and its consequent release into the milk.

Table 4 shows the relative abundance of the different volatile compounds detected after the growth of the mixtures in milk at 32ºC for 48 h. In agreement with the results obtained for organic acids, the greatest acetic acid content was obtained with the Flora Danica commercial starter. The same was true for the contents of diacetyl and acetoine.

The experimental mixtures produced more ethanol than the Flora Danica starter. Small differences were observed between the different mixtures for all other compounds, but no clear correlation between odour compounds and bacterial types was detected.

The content of free amino acids in milk after 48 h of incubation was analysed as a measure of the proteolytic capacity of the mixtures. Table 5 shows the content in amino acids and related compounds for the milk samples after fermentation with the different starter mixtures. Most amino acids increased with all mixtures, especially glutamic acid (Glu), lysine (Lys), and proline (Pro). The aspartic acid (Asp) content, however, was similar before and after fermentation, and glycine (Gly) concentrations were always

lower in the fermented milks. The free amino acid content of cheese is used as an index of maturation (Vicente et al., 2001). In addition, proteolysis during cheese ripening strongly affects the sensory properties of cheeses; free amino acids are major precursors of many aroma and taste compounds (Smit et al., 2005). Compared to commercial starters, wild lactococci strains have been reported to produce large amounts of volatile compounds via the degradation of branched-chain amino acids (Leu, Ile, Val) (Urbach et al., 1997; Ayad et al., 1999). These compounds have a very low taste threshold and have been associated with malty and burnt notes in dairy products (Smit et al., 2005). However, when properly balanced, these compounds seem to be critical for generating the aroma profile of certain traditional cheeses (Curioni and Bosset, 2002). Compounds derived from branched-chain were the majority in the present work.

Small and similar quantities of biogenic amine histamine (Him), a decarboxylated harmful compound produced from histidine (His), were detected in all samples, including the control milk samples. Tyramine and putrescine, however, were never detected. This strongly suggests that the mixtures carry no biogenic amine producers. This is not surprising since of the many of the present strains have already been shown not to produce these compounds (Ladero et al., unpublished). Interestingly, all the mixtures produced certain levels of γ-aminobutyric acid, a functional inhibitory neurotransmitter associated with relaxing and anti-anxiety effects (Abdou et al., 2006).

The analysis of volatile compound and free amino acid production was repeated in the presence of small quantities of calf rennet (similar to that retained in most cheese types). Since rennet is responsible for the majority of primary proteolysis (Irigoyen et al., 2000), the aim was to test whether its presence in milk modified the formation of these compounds during fermentation. The results are summarised in Supplementary Tables S1 and S2. Under these conditions, the production of volatile compounds was very similar to that recorded without rennet (no significant differences). The free amino acid contents obtained in the presence of rennet (Table S2) were also very similar to those obtained without rennet, although most amino acids concentrations were slightly higher under the latter condition. Though rennet is responsible for primary proteolysis in cheese (van der Berg and Exterkate, 1993), the process may be too slow for it to be measured over a short incubation time. It should be noted that carbon disulphide was identified in the trial without rennet, while chloroform appeared in its place when it was present. The results obtained for the controls, however, suggested that both compounds were already present in the uninoculated milk.

Even though they may have been technologically characterised, starter mixtures have to be assayed under real cheesemaking conditions to determine whether they meet all manufacturing and ripening requirements. Four mixtures were selected for their use as starters in experimental cheese trials: S1, S4, S6, and S8. Table 1 shows their component bacterial strains. These were uses as starters for the making of a Gouda-like cheese following the diagram shown in Figure 1. Experimental cheeses were produced on two different occasions (involving two independent samples of bulk cow's milk). Starters were added to pasteurised milk samples at an approximate concentration of 7 $\log_{10}$ cfu g^{-1} of cheese. Cheesemilk, curd and cheeses aged 3, 7, 15, and 30 days were then subjected to basic microbial and chemical analysis. Thirty-day old cheeses were also subjected to sensory analysis by a trained panel. Table 6 shows the composition and development of the majority and indicator bacterial groups during manufacturing and ripening of the cheeses made with mixture S1. Table 7 shows the values for the basic chemical variables recorded for this experimental cheese. Supplementary Tables S3, S4, S5, S6, S7 and S8 show the microbial and chemical analyses of all the other

experimental cheeses. With the exception of the cheesemilk of batches made with mixtures S6 and S8, which proved to be of poor microbial quality, the numbers and types of microorganisms found were normal for small-scale, artisanal products. Though pasteurised milk was used, the cheeses became contaminated during manufacturing by small numbers of enterococci, staphylococci (including *Staphylococcus aureus* in some samples), coliforms and fungi. Their populations reached only discrete numbers compared to those of lactococci (starter), (>9 $\log_{10}$ cfu g^{-1}) between days 3 and 7. Lactobacilli are the subdominant populations found in all mature cheeses. They were undetectable in the pasteurised milk, but they developed slowly, reaching cell densities of around 7.0 $\log_{10}$ cfu g^{-1} of cheese. A comparison of microbial development in the cheese batches made with the second bulk milk sample, and using mixtures S6 and S8 (nisin-producers), showed all the non-starter Gram-positive populations (lactobacilli, enterococci, and staphylococci) to suffer delayed growth. Even smaller numbers were recorded at the same ripening time when nisin producers were used as starter components (Tables S4 and S5). Direct comparisons of these microbial counts with those of batches made with mixtures S1 and S4 could not be made due to differences in the microbial (and chemical) quality of the milk samples. The inhibition of Gram-positive bacteria (including experimentally added pathogens) by nisin-producing starters has been reported (Roberts et al., 1992; Rodríguez et al., 1998; Rilla et al., 2004). Lactococci that produce bacteriocins other than nisin have also been used as starters or adjunct cultures to improve quality and food safety (O'Sullivan et al., 2003; Dal Bello et al., 2012), which argues for the experimental evaluation of mixture S9 (lactococcin 972 producers) in future work.

The chemical variables, including the nitrogen fractions, used as indices of ripening, showed similar trends for all mixtures, their values falling within the normal limits for artisanal cheeses (Estepar et al., 1999; Arenas et al., 2004; Horne et al., 2005; Ballesteros et al., 2006). The poor chemical quality of the cheese milk for the batches made with mixtures S6 and S8 (total solids and fat content 8 and 2% w/w respectively), had a great influence on the yield of cheese, but not on the chemical variables analysed nor on how they changed over ripening.

The sensorial properties of the cheeses produced in the four manufacturing trials were scored by a panel of 20 experts. The results are shown in Figure 3. Except for the cheese made with mixture S9, all looked similar and had a similar aroma. The texture of all was also very similar, although those made with S6 and S9 received slightly lower scores. Large differences were found, however, in terms of taste and global impression, for which cheeses made with mixtures S1 and S4 scored better. This suggests that these two mixtures could be confidently used as starters, at least in artisanal-scale cheese making. Further work should make comparisons using the same milk bulk sample with all mixtures to avoid differences attributable to milk quality.

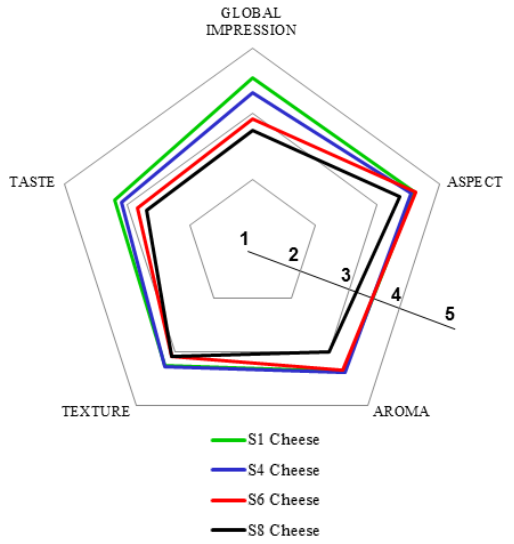

Figure 3.- Radial diagram showing the average scores for the five main cheese descriptors during evaluation of sensory characteristics of experimental cheeses. The five points hedonistic scale is indicated

In conclusion, the present results, and those reported by other authors (Ayad et al., 2000; Topisirovic et al., 2006; Brandsma et al., 2008), show that technologically useful LAB strains can be obtained from traditional cheeses. Large-scale cheese manufacturing trials using mixtures S1 and S4 will shortly be undertaken. Native bacterial strains from prestigious cheeses with protected designation of origin status, such as Cabrales (coded as A or AA) and Casín (coded as CAS), might be added to strain mixtures to create specific starters, investing these products with added value.

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Table 1.- Bacterial composition of the nine experimental starter mixtures designed.

Starter mixture	Bacterial species/types	Component strains
S1	*Lactococcus lactis* subsp. *lactis*	1A36, 2A88, 3AA15, 3AA22, 3AA29, 4AA26, 4AA2, CAS3, GE1
S2	*L. lactis* subsp. *lactis*	3AA15, 3AA29, 4AA2, 4AA26, CAS3, GE1
	L lactis subsp. *lactis* biovar *diacetylactis*	1AA6, 3AA27, L74
S3	*L. lactis* subsp. *cremoris*	1AA23, 1C8d, 206b, 3AA9, 3AA23, GE14, L39, LC144, P1A12, P2C1, P2E10
S4	*L. lactis* subsp. *lactis*	3AA15, 3AA29, 4AA2, 4AA26, CAS3, GE1
	L. lactis subsp. *cremoris*	1AA23, 3AA9, 3AA23, GE14, LC144, L39
S5	*L. lactis* subsp. *lactis*	3AA15, 3AA29, 4AA2, 4AA26, CAS3, GE1
	L lactis subsp. *lactis* biovar *diacetylactis*	1AA6, 3AA27, L74
	L. lactis subsp. *cremoris*	1AA23, 3AA9, 3AA23, GE14, LC144, L39
S6	*L. lactis* subsp. *lactis*	3AA15, 3AA29, 4AA2, 4AA26, CAS3, GE1
	L lactis subsp. *lactis* biovar *diacetylactis*	1AA6, 3AA27, L74
	L. lactis subsp. *cremoris*	1AA23, 3AA9, 3AA23, GE14, LC144, L39
	Leuconostoc spp.	1E6c, 2BC7, 2E3a, 4AB2, 4AC4, 7G3a
S7	*L. lactis* subsp. *lactis*	3AA15, 3AA29, 4AA2, 4AA26, CAS3, GE1
	L lactis subsp. *lactis* biovar *diacetylactis*	1AA6, 3AA27, L74
	L. lactis subsp. *cremoris*	1AA23, 3AA9, 3AA23, GE14, LC144, L39
	Leuconostoc spp.	1E6c, 2BC7, 2E3a, 4AB2, 4AC4, 7G3a
	Lactobacillus spp.	GA102, VI19
S8	*L. lactis* subsp. *lactis* (nisin producers)	1A6, 1A8, 1A16, 1A38, 1AA16, 1AA17, 1AA48, 2BB9, 3A28, L30, LC83A
S9	*L. lactis* subsp. *lactis* (lactococcin 972 producers)	Q12, Q16, Q18, T226, T243
FD	Lactococci, Leuconostoc, Lactobacilli	

FD, Flora Danica (Chr. Hansen, Denmark), a complex, undefined commercial starter.

Table 2.- Acidification of UHT milk and growth recoveries of the nine starter mixtures in different culture media.

Starter mixture	pH of milk[a]		Microbial counts log_{10} ufc g or ml^{-1}											
			PCA	M17A		KMA				MSEA		MRSA	
	24 h	48 h	Lyophilized	24 h	48 h	24 h *L. lactis*[b]	24 h *L. diacetylactis*[b]	48 h *L. lactis*	48 h *L. diacetylactis*	24 h	48 h	24 h	48 h
S1	4.14	4.10	<7.00	9.31	6.60	na	na	na	na	na	na	na	na
S2	4.12	4.09	7.40	9.85	7.70	9.12	8.51	7.30	6.70	na	na	na	na
S3	4.14	4.12	7.00	9.06	6.78	na	na	na	na	na	na	na	na
S4	4.12	4.08	7.30	9.26	7.32	na	na	na	na	na	na	na	na
S5	4.16	4.15	8.65	8.88	7.88	8.33	7.52	7.80	6.74	na	na	na	na
S6	4.13	4.10	9.11	9.32	8.56	8.09	7.04	7.39	5.93	3.99	3.78	na	na
S7	4.14	4.11	8.73	9.27	7.48	8.62	7.26	6.74	6.00	4.83	4.34	3.60	5.21
S8	4.15	4.13	9.56	9.17	8.08	na	na	na	na	na	na	na	na
S9	4.37	4.26	9.74	8.87	8.64	na	na	na	na	na	na	na	na

[a]pH of uninoculated milk 6.68

[b]*L. lactis, Lactococcus lactis* subsp. *lactis; L. diacetylactis, L. lactis* subsp. *lactis* biovar *diacetylactis.*

na, not analyzed.

Table 3.- Presence of sugars and organic acids detected by HPLC after growth of the starter mixtures at 32ºC for 48 h in UHT-treated milk.

Starter mixture	Sugar[a]			Organic acid[a]								
	Lactose	Glucose	Galactose	Orotic acid	Citric acid	Pyruvic acid	Lactic acid	Formic acid	Uric acid	Acetic acid	Butyric acid	Hippuric acid
S1	693.98	0.00	5.43	0.46	26.65	1.69	154.15	1.10	0.27	1.47	0.71	0.00
S2	674.21	0.00	4.77	0.47	25.89	1.75	149.30	1.07	0.26	1.70	0.54	0.00
S3	697.12	0.00	4.51	0.61	26.53	1.69	148.75	0.79	0.26	1.31	0.46	0.00
S4	707.47	0.00	5.10	0.41	27.04	1.59	155.87	1.05	0.28	1.27	0.62	0.00
S5	715.43	0.00	4.61	0.64	27.91	1.22	155.65	1.05	0.28	1.46	0.56	0.00
S6	714.71	0.00	4.72	0.38	27.72	2.50	155.48	1.09	0.28	1.43	0.88	0.00
S7	636.42	0.00	4.17	0.31	24.71	2.37	137.65	0.97	0.24	1.32	0.74	0.00
S8	690.77	0.00	4.15	0.73	26.63	2.17	143.02	1.19	0.27	1.52	0.53	0.00
S9	667.00	0.00	5.69	0.60	25.24	1.80	123.30	0.95	0.25	1.91	0.29	0.38
FD	596.86	0.00	6.49	1.04	0.00	0.96	162.77	0.29	0.24	8.52	0.73	0.00
Milk[b]	851.20	0.94	1.91	1.23	28.97	0.04	1.50	0.00	0.21	0.00	0.00	0.61

[a]Concentration in mg 100 mL^{-1}.

[b]Milk data showed are an average of the results obtained from three different milk samples used for growing the starters.

Table 4.- Relative abundance of volatile compounds after growth of the starter mixtures at 32ºC for 48 h in UHT-treated milk.

Starter mixture	Volatile compound[a]										
	Acetaldehyde	Carbon disulfide	2-Methyl propanal	2-Propanone	3-Methyl butanal	Ethanol	Diacetyl	2-Methyl-1-propanol	3-Methyl-1-butanol	Acetoine	Acetic acid
S1	nd	1.30	nd	0.30	0.14	3.71	0.08	nd	nd	0.04	0.46
S2	nd	3.72	nd	0.37	0.68	3.66	0.08	0.04	0.09	0.03	0.35
S3	nd	1.98	0.37	0.12	1.25	2.65	0.04	0.08	0.34	0.03	0.26
S4	nd	1.15	nd	0.32	0.05	3.69	0.05	nd	nd	0.04	0.55
S5	0.20	nd	0.56	0.17	1.81	3.23	0.07	0.08	0.53	0.06	0.43
S6	nd	nd	nd	0.38	0.27	3.86	0.13	nd	nd	0.07	0.64
S7	0.15	nd	nd	0.39	0.11	4.14	0.24	nd	nd	0.13	0.69
S8	0.23	nd	nd	0.29	0.07	3.95	0.02	nd	nd	0.03	0.17
S9	0.52	nd	nd	0.36	0.44	3.97	0.04	nd	0.09	0.08	0.54
FD	nd	nd	nd	0.42	0.10	1.26	0.35	nd	nd	2.44	1.38
Milk S1-S4	nd	1.62	nd	nd	nd	nd	nd	nd	nd	nd	0.27
Milk S5-S9	0.46	nd	nd	0.26	0.12	2.15	0.16	nd	0.02	0.20	0.20
Milk FD	0.11	nd	nd	0.29	nd	0.40	nd	nd	nd	0.03	0.29

[a]Concentration referred to the internal standard (Cyclohexanone 0.38 mg mL^{-1}), to which a value of 1.00 was given.
nd, not detected

Table 5.- Presence of amino acids and related compounds detected by HPLC after growth of the starters at 32ºC for 48 h in UHT-treated milk. Results are expressed as compound µg/mL of sample.

Starter mixture	Amino acid																				Derived compound		
	αAla	βAla[a] +Arg	Asn	Asp	Cys	Cys-Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met[b]	Phe	Pro	Ser	Thr	Trp	Tyr	Val	NH_4^+	Gaba	Him
S1	1.05	0.46	0.41	0.38	0.21	14.65	0.10	7.37	0.17	1.19	0.24	1.20	3.77	0.14	0.92	4.13	0.70	1.01	nd	1.97	0.70	4.75	1.80	0.09
S2	0.91	0.44	0.45	0.43	0.47	14.39	0.16	1.50	0.14	1.17	0.25	1.11	3.88	0.13	0.91	3.60	0.69	1.01	nd	1.95	0.66	4.53	6.29	0.09
S3	0.55	0.48	0.34	0.36	0.67	10.47	0.12	6.22	0.13	0.61	0.17	0.82	2.73	0.10	1.23	2.71	0.69	0.83	nd	1.63	0.43	4.60	1.33	0.06
S4	1.20	0.52	0.54	0.41	0.21	17.14	0.17	8.47	0.20	1.28	0.44	1.45	4.20	0.20	1.13	3.87	0.76	1.12	nd	2.01	0.86	4.80	1.70	0.12
S5	0.83	0.53	0.48	0.39	0.45	12.39	0.15	2.34	0.17	0.92	0.26	1.10	3.67	23.44	1.37	3.93	0.93	1.23	nd	2.59	0.67	4.99	5.81	0.07
S6	1.42	0.56	0.60	0.44	0.29	20.00	0.10	8.48	0.22	1.50	0.30	1.33	4.70	23.50	1.22	5.01	0.99	1.21	nd	2.82	0.76	5.24	3.29	0.11
S7	1.20	0.48	0.49	0.42	0.19	16.94	0.12	9.15	0.18	1.31	0.24	1.19	3.95	20.15	0.98	5.11	0.82	0.99	nd	2.38	0.66	4.46	1.27	0.08
S8	0.96	0.51	0.36	0.38	0.16	9.38	nd	7.51	0.16	0.78	0.11	0.49	2.73	19.14	0.77	2.97	0.82	1.06	nd	1.44	0.40	3.64	1.06	0.07
S9	3.59	0.49	0.93	0.40	0.15	8.76	0.17	7.27	0.37	0.56	0.05	0.04	2.81	21.12	0.83	2.52	1.07	1.02	nd	1.53	0.03	1.44	0.09	0.22
FD	0.63	0.55	0.36	0.40	0.34	19.66	0.40	7.94	0.09	0.98	0.02	0.18	3.60	21.29	0.92	5.68	0.59	0.44	0.12	1.62	0.18	35.23	1.02	0.09
Milk 1-4	0.35	0.74	nd	0.42	0.62	1.23	0.08	3.25	0.67	0.08	0.07	0.06	0.45	nd	0.05	0.22	0.16	0.16	nd	0.06	0.14	2.59	nd	0.14
Milk 5-9	0.31	0.69	nd	0.53	0.14	0.88	0.10	2.77	0.67	0.05	0.06	0.05	0.45	18.46	0.05	0.23	0.12	0.14	nd	0.22	0.12	1.88	nd	0.10
Milk FD	0.33	0.70	0.04	0.57	0.15	1.26	0.07	2.73	0.66	nd	0.04	0.06	0.46	20.34	0.04	0.28	0.17	0.14	nd	0.17	0.14	3.35	nd	0.12

nd, not detected
FD, milk incubated with the commercial starter Flora Danica (Chr. Hansen).
Milk 1-4, 5-9; mixed samples of milk from the uninoculated blanks of the different assays of the corresponding starter mixtures.
Gaba: gamma-aminobutyric acid; Him, histamine.
The biogenic amines tyramine and putrescine were also analyzed but never recorded.
[a]βAla is an enantiomer produced during processing of the sample of the natural protein constituent amino acid L-α-alanine (αAla). In the conditions of analysis, βAla elutes together with the peak of arginine (Arg).
[b]Quantification of Met (methionine) is not accurate, as its peak elutes as part of a peak including buffer and internal standard reagents.

Table 6.- Microbial counts (in Log^{10} cfu per g or ml) of diverse microbial groups along manufacturing and ripening stages of the experimental cheese made with starter mixture S1.

Microbial group (counting medium)	Stage of manufacturing and ripening					
	Milk	Curd	3 days	7 days	15 days	30 days
Total aerobic counts (PCA)	5.62	9.02	9.41	9.37	9.33	8.92
Lactococci (M17A)	5.62	9.07	9.46	9.35	9.25	8.90
Lactobacilli (MRSA pH5.4)	< 1.00	< 2.00	3.30	4.78	4.89	5.86
Leuconostoc (MSEA)	< 1.00	< 2.00	< 2.00	3.70	3.70	3.70
Enterococci (SBA)	< 1.00	1.70	2.48	< 2.00	2.00	2.18
Staphylococci (BPA)	< 1.00	4.19	4.19	4.19 (2.90)[a]	4.19 (2.00)	4.19
Enterobacteriaceae (VRBGA)	< 1.00	5.34	4.85	4.34	4.53	4.41
Coliforms (VRBLA)	< 1.00	5.30	5.64	4.01	4.36	3.84
Yeasts and moulds (YGCA)	< 1.00	3.90 (3.18)[b]	3.80 (3.76)	2.88 (2.40)	2.60	2.92

[a]In brackets, numbers of egg yolk clearing colonies in BPA (presumably, *Staphylococcus aureus*).
[b]Numbers of yeast-like colonies in YGCA.

Table 7.- Biochemical parameters along the manufacturing and ripening stages of the experimental cheeses made with starter mixture S1.

Biochemical parameter	Manufacture and ripening stage					
	Milk	Curd	3 Days	7 Days	15 Days	30 Days
Total Solids % (w/w)	12.11	39.60	49.44	52.98	56.70	61.18
Fat % (w/w)	3.60	16.50	21.50	22.00	23.50	25.50
Fat/Total Solids Index	29.73	41.67	43.49	41.53	41.45	41.68
Salt % (w/w)	0.04	0.20	1.15	1.40	1.55	1.60
Nitrogen content						
Total Protein	3.05	16.28	21.93	21.45	22.32	22.71
Casein	2.55	15.55	20.85	19.84	20.33	20.25
Soluble nitrogen	0.50	0.73	1.08	1.61	1.99	2.46
Non-protein nitrogen	0.16	0.20	0.82	1.20	1.76	2.31

Table S1.- Relative abundance of volatile compounds after growth of the starter mixtures at 32ºC for 48 h in UHT-treated milk in presence of rennet.

Starter mixture	Volatile compound[a]										
	Acetaldehyde	Chlorophorm	2-Methyl propanal	2-Propanone	3-Methyl butanal	Ethanol	Diacetyl	2-Methyl-1-propanol	3-Methyl-1-butanol	Acetoine	Acetic acid
S1	nd	nd	nd	0.37	0.18	4.82	0.07	nd	nd	0.07	0.71
S2	0.29	0.02	0.12	0.32	0.72	5.17	0.06	nd	0.10	0.03	0.40
S3	0.34	nd	0.60	0.23	1.88	3.54	nd	0.11	0.48	0.03	0.33
S4	0.08	nd	nd	0.44	0.11	5.41	0.03	nd	nd	0.02	0.48
S5	0.37	nd	0.69	0.40	2.27	3.96	0.07	0.17	0.74	0.10	1.36
S6	0.09	0.35	nd	0.45	0.38	3.95	nd	nd	nd	0.09	0.92
S7	nd	0.83	nd	0.37	0.12	4.33	0.21	nd	nd	0.17	0.60
S8	0.43	0.97	nd	0.30	0.10	4.63	nd	nd	nd	0.02	0.49
S9	0.29	0.61	nd	0.30	0.20	3.25	0.08	nd	0.05	0.06	0.23
FD	nd	0.52	nd	0.35	0.12	1.37	0.34	nd	nd	2.88	1.51
Milk S1-S4	nd	nd	nd	0.10	nd	0.06	nd	nd	nd	nd	0.68
Milk S5-S9	0.73	0.76	nd	0.28	0.20	3.21	nd	nd	nd	0.10	0.95
Milk FD	nd	nd	nd	0.27	nd	nd	nd	nd	nd	0.03	0.51

[a]Concentration referred to the internal standard (Cyclohexanone 0.38mg/mL), to which a value of 1.00 was given.
nd, not detected.

Table S2.- Presence of amino acids and related compounds detected by HPLC after growth of the starters at 32ºC for 48 h in UHT-treated milk in the presence of rennet.

Starter mixture	Amino acid																				Derived compound			
	αAla	βAla[a] +Arg	Asn	Asp	Cys	Cys-Cys	Glu	Gln	Gly	Ile	His	Leu	Lys	Met[b]	Phe	Pro	Ser	Thr	Trp	Tyr	Val	NH4+	Gaba	Him	Tym
S1	1.32	0.42	0.59	0.53	0.50	14.11	3.15	nd	2.11	0.17	1.22	1.05	3.59	14.90	0.89	4.26	0.96	1.34	nd	5.95	1.03	5.71	4.81	0.08	nd
S2	1.13	0.34	0.46	0.49	0.57	12.01	0.68	nd	0.23	0.11	1.12	0.83	3.17	9.56	0.71	3.96	0.88	1.27	nd	6.34	0.86	4.96	5.38	0.11	nd
S3	0.75	0.39	0.42	0.63	0.39	9.61	2.74	0.06	0.23	0.17	0.71	0.94	2.59	nd	1.12	3.48	1.00	1.14	nd	6.01	0.89	5.17	3.04	0.21	0.06
S4	1.61	0.55	0.70	0.65	0.31	18.75	7.69	0.07	0.24	0.22	1.51	1.27	4.31	12.14	1.09	5.58	1.14	1.50	nd	7.37	1.03	6.71	2.10	0.21	nd
S5	1.09	0.52	0.61	0.99	0.81	11.52	0.90	0.09	0.23	0.30	1.05	1.44	3.44	10.65	1.59	4.57	1.45	1.72	nd	7.93	1.43	6.91	6.13	0.26	0.09
S6	1.53	0.48	0.81	0.88	0.31	19.91	2.53	0.22	0.24	0.25	1.25	1.55	4.50	0.05	1.51	7.72	1.13	1.25	0.22	2.19	0.80	7.07	7.64	0.34	nd
S7	1.53	0.54	0.71	1.01	0.39	20.40	10.45	0.18	0.21	0.24	1.41	2.16	4.62	0.09	1.53	8.47	1.18	1.30	0.24	2.43	1.02	7.88	2.49	0.09	nd
S8	1.03	0.37	0.49	0.85	0.10	6.93	1.82	nd	0.15	0.11	0.76	0.78	2.21	0.02	1.08	4.53	0.85	1.27	0.09	0.90	0.68	4.63	5.50	0.06	nd
S9	1.45	0.34	1.13	0.40	0.14	8.99	5.57	0.08	0.23	0.13	0.45	0.76	1.13	0.01	1.21	3.95	0.23	0.55	0.05	0.67	0.34	2.69	0.46	0.12	nd
FD	0.83	0.30	0.37	0.40	0.54	15.28	4.31	0.43	0.10	0.07	0.57	0.66	2.60	0.01	0.97	4.08	0.67	0.66	0.04	1.06	0.45	25.03	3.14	0.04	nd
Milk 1-5	0.36	0.70	nd	0.44	0.08	1.02	2.78	0.06	0.60	0.06	0.06	0.09	0.56	11.59	0.05	0.27	0.13	0.16	nd	2.33	0.16	2.73	0.10	0.10	nd
Milk 6-9	0.31	0.63	nd	0.43	0.10	0.97	3.40	nd	0.62	0.06	0.03	0.09	0.55	0.01	0.06	0.30	0.08	0.15	nd	0.07	0.13	2.61	0.03	0.07	nd

nd, not detected
FD, milk incubated with the commercial starter Flora Danica (Chr. Hansen).
Milk 1-4, 5-9; mixed samples of milk from the uninoculated blanks of the different assays of the corresponding starter mixtures.
Gaba: gamma-aminobutyric acid; Him, histamine; Tym, tyramine.
The biogenic amine putrescine was also analyzed but not recorded.
[a]βAla is an enantiomer produced during processing of the sample of the natural protein constituent amino acid L-α-alanine (αAla). In the conditions of analysis, βAla elutes together with the peak of arginine (Arg).
[b]Quantification of Met (methionine) is not accurate, as its peak elutes as part of a peak including the reactive buffer and the internal standard reagent.

Table S3.- Microbial counts (in Log10 cfu per g or ml) of diverse microbial groups along manufacturing and ripening stages of the experimental cheese made with starter mixture S4.

Microbial group (counting medium)	Stage of manufacturing and ripening					
	Milk	**Curd**	**3 days**	**7 days**	**15 days**	**30 days**
Total aerobic counts (PCA)	5.62	9.08	9.68	9.48	9.45	9.27
Lactococci (M17A)	5.62	8.99	9.69	9.45	9.42	9.19
Lactobacilli (MRSA pH5.4)	< 1.00	< 2.00	3.30	4.19	5.58	6.85
Leuconostoc (MSEA)	< 1.00	< 2.00	< 2.00	< 2.00	< 2.00	2.60
Enterococci (SBA)	< 1.00	< 2.00	2.40	< 2.00	< 2.00	2.70
Staphylococci (BPA)	< 1.00	2.88 (2.00)[a]	4.53	3.57 (2.60)	3.70	2.88
Enterobacteriaceae (VRBGA)	< 1.00	4.80	4.76	5.04	4.67	4.24
Coliforms (VRBLA)	< 1.00	4.92	5.34	4.59	4.41	4.21
Yeasts and moulds (YGCA)	< 1.00	2.90	3.49	3.64	2.74	2.30

[a]In brackets, numbers of egg yolk clearing colonies in BPA (presumably, *Staphylococcus aureus*).

Table S4.- Microbial counts (in Log10 cfu per g or ml) of diverse microbial groups along manufacturing and ripening stages of the experimental cheese made with starter mixture S6.

Microbial group (counting medium)	Stage of manufacturing and ripening					
	Milk	**Curd**	**3 days**	**7 days**	**15 days**	**30 days**
Total aerobic counts (PCA)	5.71	9.02	9.50	9.56	9.45	9.17
Lactococci (M17A)	5.84	9.06	9.59	9.22	9.49	8.92
Lactobacilli (MRSA pH5.4)	< 1.00	< 2.00	4.00	4.08	6.43	7.76
Leuconostoc (MSEA)	< 1.00	3.30	5.74	5.86	5.76	5.82
Enterococci (SBA)	1.00	1.70	2.65	2.78	2.48	2.74
Staphylococci (BPA)	1.78	3.52 (2.60)[a]	5.64	5.31 (2.90)	5.00	5.06
Enterobacteriaceae (VRBGA)	2.20	4.60	4.70	5.41	4.89	4.88
Coliforms (VRBLA)	1.70	4.98	5.17	5.29	4.81	4.58
Yeasts and moulds (YGCA)	1.70 (1.70)[b]	3.36 (3.34)	2.00	2.81 (2.70)	2.40 (2.18)	2.40

[a]In brackets, numbers of egg yolk clearing colonies in BPA (presumably, *Staphylococcus aureus*).
[b]Numbers of yeast-like colonies in YGCA.

Table S5.- Microbial counts (in Log_{10} cfu per g or ml) of diverse microbial groups along manufacturing and ripening stages of the experimental cheese made with starter mixture S8.

Microbial group (counting medium)	Stage of manufacturing and ripening					
	Milk	**Curd**	**3 days**	**7 days**	**15 days**	**30 days**
Total aerobic counts (PCA)	5.71	8.34	9.29	9.47	9.34	8.96
Lactococci (M17A)	5.84	8.42	9.28	9.54	8.98	8.78
Lactobacilli (MRSA pH5.4)	< 1.00	2.30	3.48	3.65	5.28	6.51
Leuconostoc (MSEA)	< 1.00	< 2.00	< 2.00	3.10	< 2.00	< 2.00
Enterococci (SBA)	1.00	2.00	< 2.00	2.30	2.40	2.18
Staphylococci (BPA)	1.78	3.61 (2.78)[a]	6.13	5.91 (4.90)	5.21	4.62
Enterobacteriaceae (VRBGA)	2.20	4.60	6.17	5.74	5.24	4.56
Coliforms (VRBLA)	1.70	4.71	6.02	5.69	4.89	4.51
Yeasts and moulds (YGCA)	1.70 (1.70)[b]	1.92 (1.92)	2.00	2.60 (2.48)	2.54 (2.48)	2.18 (2.18)

[a]In brackets, numbers of egg yolk clearing colonies in BPA (presumably, *Staphylococcus aureus*).
[b]Numbers of yeast-like colonies in YGCA.

Table S6.- Biochemical parameters along the manufacturing and ripening stages of the experimental cheese made with starter mixture S4.

Biochemical parameter	Manufacture and ripening stage					
	Milk	**Curd**	**3 Days**	**7 Days**	**15 Days**	**30 Days**
Total Solids % (w/w)	12.11	40.39	50.29	51.14	55.79	56.87
Fat % (w/w)	3.60	17.50	22.00	23.00	24.00	24.50
Fat/Total Solids Index	29.73	43.33	43.75	44.97	43.02	43.81
Salt % (w/w)	0.04	0.20	0.90	1.20	1.40	1.45
Nitrogen content						
Total protein	3.05	15.18	21.81	22.14	21.92	22.14
Casein	2.55	14.42	20.60	20.53	19.81	19.31
Soluble nitrogen	0.50	0.76	1.21	1.61	2.11	3.83
Non-protein nitrogen	0.16	0.23	0.80	1.37	1.77	2.35

Table S7.- Biochemical parameters along the manufacturing and ripening stages of the experimental cheeses made with starter mixture S6.

Biochemical parameter	Manufacture and ripening stage					
	Milk	**Curd**	**3 Days**	**7 Days**	**15 Days**	**30 Days**
Total Solids % (w/w)	7.99	33.27	45.67	46.69	50.89	51.34
Fat % (w/w)	2.00	14.50	19.00	21.00	24.00	24.50
Fat/Total Solids Index	25.03	43.58	41.60	44.98	47.17	47.72
Salt % (w/w)	0.03	0.10	0.90	1.30	1.65	1.80
Nitrogen content						
Total Protein	2.22	15.47	20.28	20.71	21.94	23.13
Casein	1.85	14.63	18.69	18.27	18.48	18.88
Soluble nitrogen	0.37	0.84	1.59	2.44	3.46	4.25
Non-protein nitrogen	0.11	0.16	0.99	1.43	1.98	2.61

Table S8.- Biochemical parameters along the manufacturing and ripening stages of the experimental cheeses made with starter mixture S8.

Biochemical parameter	Manufacture and ripening stage					
	Milk	**Curd**	**3 Days**	**7 Days**	**15 Days**	**30 Days**
Total Solids % (w/w)	7.99	36.21	47.60	49.22	52.91	54.76
Fat % (w/w)	2.00	16.00	20.00	20.50	22.50	23.00
Fat/Total Solids Index	25.03	44.19	42.02	41.65	42.53	42.00
Salt % (w/w)	0.03	0.15	0.65	1.10	1.20	1.70
Nitrogen content						
Total Protein	2.22	17.51	23.46	21.34	21.4	23.04
Casein	1.85	16.62	21.39	18.5	16.69	18.00
Soluble nitrogen	0.37	0.89	2.07	2.84	4.71	5.04
Non-protein nitrogen	0.11	0.14	0.75	1.14	1.69	2.54

Discusión general

Discusión general

A lo largo de los artículos que componen los resultados de esta Tesis Doctoral, se describen detalladamente los objetivos de cada uno de los trabajos parciales, la metodología que se utiliza en los distintos experimentos y se discuten los resultados más sobresalientes en el ámbito de los resultados obtenidos por otros autores en el campo. El propósito que se persigue con este capítulo de Discusión General es situar las principales conclusiones alcanzadas en este estudio bajo la perspectiva global de los productos lácteos tradicionales en el panorama actual de los mercados y recoger el papel que los cultivos iniciadores pueden tener en la elaboración y maduración de los productos tradicionales y no tradicionales de una manera más general de la que se relaciona en cada uno de los trabajos por separado.

Los productos tradicionales en el mundo actual

Encontrar una definición de producto tradicional que abarque toda la gama de productos existentes es una tarea difícil. En todo caso, según varios estudios de percepción recientes, el consumidor europeo tiene en general una opinión muy positiva de las cualidades de los alimentos tradicionales (Vanhonacker y col., 2010, Guerrero y col., 2012). Aún más, el consumo de estos productos suele asociarse a menudo con efectos beneficiosos para la salud (Trichopoulou y col., 2007). Sin embargo, está claro que los consumidores modernos son cada vez más consciente de su responsabilidad para exigir productos con buenas cualidades organolépticas e

higiénicamente seguros. De manera que, en un mundo cada vez más globalizado, los productos tradicionales sólo podrán afrontar con seguridad su futuro si son capaces de adaptarse a las nuevas y exigentes demandas de los mercados. Los dos retos principales de los productos tradicionales elaborados a pequeña escala (artesanos) son, sin duda, mejorar la regularidad en todas las elaboraciones y en todas las épocas del año y asegurar la salubridad y seguridad higiénica de los mismos. Todo ello, sin pérdida de al menos parte de la tipicidad y originalidad que diferencia a este tipo de productos de los productos industriales de gran consumo (Garabal, 2007).

Hay un consenso en que los productos tradicionales en general, y los productos lácteos en particular, son una pieza clave de la herencia cultural europea (Reglamento CE nº 510/2006). La estrategia conjunta de la ONU y la Unión Europea para la comercialización de los productos tradicionales pasa por incrementar la competitividad y la mercadotecnia, con el objetivo global de mejorar el desarrollo rural, generar empleo, crecimiento y bienestar social en las zonas de elaboración (FAO, 2004). De su producción y comercialización depende a día de hoy la economía de muchas regiones. En muchos casos estas regiones se encuentran en áreas geográficas aisladas y tradicionalmente deprimidas en las que los productos tradicionales son un motor de dinamización económica. Estas regiones han sabido mantener a través de las generaciones un conocimiento y unos procedimientos ancestrales compartidos por toda la comunidad, cuyos productos resultantes son muy apreciados en la actualidad. Este conocimiento colectivo local ha dado lugar a productos universales como ocurre con los quesos Roquefort, Parmesano, Cheddar, etc., o las leches fermentadas como el yogurt o el kéfir. Los productos tradicionales tienen una relación muy fuerte entre el componente humano y el medio natural, incluyendo en este medio ambiente las especies y razas de animales y la alimentación a la que éstos tienen acceso en función de la localización geográfica de las zonas de elaboración. Los microorganismos que colonizan el producto y el entorno de elaboración podrían situarse también dentro de este medio ambiente esencial para la tipicidad de los productos lácteos tradicionales (Garabal, 2007).

Los productos lácteos ocupan un lugar destacado en la tradición europea, española y especialmente asturiana. En nuestra región la ganadería asociada al sector

lácteo da a muchas áreas rurales su carácter distintivo, formando parte de su atractivo cultural, ayudando a moldear incluso sus paisajes y constituyendo una parte importante de su economía y desarrollo (Izquierdo, 2008). En Asturias, la diversidad de quesos existente es un reflejo de una gran variedad de tradiciones y métodos de elaboración. La mayoría de nuestros quesos se elaboran todavía de manera artesana siguiendo procedimientos ancestrales con poca o ninguna innovación. La materia prima incluye leche cruda, fundamentalmente de vaca, a la que no se le añaden fermentos, ni se inocula de ninguna otra manera, lo que sin duda propicia que las características organolépticas de los productos finales varíen de forma notable entre lotes, épocas del año y productores.

Por este motivo, el registro y la protección de los productos tradicionales más apreciados son claves para (1) protegerlos de imitaciones, (2) aumentar su calidad media y (3) que se ajusten a una legislación alimentaria cada vez más exigente (Trichopuolou y col., 2007). El estudio y la documentación de los productos tradicionales se pueden utilizar también como forma de certificar su calidad y asegurar su autenticidad mediante el desarrollo de estándares que servirán de referencia para consumidores y legisladores. Autores como Cayot (2007) defienden que la caracterización organoléptica de los productos tradicionales es esencial para su producción industrial, la generación de una normativa adecuada que los regule y el desarrollo de procesos de mejora e innovación. Sin embargo, tradición e innovación son conceptos en apariencia contrarios, por lo que innovar en alimentos tradicionales resulta una tarea difícil (Amilien y col., 2005; Gellynck y Kühne, 2008; Jordana, 2000). Sin embargo diversos estudios realizados en países de la Unión Europea muestran que gran parte de los consumidores están dispuestos a acoger la innovación en una amplia variedad de alimentos tradicionales como quesos, productos cárnicos, bebidas fermentadas y otros (Kuhne y col., 2010). La innovación en los productos tradicionales es necesaria porque, si bien se han de mantener sus propiedades típicas y distintivas más originales, han de ser capaces de adaptarse a los gustos de los nuevos consumidores que, aunque de forma casi imperceptible, van cambiando a lo largo del tiempo.

Salvaguardar la originalidad y tipicidad de quesos con etiquetas de calidad

Para el proteger las características distintivas de los productos tradicionales, la Unión Europea dispone de tres sellos de calidad: la especialidad tradicional garantizada (ETG), en la que se resalta el carácter tradicional de un producto, bien sea por su composición o por la forma en que es producido; la indicación geográfica protegida (IGP); y la denominación de origen protegida (DOP). Estas dos últimas marcas se aplican a alimentos que son producidos en un área geográfica concreta utilizando procedimientos tradicionales y en el caso de los productos PDO empleando materias primas procedentes de manera exclusiva de la propia área geográfica (Reglamento CE No 510/2006). En Europa, hay más de 200 variedades distintas de quesos con denominación de origen protegida y más de 50 con indicación geográfica protegida (DOOR, 2013). Los fermentos específicos podrían contribuir a mantener la tipicidad y la originalidad de los productos tradicionales con sellos de calidad, reforzando la ligazón entre medio natural y producto, lo que sirve para revalorizar las marcas.

La caracterización microbiana como oportunidad de mejora

El queso y otros productos lácteos tradicionales se consiguen tras una fermentación en la que algunos grupos microbianos juegan un papel clave en su elaboración y maduración. Por esta razón, los productos lácteos fermentados son productos dinámicos desde el punto de vista microbiológico y bioquímico, sujetos a muchos cambios durante su maduración hasta dar lugar al producto final de consumo. Estos cambios se deben en gran medida a la acción de los microorganismos que se desarrollan en su seno. El efecto de la microbiota y las interacciones entre los microorganismos y los componentes de la materia prima aportan a cada variedad sus propiedades organolépticas definitorias (Garabal, 2007). Por ello, para controlar y homogenizar la producción de estos productos es esencial conocer las poblaciones microbianas presentes y su comportamiento y dinámica durante las etapas de elaboración y maduración.

En esta memoria reportamos la caracterización microbiana de tres productos lácteos tradicionales: dos quesos con DOP y una leche fermentada natural (Segunda parte, artículos II, III y IV). Tal y como esperábamos, los microorganismos más

abundantes en todos estos productos pertenecen a diversas especies de BAL. Sin embargo, como queda claro en los artículos, hemos encontrado resultados sorprendentes, entre los que cabe mencionar la identificación de *Lc. garvieae* como el lactococo dominante en la etapa de fermentación del queso Casín, la presencia abundante y extendida de *S. thermophilus* a lo largo de la elaboración y maduración de muchos de los quesos analizados, la increíble estabilidad microbiana del consorcio establecido en la leche fermentada natural y la presencia de (al menos) ADN de microorganismos no detectados con anterioridad en queso mediante técnicas de cultivo.

Diversidad microbiana en productos lácteos

Cada uno de los productos tradicionales puede considerarse un ecosistema único, atendiendo a la enorme variedad de especies y cepas de bacterias del ácido láctico (BAL) y de otros tipos microbianos (incluyendo mohos y levaduras) que en ellos se desarrollan y que, en gran medida, perfilarán el aroma, el sabor y en algunos casos el aspecto de los productos finales. Todos estos microorganismos se vienen utilizando desde hace milenios en la elaboración de productos lácteos fermentados. La efectividad de su acción y la inocuidad de su consumo promueven su utilización masiva y controlada por la industria; no solo para la trasformación de los alimentos, sino que también -sobre todo en los últimos tiempos- para otras muchas aplicaciones biotecnológicas (Steidler y Rottiers, 2007; Wells, 2011). Como no podía ser de otra manera, los productos lácteos tradicionales de calidad elaborados con leche cruda son, sin duda, una inagotable fuente de nuevas cepas de BAL o de cepas con nuevas propiedades.

Por este motivo, a lo largo de las pasadas décadas, se han dedicado muchos esfuerzos a la caracterización en profundidad de la microbiota de varios de estos productos lácteos tradicionales. Como ya se apuntara en la Introducción de esta memoria, muchos son los quesos tradicionales españoles que han sido caracterizados microbiológicamente. Sin ser exhaustivos, podemos mencionar el queso de Cabrales (Núñez, 1978; Flórez y col., 2006), el queso Manchego (Ordóñez y col., 1978; Ballesteros y col., 2006), el queso de la Serena (Del Pozo y col., 1988),

el queso de La Armada (Tornadijo y col., 1995), el queso Afuega'l Pitu (Cuesta y col., 1996), Arzúa (Centeno y col., 1996), Peñamellera (Estepar y col., 1999), etc. Bien sea en composición de especies o en su frecuencia relativa, la diversidad microbiana de cada producto es única. Además de las especies de BAL, algunos quesos presentan niveles elevados de otros tipos microbianos cuya función es esencial para su maduración (*Penicillium, Geotrichum, Corynebacterium, Micrococcus,* etc.). La diversidad microbiana en estos productos, tanto en número de especies como de cepas dentro de cada especie, la podemos ilustrar con los resultados de un amplio estudio realizado por Cogan y col. (1997). En este trabajo se analizaron numerosos productos lácteos europeos a partir de los cuales se aislaron e identificaron más de 4000 cepas. Los microorganismos más abundantes pertenecían a diversos grupos de BAL (38% lactococos, 17% enterococos, 14% *Streptococcus thermophilus*, 12% lactobacilos termófilos, 10% leuconostocs y 9% lactobacilos mesófilos); bacterias mayoritarias en todos los productos lácteos fermentados. La diversidad fenotípica y genética que se detectó entre los aislados en éste y otros trabajos es sorprendente, constituyendo el reservorio que hemos mencionado de donde seleccionar nuevos tipos (Ayad y col., 2000; Ayad y col., 2001; Topisirovic y col., 2006).

Las técnicas empleadas en los estudios que se referencian en el párrafo precedente presentan, sin embargo, limitaciones asociadas al cultivo de los microorganismos, lo que dificulta el aislamiento y la posterior caracterización de algunos tipos microbianos que no se multiplican en los medios de cultivo utilizados o de otros para los que, por inesperados, no se utilizan condiciones adecuadas para su desarrollo. La llegada de las técnicas independientes de cultivo está suponiendo una considerable mejora en la descripción microbiana de los ecosistemas lácteos llegando incluso a detectar la presencia de ADN correspondiente a especies no detectadas con anterioridad (Giraffa y Neriani, 2011; Jany y Barbier, 2008). En los últimos años se han empezado a aplicar a las fermentaciones tradicionales las nuevas técnicas de secuenciación (Next Generation Sequencing, NGS) para estudiar la diversidad microbiana presente en productos lácteos fermentados (Dobson y col., 2011; Masoud y col., 2011; Leite y col., 2012; Lusk y col. 2012; Ercolini y col., 2013). En un reciente y amplio trabajo, Quigley y cols. (2012) utilizan la pirosecuenciación

para describir la microbiota presente en 62 tipos de quesos tradicionales irlandeses. Con esta técnica se ha detectado ADN perteneciente a géneros bacterianos nunca antes descritos en quesos (*Faecalibacterium*, *Prevotella* y *Helcococcus*). La detección de nuevos tipos microbianos representa un primer paso en la búsqueda de microorganismos para el diseño de nuevos y mejores fermentos. Una vez detectada su presencia, es necesario profundizar en su estudio para conocer que rol cumplen los microorganismos en la producción o maduración de los productos lácteos y valorar su posible inclusión en futuros y mejorados fermentos específicos siempre que sea posible su aislamiento y caracterización.

En los trabajos que conforman esta memoria se han utilizado de manera conjunta técnicas convencionales de cultivo en medios selectivos y/o diferenciales junto a técnicas moleculares independientes de cultivo como la DGGE (Primera parte artículo I y Segunda parte, artículos II, III y IV) y la pirosecuenciación (Segunda parte, artículo III). Estas últimas técnicas revelaron, en los productos estudiados en esta memoria, la presencia de poblaciones de especies poco corrientes como *Lc. garvieae, Lc. raffinolactis, S. thermophilus, Bifidobacterium* spp., y otras. Tras conocer su abundancia, nuestro grupo ha desarrollado una estrategia para el aislamiento selectivo de *S. thermophilus*. Las cepas aisladas se están caracterizando y comparando con otras de la misma especie procedentes de fermentos comerciales. Tanto las técnicas de cultivo como las cultivo-independientes tienen ventajas e inconvenientes (Giraffa y Neviani, 2001; Jany y Barbier, 2008; Bokulich y Mills, 2012). La la utilización combinada de ambos tipos de técnicas podría ser la opción más indicada para una mejor caracterización microbiana del ecosistema del queso y los productos lácteos.

Desde su aparición en el año 2004, las NGS están cambiando la investigación en diversos campos de la Microbiología. Las técnicas NGS no sólo permiten describir con mayor detalle las poblaciones microbianas de un ecosistema complejo como el que se desarrolla en las fermentaciones naturales, sino que, además, están disparando el conocimiento de los genomas de cada bacteria de manera individual (Mills y col. 2010). En la actualidad se han publicado los genomas completos de 87 cepas de BAL y hay más de 300 en proceso de secuenciación. Este conocimiento

permitirá a la industria alimentaria una mejor explotación de las capacidades fermentativas y transformadoras de las BAL (Liu y col., 2008; Branco dos Santos y col., 2013), lo que abre las puertas a nuevos métodos de caracterización de cepas de BAL mediante comparación genómica, incluyendo el estudio de aspectos tecnológicos (fenotípicos) de máximo interés para la industria láctea (Liu y col., 2008; Passerini y col., 2010; Siezen y col., 2010; Tan-a-ram y col., 2011; Siezen y col., 2011). De hecho, con la secuenciación genómica de la primera bacteria láctica *Lactococcus lactis* IL 1403 (Bolotin y col., 1999) se descubrió la posibilidad de que ésta y otras cepas de BAL fermentadoras pudieran respirar siempre que se le proporcionara una fuente del grupo hemo (Vido y col., 2004). Esta capacidad se aprovechó de inmediato para diseñar un nuevo método de producción de fermentos con un incremento sustancial de la masa microbiana celular respecto a la que ofrece el cultivo convencional (Pedersen y col., 2005).

En este contexto, aunque no se relaciona en la presente memoria, nuestro grupo llevó a cabo la secuenciación y el análisis genómico de una cepa de *Lc. garvieae* procedente del queso Casín (Flórez y col., 2012). La secuenciación se abordó con dos objetivos principales: analizar la seguridad de las cepas y estimar sus propiedades tecnológicas. El análisis de las secuencias no reveló la presencia de genes específicos de patogenicidad o virulencia, aunque la cepa secuenciada porta un gen de resistencia a tetraciclina codificado en un transposón que la inhabilita para ser utilizada como fermento. Tampoco se encontraron genes que codificaran para proteinasas caseinolíticas extracelulares similares a las de *Lc. lactis*, lo que por otra parte explicaba el escaso desarrollo en leche de las cepas de esta especie cuando se cultivan individualmente (Fernández y col., 2010).

La secuenciación genómica y el análisis de otras cepas de BAL procedentes de los quesos tradicionales podría proporcionar una información valiosa sobre sus propiedades, y servirá también para conocer las relaciones evolutivas de estos microorganismos con las cepas que componen los fermentos comerciales, a las que comúnmente se denomina cepas "domesticadas" (perfectamente adaptadas a un crecimiento rápido en leche) (Passerini y col., 2010; Bachmann y col., 2012).

El uso de fermentos y fermentos específicos

Debido al continuo crecimiento del sector lácteo, los fermentos comerciales se han impuesto como opción principal para la producción de queso a gran escala. Muchas variedades de queso en todo el mundo se elaboran utilizando fermentos comerciales genéricos que han sido seleccionados para producir grandes cantidades de manera homogénea y rápida. Las cualidades organolépticas de sabor y aroma de las variedades tradicionales difícilmente se pueden encontrar en los quesos industriales; productos que tampoco llevan asociados los valores positivos del concepto tradicional (artesano, pequeña escala, respeto al medio ambiente, etc.). Además, muchos quesos tradicionales se elaboran aún con leche cruda, lo que permite el desarrollo de una compleja y variada microbiota cuyos componentes evolucionan e interactúan a lo largo de la maduración (Beresford et al., 2001). De una u otra forma, todos los componentes de esta microbiota participan en la generación de los originales y complejos perfiles organolépticos de los quesos maduros. Esta complejidad hace también difícil su reproducción por medio de la utilización de fermentos comerciales, compuestos como norma general por una o muy pocas cepas de una o muy pocas especies.

En esta memoria relacionamos tres trabajos dedicados a la caracterización de cepas de BAL procedentes de productos lácteos tradicionales (Tercera parte, artículos V, VI y VII), y un tercer trabajo encaminado a estudiar el comportamiento en condiciones reales de elaboración de una serie de mezclas diseñadas con algunas de las cepas seleccionadas (Cuarta parte, artículo VIII). La caracterización de cepas es necesaria porque, junto a propiedades positivas, algunas cepas presentan otras indeseables como la producción de aminas biógenas o la presencia de genes de resistencia a antibióticos. En todo caso, como principal conclusión podemos decir que los productos lácteos tradicionales contienen un reservorio de cepas con buenas aptitudes tecnológicas que se pueden emplear como fermentos y/o cultivos adjuntos. Es preciso identificar y preservar esta diversidad microbiana ya que constituye, junto a las razas animales y la alimentación del ganado, uno de los pilares de la calidad organoléptica y sensorial de los productos tradicionales.

Los fermentos específicos están diseñados para cada producto concreto, con el fin de conservar los microorganismos propios del producto y mantener sus proporciones relativas durante las fases de fermentación y maduración (Parente y Cogan, 2004). Este tipo de fermentos se supone capaz de preservar el *flavor* típico de los productos tradicionales. Además, el uso de fermentos específicos permitiría reproducir las cualidades organolépticas de productos fermentados tradicionales utilizando leche pasteurizada, y reducir los accidentes tecnológicos (falta de acidificación, sabores extraños). De hecho, uno de los principales objetivos de los trabajos que describen la microbiota de los quesos tradicionales es la identificación y selección de cepas para el diseño de fermentos específicos (Núñez, 1978; Ordóñez y col., 1978; Del Pozo y col., 1988; Tornadijo y col., 1995; Centeno y col., 1996; Cuesta y col., 1996; Estepar y col., 1999; Ballesteros y col., 2006; Flórez y col., 2006). Las cepas aisladas pueden servir también para sustituir o complementar las cepas de los fermentos comerciales, dado que la industria busca constantemente cepas con nuevas características bioquímicas, aromáticas y de resistencia a los bacteriófagos (Parente y Cogan, 2004; van Hylckama Vlieg y col., 2006). Estas nuevas cepas han de provenir de productos elaborados sin la adición de fermentos. Entre ellas se ha encontrado una enorme diversidad metabólica (van Hylckama Vlieg y col., 2006), incluyendo la producción de sustancias aromáticas diferentes a las que producen las cepas comerciales (Ayad y col., 2000; Ayad y col., 2001; Hanniffy y col., 2009).

Entre los estudios que apoyan la utilidad de los fermentos compuestos por cepas salvajes, podemos citar el aumento del aroma en el queso Cheddar y la producción de aminoácidos funcionales (Lacroix y col., 2010); la implantación y dominancia de las cepas autóctonas sobre las cepas de los fermentos en un queso francés de maduración superficial (Feurer y col., 2004), lo que en la práctica indica la inutilidad del empleo de dichos fermentos; o los resultados obtenidos en el queso alemán de pasta lavada Limburguer, en los que se vio que los fermentos comerciales de maduración que se utilizan (*Brevibacterium aurantiacun*, *Debaryomyces hansenii, Galactomyces geotrichum* y *Arthrobacter arilaitensis*) no son capaces de establecerse en la masa, cediendo el nicho ecológico a los microorganismos endógenos que se desarrollan de manera espontánea (Goerges y col., 2008). El aislamiento y la

caracterización de estos microorganismos nativos han posibilitado el diseño de exitosos fermentos específicos.

En este trabajo no se han desarrollado fermentos específicos. Sin embargo, contamos con un número suficiente de cepas identificadas, bien caracterizadas y con buenas aptitudes tecnológicas de cada uno de los productos estudiados, con lo que estamos en condiciones de desarrollarlos en el futuro siempre y cuando haya una demanda por parte de los sectores productivos.

Conclusiones

Conclusiones:

PRIMERA: La utilización combinada de técnicas de cultivo y técnicas moleculares independientes de cultivo como la DGGE y la pirosecuenciación permiten obtener un conocimiento más profundo de la diversidad microbiana del queso y otros productos lácteos fermentados y un mejor seguimiento de la evolución de las poblaciones a lo largo de los procesos de elaboración y maduración.

SEGUNDA: Las técnicas de cultivo son indispensables e insustituibles para el aislamiento de cepas de bacterias lácticas (y otros tipos microbianos) con la finalidad de emplearlas después en la fermentación de la leche o para otras aplicaciones. Para su empleo como cultivos iniciadores o como cultivos adjuntos es necesaria la caracterización de los aislados y la selección de los más adecuados para cada aplicación.

TERCERA: Las técnicas independientes de cultivo permiten identificar poblaciones géneros y especies bacterianos no detectadas mediante las técnicas convencionales de cultivo. La presencia de una población numerosa de *Streptococcus thermophilus* parece ser corriente en muchos tipos quesos, tal y como se ha revelado repetidamente en este trabajo mediante las técnicas independientes de cultivo. En los quesos tradicionales son corrientes también especies de *Lactococcus* distintas a *Lc. lactis* como *Lc. garvieae* y *Lc. raffinolactis,* que no siempre se detectan entre los aislados.

CUARTA: Los productos lácteos tradicionales elaborados sin adición de fermentos son una buena fuente de nuevos microorganismos con propiedades tecnológicas deseables, incluyendo cepas acidificadoras de *Lc. lactis* que pudieran constituir cultivos iniciadores en sentido estricto y cepas con capacidad aromatizante como *Lc. lactis* subsp. lactis biovar *diacetylactis*, *Leuconostoc* spp., y *Lactobacillus* spp. con utilidad como cultivos adjuntos.

QUINTA: Los microorganismos procedentes de la caracterización microbiana de los tres productos tradicionales estudiados en esta Tesis constituyen una amplia colección, formada por bacterias lácticas en su mayoría, con gran potencial e interés tecnológico. En total han reunido más de 450 aislados pertenecientes a los géneros *Lactococcus* (50%), *Lactobacillus* (25%), *Leuconostoc* (12,5%) y otros (12,5%).

SEXTA: La caracterización *in vitro* de los aislados se considera un método valioso y apropiado para la selección de aquellos que cumplan los requisitos definidos para cada tipo microbiano y para cada aplicación. Se considera también adecuada para no utilizar en los sistemas alimentarios microorganismos con propiedades indeseables, como la producción de aromas y/o sabores extraños, la producción de compuestos dañinos o la presencia de genes de resistencia a antibióticos que se puedan extender a través de la cadena alimentaria.

SÉPTIMA: La caracterización *in vitro* de los aislados, sin embargo, no puede suplir el ensayo de las cepas seleccionadas en las condiciones reales de elaboración. Serán estas pruebas experimentales las que determinen de manera definitiva la utilidad de las cepas aisladas o sus mezclas definidas como cultivos iniciadores y adjuntos para un determinado producto.

OCTAVA: Las cepas de bacterias ácido-lácticas caracterizadas y las mezclas ensayadas con buenos resultados están listas para su transferencia al sector industrial. Algunas cepas ya se han transferido a empresas como Chr. Hansen, Biogés Starters SA y Proquiga SA. Disponemos, además, de un importante reservorio de aislados para la caracterización y selección de más cepas y/o para la formulación de fermentos específicos.

Conclusions

Conclusions:

FIRST: The combined application of culture-dependent and culture-independent techniques such as DGGE and pyrosequencing provide a deeper knowledge of the microbial diversity of cheese and other fermented dairy products, as well as a better description of the evolution of the microbial populations through manufacturing and ripening.

SECOND: Culture techniques are essential and irreplaceable for the isolation of new bacterial strains with the aim of using them in dairy fermentations or for other technological applications. The characterization of the isolates and the selection of the most suitable ones for each application allow their utilization as starter or adjunct cultures.

THIRD: Culture-independent techniques allow the identification of microbial populations (genus and species) not detected previously by conventional culture techniques. Numerous populations of *Streptococcus thermophilus* seem to be widely spread in many cheese types, as the culture independent techniques revealed throughout this work. Moreover, species of *Lactococcus* different from *L. lactis*, such as *Lc. garvieae* y *Lc. raffinolactis* seem to be commonly present in traditional cheeses.

FOURTH: Traditional dairy products made without the addition of starter cultures are a good source of new microorganisms with interesting technological properties, including high acidifying strains of *Lactococcus lactis* which can act as

starter cultures or microorganisms with aromatizing capabilities such as *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* spp., and *Lactobacillus* spp., which can then be used as adjunct cultures.

FIFTH: The microorganisms obtained from the three traditional dairy products studied in this work make up a great microbial collection, composed mainly by lactic acid bacteria, whose components are of great interest and technological potential. The total number of isolates, belonging to the genus *Lactococcus* (50%), *Lactobacillus* (25%), *Leuconostoc* (12.5%) and others (12.5%), raises up to more than 450..

SIXTH: The *in vitro* characterization of isolates is a very convenient way to select appropriate microorganisms for every application. In the food industry, it is considered useful too in order to avoid microorganisms having undesirable traits such as the production of bad flavor or aroma, the production of toxic compounds or the presence of antibiotic resistance genes that could spread through the food chain.

SEVENTH: The *in vitro* characterization of isolates cannot replace the assay of the selected microorganisms under the actual production of manufacturing conditions. These experiments will ultimately determine the usefulness of single strain or strain mixtures as starter and adjunct cultures.

EIGHTH: The lactic acid bacteria strains which were characterized and the strain mixtures of strains assayed with satisfactory results, are ready to transfer to the food fermentation industry. In fact, some of the strains have already been transferred to culture-producing companies such as Chr. Hansen, Biogés Starters S.A. and Proquiga S.A. We also keep an important collection of other isolates, for further characterization and for the development of new specific starters.

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Infome sobre la calidad de las publicaciones

Informe sobre la calidad de las publicaciones

La información sobre las revistas ha sido recogida de la Web Of Knowledge de FECYT (Fundación Española para la Ciencia y la Tecnología) (http://www.accesowok.fecyt.es/) en una consulta realizada en Abril de 2013.

El factor de impacto (FI) de cada revista corresponde a los últimos datos publicado por Journal Citation Reports (año 2011). También se indica la categoría o categorías a las que está asociada la revista, así como su posición (Pos) y cuartil (Q) dentro de la categoría calculados en función de su factor de impacto.

El número de veces que ha sido citado cada artículo por publicaciones SCI (Citado Por) se ha consultado utilizando el motor de búsqueda Google Académico (http://scholar.google.es) en Abril de 2013.

Artículo I

- Alegría, A., R. González, M. Díaz, and B. Mayo. 2011. Assessment of microbial populations dynamics in a blue cheese by culturing and denaturing gradient gel electrophoresis. *Current microbiology* 62: 888-893.

Categoría	Total	Pos	Q	FI (2011)	Citado Por
Microbiology	114	76	Q3	1.815	2

Artículo II

- Alegría, A., P. Álvarez-Martín, N. Sacristán, E. Fernández, S. Delgado, and B. Mayo. 2009. Diversity and evolution of majority microbial populations during manufacturing and ripening of Casín, a Spanish traditional, starter-free cheese made of raw cow's milk. *International Journal of Food Microbiology* 136: 44-51.

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Food Science and Technology	128	9	Q1	3.327	24
Microbiology	114	35	Q2		

Artículo III

- Alegría, A., P. Szczesny, B. Mayo, J. Bardowski and M. Kowalczyk. 2012. Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and -independent approaches. *Applied and Environmental Microbiology* 78: 1890-8.

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Biotechnology and Environmental Microbiology	158	29	Q1	3.829	10
Microbiology	114	28	Q1		

Artículo IV

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Artículo V

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Microbiology	114	28	Q1		

Artículo VI

- Alegría, A., S. Delgado, C. Roces, B. López and B. Mayo. 2010. Bacteriocins produced by *wild Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk. *International Journal of Food Microbiology* 143: 60-66.

Categoría	Total	Pos	Q	FI (2011)	Citado Por
Food Science and Technology	128	9	Q1	3.327	10
Microbiology	114	35	Q2		

Artículo VII

- Alegría, A., S. Delgado, A.B. Flórez and B. Mayo. 2013. Characterization of *Leuconostoc* strains isolated from raw milk traditional cheeses and evaluation of their suitability as adjunct cultures for dairy. *Dairy Science and Technology* Aceptado en Abril de 2013. DOI 10.1007/s13594-013-0128-3

Categoría	Total	Pos	Q	FI (2011)	Citado Por
Food Science and Technology	128	63	Q2	1.183	-

Artículo VIII

- Technological characterization of mixtures of mesophilic lactic acid bacteria strains isolated from traditional raw milk cheeses and their experimental evaluation as cheese starters.

Artículo en preparación para su publicación.