MEMORIA DE TESIS DOCTORAL

"ESTUDIO BACTERIOLÓGICO DE LA LECHE DE TANQUE DE REBAÑOS OVINOS Y CARACTERIZACIÓN DE CEPAS DE COCOS GRAM-POSITIVOS CATALASA-NEGATIVOS Y DE *Mycoplasma agalactiae* "

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"La ciencia es el alma de la prosperidad de las naciones y la fuente de vida de todo progreso".

Louis Pasteur

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"Las doctrinas suceden a las doctrinas, los sistemas a los sistemas, las escuelas a las escuelas. Y en este perpetuo renacimiento sobre ruinas, y de esta palingenesia sucesiva, siempre va quedando alguna verdad que en lenta sedimentación va formando lo que al cabo constituirá la base incontrastable de la Ciencia, pero que no puede ser su edificio entero en cada uno de los momentos en que lo exigen y lo desean, por rara combinación, simultáneamente, el egoísmo y el altruismo humanos."

Dr. D. Carlos María Cortezo.

Fragmento del discurso *Los grandes remedios*.

Sesión inaugural en la Real Academia de Medicina.

Madrid, 1905.

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RESUMEN

La memoria de Tesis doctoral titulada "**ESTUDIO BACTERIOLOGICO DE LA LECHE DE TANQUE DE REBAÑOS OVINOS Y CARACTERIZACIÓN DE CEPAS DE COCOS GRAM-POSITIVOS CATALASA-NEGATIVOS Y DE *Mycoplasma agalactiae* "** comprende seis estudios diferentes llevados a cabo durante el periodo 2009-2013 y financiados por el Ministerio de Economía y Competitividad, Gobierno de España, a través del proyecto AGL2008-00422 (ayuda no. BES-2009-025401). El trabajo fue realizado en la Universidad de León (León, España), la Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, Reino Unido) y el Centro Nacional de Microbiología (ISCIII, Majadahonda, España).

Se diseñó un primer estudio para analizar los efectos del almacenamiento y la conservación sobre los recuentos de microorganismos mesófilos, termodúricos, psicrotrofos, coliformes, *Escherichia coli*, *Streptococcus agalactiae* y *Staphylococcus aureus*. Se llevaron a cabo un total de 910 determinaciones analíticas a partir de alícuotas de leche de oveja tomadas de 10 muestras de silo. Las condiciones evaluadas fueron: leche cruda y conservada con azidiol a 4 ºC y congelada a -20 ºC. La leche de las alícuotas refrigeradas fue almacenada durante 2, 24, 48, 72 y 96 h después de su recogida y la de las alícuotas congeladas durante 7, 15 y 30 días desde su recogida.

Los factores silo y condiciones de almacenamiento contribuyeron significativamente a la variación de todas las variables microbiológicas, aunque la edad de la leche dentro del almacenamiento fue únicamente significativa para los recuentos de microbiota mesófila, psicrotrofa y microorganismos coliformes. En la leche cruda refrigerada, los recuentos de bacterias mesófilas, psicrotrofas y coliformes se incrementaron significativamente tras 96 h post-recogida, mientras que otros grupos y especies bacterianos evaluados mantuvieron su concentración inicial.

En todos los casos, la conservación con azidiol mantuvo la concentración bacteriana inicial en la leche cruda de oveja. Los menores recuentos fueron registrados en la leche congelada, particularmente para coliformes, *E. coli*, *Str. agalactiae* y *S. aureus*. Las estimaciones de organismos mesófilos, termodúricos y psicrotrofos mostraron valores similares tanto en las muestras de leche conservadas con azidiol como en las congeladas. Los recuentos de coliformes y *E. coli* disminuyeron significativamente después de la congelación. En consecuencia, la congelación a -20 ºC podría ser también apropiada para el análisis de los grupos bacterianos mesófilos, psicrotrofos y termodúricos, pero no para los coliformes o los patógenos mamarios.

Para analizar la relación entre el recuento de diferentes organismos y el recuento bacteriológico total (RBTLT) y el recuento de células somáticas (RCSLT) según se determinan en leche de oveja en los laboratorios, un segundo trabajo fue llevado a cabo en 751 muestras de leche de tanque procedentes de 205 rebaños de ovino lechero del consorcio de Promoción del Ovino (CPO, Villalpando, Zamora) recogidas entre enero y

diciembre de 2011. Se realizaron cuatro muestreos en cada rebaño, uno por estación, a lo largo de un año. Las variables analizadas fueron los recuentos en tanque de microorganismos termodúricos, psicrotrofos, coliformes y cocos Gram-positivos catalasa-negativos (CGPCN). Los microorganismos termodúricos, psicrotrofos y coliformes se relacionaron significativamente con RBTLT, mientras que los CGPCN se relacionaron tanto con la variable RBTLT como con la variable RCSLT. Los mayores recuentos se dieron en el caso de psicrotrofos y coliformes, y una correlación de moderada a alta (r=0.51) se encontró entre ambas variables, indicando que las prácticas de limpieza deficientes en los rebaños tienden a seleccionar organismos menos resistentes, tales como los bacilos Gram negativos. Además RBTLT se correlacionó con RCSLT (r= 0.42). Algunos factores de variación para recuentos bacterianos específicos, tales como la raza, la estación, el tipo de ordeño, la terapia de secado y la producción láctea fueron también analizados. La información del rebaño fue recogida en los libros de explotación, de las auditorías anuales y del sistema de trazabilidad del CPO. Los psicrotrofos y los coliformes tuvieron recuentos elevados en invierno, mientras que los de los CGPCN fueron mayores en verano y en los rebaños ordeñados manualmente. La terapia de secado contribuyó a la reducción de la microbiota psicrotrofa, por lo que algunos patógenos mamarios podrían ser también psicrotrofos. Los resultados de este trabajo serían de utilidad en el afrontamiento de problemas de calidad de leche y en el desarrollo de mejores sistemas de pago por calidad en el ovino lechero.

La caracterización de CGPCN aislados en los dos trabajos anteriores llevó a la realización de los siguientes dos estudios. La diversidad específica de 192 aislados de una colección de CGPCN de leche ovina de tanque fue investigada por medio de la tecnología del ADNr. El enfoque molecular permitió la identificación de 23 especies de interés sanitario y tecnológico tanto para el hombre como para los animales dentro de los géneros *Enterococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus* y *Trichococcus*. Un estudio de la virulencia y resistencia a glicopéptidos se llevó a cabo en varios aislados de *Enterococcus faecalis* dada la predominancia mostrada por esta especie y su importancia como patógeno oportunista y portador de genes de resistencia. Todos los *E. faecalis* analizados albergaban al menos un gen asociado a factor de virulencia y una concentración mínima inhibidora (CMI) ≥ 4 mg/L a la vancomicina, punto de corte establecido por el CLSI (Clinical and Laboratory Standards Institute) en 2012, pese a no existir evidencia molecular de resistencia a la vancomicina en la mayoría de las cepas. Un único gen *vanC1* fue detectado en la cepa CNM460_12. El amplicón de este gen se secuenció y comparó con las secuencias disponibles en la base de datos BLAST del NCBI (National Center for Biotechnology Information, Basic Local Alignment Search tool, www.ncbi.nlm.nih.gov) corroborando su identidad con la secuencia *vanC1* de la cepa *E. gallinarum* eS464 (no. Acceso GenBank EU151772) y con otras cepas portadoras de *vanC1*. El ensayo fenotípico de susceptibilidad a antibióticos demostró sensibilidad a ampicilina, macrólidos, aminoglicósidos y fluoroquinolonas, al contrario que otros enterococos vancomicina resistentes (EVR); y confirmó una resistencia moderada a la vancomicina (CMI=12 mg/L) y susceptibilidad a la teicoplanina. Los perfiles genéticos de virulencia y MLST (MultiLocus Sequence Typing) fueron los siguientes: *asa1*+ (sustancia de agregación)/ *gelE* + (gelatinasa) /*cylA*+ (activador citolisina) y secuenciotipo (ST) 168 [perfil alélico: *gdh*-20, *gyd*-1,*psts*-7, *gki*-25, *aroE*-23, *xpt*-2,

yqil-2]. Este es el primer caso, según nuestros conocimientos, de detección del gen *vanC1* en un *E. faecalis* con fenotipo vancomicin-resistente aislado a partir de leche de oveja de tanque. Esto es relevante puesto que el gen de resistencia *vanC1* es un marcador de especie usado para la identificación de *E. gallinarum*. La presencia del gen *asa1* sugiere la transferencia horizontal del gen entre las dos especies de la leche. La población de CGPCN de la leche de oveja destinada a la producción de queso debe ser caracterizada debido a la creciente preocupación en relación a la mamitis, los sistemas de gestión de la calidad, la seguridad alimentaria y el desarrollo de nuevos productos. La detección de genes de virulencia y resistencia a la vancomicina en *E. faecalis* de leche de oveja destinada a la fabricación de productos lácteos enfatiza la necesidad de evaluar la presencia de genotipos de virulencia y resistencia, ya que *E. faecalis* es la especie enterocócica predominante en los quesos mediterráneos (recuentos entre 10^4-10^7 ufc/g) pudiendo ser potencialmente transferidos al hombre.

El propósito de los dos trabajos restantes fue la caracterización del agente causal de la Agalaxia Contagiosa (AC), un síndrome que provoca importantes pérdidas en las granjas de ovino lechero. Se obtuvieron trece cepas a partir de leche de oveja almacenada en tanques y silos de gran tamaño. Se identificaron como *Mycoplasma agalactie* por PCR con electroforesis desnaturalizante en gel con gradiente (PCR-Denaturing Gradient Gel Electrophoresis o PCR-DGGE). Se analizó la actividad *in vitro* frente a trece antimicrobianos de interés veterinario y los resultados mostraron que la clindamicina y las quinolonas, estos últimos utilizados como tratamientos estándar frente a AC, fueron los compuestos más efectivos *in vitro* frente a *Myc. agalactiae* con una CMI < 0.12 μg/mL y < 0.12 - 0.5 μgmL^{-1} respectivamente. Basándose en el mismo examen *in vitro*, la tilosina y la tilmicosina serían asimismo compuestos adecuados para su tratamiento. Todos estos aislados fueron resistentes a la eritromicina, indicando que ésta no sería una opción apta para la terapéutica. Las cepas mostraron un perfil genético y proteico común por electroforesis en campo pulsado (PFGE) y electroforesis desnaturalizante en gel de poliacrilamida con dodecil sulfato sódico (SDS-PAGE), y diferencias menores observadas en los perfiles immunoblot; sugiriendo una relación clonal entre ellas. Esta similitud proteica y genética sugiere la persistencia y cronicidad de *Myc. agalactiae* en un área endémica. Por otra parte, el uso del análisis multilocus de secuencias (MLST) en las mismas cepas reveló una inesperada diversidad, identificando tres aislados dentro de dos secuenciotipos de nueva descripción (ST-16 y ST-17) y dejando el resto de cepas incluidas dentro del conjunto europeo mayoritario (ST-5). La identificación de la sensibilidad a antimicrobianos de *Myc. agalactiae* y la caracterización molecular, proteica y antigénica es de gran ayuda en estudios epidemiológicos, el establecimiento de pautas terapéuticas y el desarrollo de nuevas vacunas en un área endémica como Castilla y León donde se declararon un total de 924 brotes de AC (931 en toda España) entre 2010 y 2011, de acuerdo con los datos de la Organización Mundial de Sanidad Animal (OIE).

SUMMARY

The report of Thesis entitled "**BACTERIOLOGICAL STUDY OF TANK MILK IN SHEEP FLOCKS AND CHARACTERIZATION OF STRAINS OF GRAM POSITIVE CATALASE NEGATIVE COCCI AND *Mycoplasma agalactiae*"** comprises six different studies carried out during the period 2009-2013 and funded by the Spanish Ministry of Economy and Competitiveness, Government of Spain, via project AGL2008-00422 (grant no. BES-2009-025401). The work was conducted at the University of León (León, Spain), the Animal Health and Veterinary Laboratories Agency (Weybridge, UK) and the National Center of Microbiology (ISCIII, Majadahonda, Spain).

A first study was designed to analyze the effects of the storage and preservation conditions on counts of mesophiles, thermodurics, psycrhotrophs, coliforms, *Escherichia coli*, *Streptococcus agalactiae*, and *Staphylococcus aureus* in silo ovine milk. A total of 910 analytical determinations were carried out from aliquots of 10 silo ovine milk samples. The conditions tested were unpreserved and azidiol-preserved milk stored at 4°C, and unpreserved milk stored at −20°C. Milk aged 2, 24, 48, 72, and 96 h post-collection for refrigerated aliquots, and 7, 15, and 30 d post-collection for frozen aliquots.

The factors silo and storage conditions significantly contributed to the variation of all microbiological variables, although milk age effect within storage was only significant for mesophilic, psychrotrophic, and coliform bacteria counts. In refrigerated raw milk, mesophilic, psychrotroph, and coliform counts significantly increased over 96 h post-collection, whereas the other groups and bacteria species tested maintained their initial concentration.

In all cases, azidiol preservation maintained the initial bacterial concentration in raw sheep milk under refrigeration throughout 96 h. Thus, azidiol was a suitable preservative for microbiological studies in sheep milk. Smallest counts were registered for frozen samples, particularly for coliforms, *E. coli*, *Str. agalactiae* and *S. aureus*. Estimates of mesophilic, thermoduric and psychrotrophic organisms showed similar values on both azidiol-preserved and frozen milk samples. Coliforms and *E. coli* counts significantly decrease over time after freezing. Consequently, freezing at −20°C could be appropriate for analysis of mesophilic, thermoduric, and psychrotrophic bacterial groups, but not for coliforms or mammary pathogens.

A second study was performed to analyze the relationship between the counts of different organisms and total bacterial count (BTTBC) and somatic cell count (BTSCC) as determined in dairy laboratories in ovine bulk tank milk. A total of 751 bulk tank milk samples from 205 dairy sheep flocks of the Consortium for Ovine Promotion (CPO, Villalpando, Zamora, Spain) were collected between January and December 2011. Four samplings were carried out in each flock, once per season, throughout one year. Variables analyzed were bulk tank counts of thermoduric, psychrotrophic, coliform and gram-positive catalase-negative cocci (GPCNC) bacterial groups. Thermoduric, psychrotrophic, and coliform species were significantly related to BTTBC, whereas GPCNC were

correlated with both BTTBC and BTSCC variables. The highest counts were for psychrotrophs and coliforms, and a moderate to high correlation (r = 0.51) was found between both variables, indicating that poor cleaning practices in the flocks tend to select for less-resistant organisms, such as gram-negative rods. In addition, BTTBC correlated with BTSCC (r = 0.42). Some variation factors for specific bacterial counts, such as breed, season, milking type, dry therapy, and milk yield, were also analyzed. Flock information was collected from flock books, annual audits, and the CPO traceability system. Psychrotrophs and coliforms had elevated counts in winter, whereas GPCNC were higher in summer and in hand-milked flocks. Dry therapy contributed to the reduction in psychrotrophic bacteria; therefore, some strains of mammary pathogens could also be psychrotrophs. Results of this study would be helpful for troubleshooting milk quality problems and developing premium payment systems in dairy sheep.

The characterization of GPCNC isolated in the two latter works led to the following two papers. The species diversity of 192 isolates of a collection of GPCNC from sheep bulk tank milk was investigated using 16S rDNA technologies. The molecular approach enabled the identification of 23 species of GPCNC of sanitary and technological importance either to humans and/or animals within *Enterococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Trichococcus* genera. A glycopetide resistance and virulence survey was carried out on several *Enterococcus faecalis* isolates given the predominance found and its relevance as human opportunistic pathogen and resistance genes carrier. All the *E. faecalis* tested harboured at least one virulence factor gene and vancomycin MIC ≥ 4 mg/L, Clinical and Laboratory Standards Institute (CLSI 2012) clinical breakpoint, although no molecular evidence of vancomycin resistance was found in most of them. A unique *van*C1 gene was found in the strain CNM460_12. This *van*C1 amplicon was sequenced and compared with sequences in the National Center for Biotechnology Information database (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov), which corroborated its similarity with the sequence of the *van*C1 gene of *E. gallinarum* strain eS464 (GenBank accession no. EU151772) and with those of other VanC1-carrying strains. Phenotypic antimicrobial susceptibilities testing demonstrated susceptibility to ampicillin, macrolides, aminoglycosides and fluoroquinolones, unlike clinical vancomycin resistant enterococci (VRE) strains, and confirmed moderate resistance to vancomycin (MIC=12 mg /L) and susceptibility to teicoplanin. Genotypic virulence and MultiLocus Sequence Typing (MLST) profiles were respectively as follows: *asa1*+ (aggregation substance)/ *gelE* + (gelatinase) /*cylA*+(cytolysin activator) and sequence type (ST)168 [allelic profile: *gdh*-20, *gyd*-1, *pst*s-7, *gki*-25, *aro*E-23, *xpt*-2, *yqi*l-2]. This is the first report, to the best of our knowledge, of a *van*C1 gene in *E. faecalis* with a vancomycin-resistant phenotype isolated from sheep bulk milk. This is relevant since the detection of the *van*C1 resistance gene is a species marker used for the identification of *E. gallinarum*. The presence of the *asa1* gene suggests horizontal transfer between the two species in milk. Sheep milk intended for cheese production GPCNC population must be characterized due to the growing concern about mastitis, quality control programmes, food safety and new products development. Detection of virulence genes and vancomycin resistance in *E. faecalis* from sheep milk intended for dairy products emphasizes the need for screening for the presence of

virulence and antimicrobial resistance genotypes due to *E. faecalis* is the predominant enterococcal species in Mediterranean cheeses (enterococcal counts range from 10^4 to 10^7 c.f.u/g) which could potentially be transferred to humans.

The purpose of the two remaining works was the characterization of the causative agent of Contagious Agalactia (CA), a syndrome that causes important economic losses in dairy sheep farms. Thirteen mycoplasma isolates were obtained from samples of sheep milk taken from bulk tank and large silos. They were identified as *Mycoplasma agalactiae* by PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The *in vitro* activity of thirteen antimicrobials of veterinary interest was tested against these isolates. Results showed that the most effective compounds against *Myc. agalactiae in vitro* were clindamycin and the quinolones, with minimum inhibitory concentration (MIC) values of < 0.12 μg/mL and < 0.12 - 0.5 μg/mL respectively; these last antimicrobials which are used as standard treatments against CA. Based on the *in vitro* assay tylosin and tilmicosin would also be appropriate antimicrobials. All these isolates were resistant to erythromycin, indicating that it would not be a suitable choice for therapy. The isolates showed common genetic and protein profiles by Pulsed Field Gel Electrophoresis (PFGE) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), with minor differences observed by immunoblot analysis, suggesting a clonal relationship among them. This molecular similarity suggests the chronic persistence of *Myc. agalactiae* in an endemic area. On the other hand, unexpected variability was found using MLST analysis on the same strains, identifying three isolates with two novel outliers (ST-16 and ST-17) and leaving the rest included in the European majority cluster (ST-5). The identification of *Myc. agalactiae* antimicrobial susceptibilities, molecular, proteic and antigenic profiles is of great help in epidemiological studies, setting appropriate therapeutic guidelines and new vaccines development in an endemic area as Castilla y León region where a total of 924 CA outbreaks (931 all over Spain) were declared in 2010-2011, according to World Organisation for Animal Health (OIE) data.

INTRODUCCIÓN GENERAL Y OBJETIVOS

ESTUDIO BACTERIOLÓGICO DE LA LECHE DE TANQUE DE REBAÑOS OVINOS Y CARACTERIZACIÓN DE CEPAS DE COCOS GRAM-POSITIVOS CATALASA-NEGATIVOS Y DE *Mycoplasma agalactiae.*

La presente Memoria de Tesis Doctoral se defiende bajo el modelo de compendio de publicaciones y opta a la mención "Doctorado Internacional" de acuerdo al *Reglamento de las Enseñanzas Oficiales de Doctorado y del Título de Doctor de la Universidad de León* (BOCYL Núm 197, 11 de Noviembre de 2012, Págs. 61447-61471). Las publicaciones que se presentan son de contrastada relevancia en las categorías de *Microbiology* y *Agricultural, Dairy and Animal Science* según su ponderación en el *Journal Citation Reports* (2011).

En España se producen 550.000 toneladas anuales de leche cruda de oveja frente a un total de 6.522.000 toneladas de leche de vaca en explotación (FAOSTAT, 2011). Castilla y León es la primera región productora de leche de oveja en España y representa el 73 % de la producción total nacional (MAGRAMA, 2011). A pesar de ser una producción minoritaria, comparativamente a la de vaca, su composición y sus propiedades nutricionales y tecnológicas convierten a la leche de oveja en materia prima para productos lácteos de alto valor añadido, bien sean a base de leche pura de oveja o formando parte de mezclas. Así, prácticamente el 100 % de la producción de leche de oveja en España se destina a la producción de quesos (MAGRAMA, 2011) muchos de ellos amparados dentro de marcas de calidad reconocidas por la Unión Europea (DOP, IGP o Ganadería Ecológica).

La demanda por parte de las distintas administraciones y por parte de los consumidores de una leche segura y de alta calidad ha supuesto un reto para los productores, empresas de transporte, industria láctea de transformación, laboratorios de análisis y facultativos veterinarios, siendo las unidades de producción (explotaciones ganaderas) el punto de partida para la obtención de leche de calidad.

Gonzalo (2013) divide la calidad de la leche de oveja en cuatro tipos: calidad higiénico-sanitaria, calidad tecnológica, calidad nutricional y calidad ética. Nuestro interés en las líneas de investigación que configuran la presente Memoria de Tesis Doctoral se centra fundamentalmente en la primera de ellas (la calidad higiénico-sanitaria) en origen, la cual constituye la base para todas las demás. La unidad muestral viene representada por la leche de tanque de los rebaños de ganado ovino lechero, si bien -en algunos casos- hemos utilizado también la leche de silo (o de grandes mezclas) para el estudio de algunos microorganismos cuya presencia no está generalizada en todos los rebaños.

El conocimiento de la calidad higiénico-sanitaria, y particularmente bacteriológica de la leche de tanque y de sus factores de variación, tiene gran importancia a diferentes niveles de la cadena alimentaria:

- En primer lugar, a nivel del productor/ganadero, ya que la calidad de la leche de tanque constituye un reflejo de la calidad del manejo del rebaño. Así, es muy frecuente el uso de parámetros de recuento de la bacteriología total y de células

somáticas por parte de los técnicos para conocer la situación de la higiene del ordeño y sanidad mamaria del rebaño, habiéndose documentado una relación significativa entre ambos tipos de recuento bacteriológico y celular (Gonzalo *et al.* 2010; Koop *et al.*, 2009). En este sentido la calidad higiénico-sanitaria de la leche de tanque representa una herramienta de monitorización de la higiene y sanidad mamaria del rebaño, y la base para la implementación de medidas de prevención y control a nivel de explotación. Desde el punto de vista económico, dicha monitorización permite también incrementar los retornos económicos de los ganaderos que, además, pueden acogerse a las primas o incentivos económicos recogidos dentro de los esquemas de pago por calidad (Pirisi *et al.*, 2007). Adicionalmente, la presencia de determinados organismos patógenos en origen puede condicionar la seguridad del resto de la cadena productiva.

- En segundo lugar, a nivel de la industria láctea, pues la mejora de la calidad tecnológica e higiénica de la leche, alarga su vida útil y proporciona productos a su vez de mejor calidad y seguridad, y

- En tercer lugar, a nivel del consumidor, que dispondrá de productos con un menor riesgo sanitario, más duraderos y de mejores características tecnológicas, aumentando el nivel de satisfacción.

No obstante, desde nuestro punto de vista, es la calidad a nivel de los operadores primarios la que más va a condicionar la calidad en el resto de la cadena y en el producto final, por lo que los estudios que configuran la presente Memoria se han centrado fundamentalmente en el primer y más heterogéneo eslabón de la cadena alimentaria, las ganaderías de ovino lechero.

Respecto a las variables definitorias de la calidad de la leche, el recuento bacteriológico total (flora mesófila viable) resulta un parámetro muy inespecífico, que no ofrece información sobre otras variables microbiológicas de interés presentes también en la leche. Por eso, una mayor profundización en el estudio y cuantificación de los diferentes grupos bacterianos de la leche de tanque de los rebaños ovinos resultaría de gran interés. Desde el punto de vista de la calidad, de acuerdo con (Robinson, 2005) podemos clasificar los microorganismos presentes la leche almacenada en tanques y silos de refrigeración como:

- Microorganismos alterantes, relacionados con la calidad tecnológica o comercial: son aquéllos que se multiplican en la leche y producen la degradación de sus componentes proteicos, glucídicos y/o lipídicos devaluando el valor comercial de la leche o los productos lácteos elaborados a partir de ella. En este grupo se encuentran bacterias psicrotrofas productoras de enzimas degradantes o las esporas butíricas.

- Microorganismos indicadores y microorganismos patógenos, relacionados con la calidad higiénico-sanitaria: son, respectivamente, aquéllos agentes cuyo recuento

permite evaluar las condiciones del ambiente y las instalaciones y sirven también de alerta -si están presentes en altas concentraciones- de la posible presencia de bacterias patógenas y/o alterantes y aquellos causantes de enfermedad en el hombre o los animales. Dentro de los microorganismos indicadores encontramos la microbiota aerobia mesófila, las bacterias psicrotrofas y termodúricas, las bacterias coliformes y *Escherichia coli*. Como patógenos en leche de oveja podemos destacar *Staphylococcus aureus*, *Streptococcus agalactiae, Enterococcus faecalis, Mycoplasma agalactia*e o *Listeria monocytogenes*, entre otros.

Todos estos organismos y grupos bacterianos proceden de la ubre (*Str. agalactiae*, *Myc. agalactiae*, *S. aureus*) y de fuentes de contaminación ambiental, tales como las heces (enterococos, coliformes y *E. coli*), la paja (esporulados, bacilos y clostridios) y el equipo e instalaciones de ordeño (psicrotrofos y termodúricos principalmente). Su estudio reviste especial interés de cara al diseño de estrategias de mejora del manejo del rebaño y del ordeño y de la propia calidad de la leche. Sin embargo, la mayoría de ellos no han sido cuantificados ni tipificados en la leche de tanque del ganado ovino, así como tampoco ha sido estudiada su relación con las variables tradicionales de calidad de la leche como el recuento celular y el recuento bacteriológico total.

Adicionalmente, merece particular atención el estudio y caracterización de dos tipos de microorganismos que tienen una gran significación en el ganado ovino. Uno de ello es *Myc. agalactiae*, como agente responsable de la agalaxia contagiosa, en una zona enzoótica de este síndrome como es Castilla y León, y del que los estudios existentes revelan una gran variabilidad genética y mutacional en determinados puntos de su cromosoma que constituyen mecanismos de evasión de la respuesta inmune del hospedador y hacen que la infección persista en los rebaños largo tiempo (Tola *et al.*, 1997; Flitman-Tene *et al.*, 2000). El segundo, viene representado por los cocos Gram positivos catalasa negativos, algunos de los cuales han sido descritos como patógenos nosocomiales para el hombre (Ruoff, 2002), constituyendo los rebaños animales verdaderos reservorios de los mismos (Bates *et al.*, 1994) y sobre los que no existe ninguna información en el ovino lechero.

Más específicamente, en el caso del *Myc. agalactiae*, el estudio de determinados perfiles moleculares de variabilidad genética, tales como los análisis VNTR (Variable Number Tandem Repeat) y MLST (MultiLocus Sequence Typing), así como de su variabilidad antigénica y de su sensibilidad antibiótica, podrían permitir una interesante aproximación a los mecanismos de variabilidad poblacional y susceptibilidad antibiótica que permitan diseñar estrategias más efectivas de control de la agalaxia contagiosa. En el caso de los cocos Gram positivos catalasa negativos interesa conocer sus recuentos en la leche de tanque, su relación con otros grupos bacterianos y su caracterización molecular y mecanismos de resistencia antibiótica, dado el carácter de patógenos potenciales para el hombre que presentan la mayoría de las especies de este grupo bacteriano.

No obstante todo lo anterior, los criterios microbiológicos vigentes para leche de pequeños rumiantes recogidos en el Reglamento (CE) 853/2004 sólo se refieren al recuento total de microbiota mesófila, cuyos límites establece en 500.000 ufc/mL si la leche no va a ser sometida a tratamiento térmico y de 1.500.000 ufc/mL si esta va a ser tratada

térmicamente antes de su uso. Además, en leche de pequeños rumiantes, dicho Reglamento no recoge ningún límite de recuento de células somáticas, reflejo de la sanidad mamaria del rebaño. Recientemente, el Real Decreto 752/2011 (*Normativa básica de control que deben cumplir los agentes del sector de leche cruda de oveja y cabra*) y cuyo fin es equiparar el sistema de control y la calidad implementados en ganado vacuno al de los pequeños rumiantes, tradicionalmente más deficiente, indica que únicamente se debe comunicar la media geométrica móvil mensual del recuento de células somáticas sin que se proponga límite alguno para esta variable. En este Real Decreto también se incluye el uso del azidiol, como conservante, y sus condiciones de uso en muestras de leche de oveja y cabra. El problema del uso del azidiol y del periodo útil de almacenamiento para el análisis de los recuentos microbiológicos de la leche plantea un problema metodológico de interés en los sistemas de pago por calidad y también en el diseño de protocolos de control e investigación analítica. Existen estudios que evidencian efectos significativos del azidiol y del tipo de almacenamiento de la leche sobre el recuento de células somáticas en la leche de oveja (Gonzalo *et al.*, 2004) y cabra (Sánchez *et al.*, 2005), por lo que el desarrollo de investigaciones relativas al efecto de las condiciones de preservación y almacenamiento sobre las variables microbiológicas resulta aconsejable con el fin de establecer metodologías y criterios válidos de uso en la leche de oveja.

Por consiguiente, se plantean las siguientes necesidades:

1. Delimitar los criterios metodológicos de muestreo, preservación y almacenamiento de la leche ovina de tanque para el análisis de los diferentes grupos bacterianos presentes en la misma,
2. Definir la relación de tales grupos bacterianos entre sí y con otras variables indicadoras de las condiciones de producción en los rebaños de ganado ovino lechero, así como estudiar la influencia de sus factores sistemáticos de variación y,
3. Caracterizar, finalmente, algunas de las especies más prevalentes relacionadas con la sanidad humana y animal.

Sobre la base del interés de las necesidades enunciadas, se establecieron los siguientes objetivos:

1. Estandarización de las condiciones de preservación y almacenamiento de la leche de silo procedente de rebaños ovinos a partir de un sistema de alícuotas que permita definir las condiciones analíticas óptimas de las variables bacteriológicas estudiadas.
2. Estudio de la relación entre los grupos bacterianos específicos de la leche de tanque y otras variables de calidad higiénico-sanitarias básicas, como el recuento bacteriológico total y el recuento celular, así como de aquellos factores de variación ambientales (por ejemplo, la estación anual) o de manejo (por ejemplo, el ordeño o la antibioterapia de secado) de interés en los rebaños de ganado ovino lechero.
3. Creación de una colección de especies patógenas presentes en leche de oveja de interés en medicina humana (estreptococos, enterococos, estafilococos), sanidad animal (*Myc agalactiae*, *Str. agalactiae*, *S. aureus*) y microbiología de los alimentos para su caracterización de cara a la obtención de información de interés relativa a su

perfil de virulencia, perfil molecular, perfil de resistencia a antimicrobianos y seguimiento epidemiológico.

Más específicamente:

3.1. Aislamiento y caracterización de cocos Gram positivos catalasa negativos en leche de tanque.
3.2. Aislamiento y caracterización de cepas de *Mycoplasma agalactiae* en leche de tanque en una zona endémica de agalaxia contagiosa (Castilla y León).

El estudio de estos objetivos permitirá mejorar el actual marco teórico e interpretativo de la calidad bacteriológica en origen de la leche de oveja, con el fin no sólo de poder optimizar la monitorización de los procesos de mejora de la calidad y seguridad de la leche de oveja, sino también de implementar nuevas herramientas de utilidad en la toma de decisiones conducentes a dicha mejora.

Todos estos objetivos se han concretado en la realización de los siguientes trabajos científicos que incluimos en la presente Memoria de Tesis Doctoral:

1. Influence of storage and preservation on microbiological quality of silo ovine milk.
   de Garnica ML, Santos JA, Gonzalo C. J Dairy Sci. 2011 Apr; 94(4):1922-7. doi: 10.3168/jds.2010-3787.

2. Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk.
   de Garnica ML, Linage B, Carriedo JA, De La Fuente LF, García-Jimeno MC, Santos JA, Gonzalo C. J Dairy Sci. 2013 Feb; 96(2):1021-9. doi: 10.3168/jds.2012-5915. Epub 2012 Nov 29.

3. Presence of the *vanC1* gene in a vancomycin-resistant *Enterococcus faecalis* strain isolated from ewe bulk tank milk.
   de Garnica ML, Valdezate S, Gonzalo C, Saez-Nieto JA.
   J Med Microbiol. 2013 Mar; 62(Pt 3):494-5. doi: 10.1099/jmm.0.052274-0. Epub 2012 Nov 15.

4. Diversity of Gram-positive catalase-negative cocci in sheep bulk tank milk by comparative 16S rDNA sequence analysis.
   de Garnica ML, Saez-Nieto JA, Gonzalez R, Santos JA, Gonzalo C.
   En fase de publicación.

5. Isolation, molecular characterization and antimicrobial susceptibilities of isolates of Mycoplasma agalactiae from bulk tank milk in an endemic area of Spain.
   de Garnica ML, Rosales RS, Gonzalo C, Santos JA, Nicholas RA. J Appl Microbiol. 2013 Feb 27. doi: 10.1111/jam.12176.

6. Multilocus sequence typing of *Mycoplasma agalactiae*.

McAuliffe L, Gosney F, Hlusek M, **de Garnica** ML, Spergser J, Kargl M, Rosengarten R, Ayling RD, Nicholas RA, Ellis RJ. J Med Microbiol. 2011 Jun;60(Pt 6):803-11. doi: 10.1099/jmm.0.028159-0. Epub 2011 Mar 3.

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Koop, G., M. Nielen, and T. Van Werven. 2009. Bulk milk somatic cell counts are related to bulk milk total bacterial counts and several herd-level risk factors in dairy goats. J Dairy Sci. 92:4355-4364.

Pirisi, A., A. Lauret, and J.P. Dubeuf. 2007. Basic and incentive payments for goat and sheep milk in relation to quality. Small Ruminant Research, 68: 167-178.

Robinson, R. K. 2005. Dairy Microbiology Handbook: The Microbiology of Milk and Milk Products. Wiley-Interscience, NJ, USA.

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Sánchez, A., D. Sierra, C. Luengo, J.C. Corrales, C.T. Morales, A. Contreras, and C. Gonzalo. 2005. Influence of storage and preservation on Fossomatic cell count and composition of goat milk. J. Dairy Sci., 88: 3095-3100.

Tola, S., Manunta, D., Cocco, M., Turrini, F., Rocchigiani, A. M., Idini, G., *et al.* 1997. Characterization of membrane surface proteins of *Mycoplasma agalactiae* during natural infection.FEMS Microbiol Lett, 154: 355-362.

INTRODUCTION AND OBJECTIVES

The Doctoral Thesis entitled " **BACTERIOLOGICAL STUDY OF TANK MILK IN SHEEP FLOCKS AND CHARACTERIZATION OF STRAINS OF GRAM POSITIVE CATALASE NEGATIVE COCCI AND *Mycoplasma agalactiae*.**" is defended under the model "compendium of publications" to apply for an "International Doctorate" according to the *Official Regulation of the Doctoral Degree of the University of León* (BOCYL no. 197, 11 November 2012, pages 61447-61471). The publications presented are of proven relevance in the categories of *Microbiology* and *Agricultural, Dairy and Animal Science* as indexed in the Journal Citation Reports (2011).

Castilla y León is the first ewe-milk-producing region in Spain and approximately constitutes the 73% of the total national production (MAGRAMA, 2011). Spain produces 550,000 tons of raw sheep's milk in farm per year versus a total of 6,522,000 tons of cow´s milk (FAOSTAT, 2011). Despite the minor production, its compositional, nutritional and technological properties make sheep milk the raw material for high added value dairy products, either based on pure sheep milk or as part of mixtures of several species milk. Thus, near 100% of sheep milk production in Spain is intended for cheese fabrication (MAGRAMA, 2011), many of its varieties recognized by the European Union quality systems (PDO, PGI or Organic Farming).

Safer and higher quality sheep milk demanded by authorities and consumers has been a challenge for producers, transportation companies, dairy industry, laboratories and veterinary practitioners. The producing units (farms) have been set as the starting point and the basis to obtain better quality milk.

Gonzalo (2013) classifies the quality of sheep milk into four types: hygienic quality, technological quality, nutritional quality and ethical quality. Our interest in the research lines part of this thesis report has been mainly focused on the first one, the hygienic quality at the source, as the basis for the rest. The sampling unit was the ovine bulk tank milk, although in some cases, silo milk (or large mixtures) was used as well for the study of some microorganisms or bacterial groups not present in every flocks.

Knowledge of hygienic quality , particularly the bacteriological quality of tank milk and its variation factors, is important at different levels of the food chain:

- First, at the producer / farmer level; because the quality of tank milk reflects the quality of herd management. Total bacteriology and total somatic cell counts are parameters frequently used by technicians to determine the status of mammary health and hygiene in herds. A significant relationship between both counts has been documented (Gonzalo *et al.* 2010; Koop *et al.*, 2009). In this sense, the hygienic quality of tank milk is a useful monitoring tool of mammary hygiene and herd health, and a basis for prevention and control measures implementation at farm level. From the economic point of view the latter monitoring program can also increase farmers´ incomes, who are also eligible for bonuses or incentives included

in quality payment schemes (Pirisi *et al.*, 2007). Additionally, the presence of specific pathogens in the farm may determine the safety of the rest of the food chain.

- Second, at the dairy industry level; because technological and hygienic milk quality improvement prolongs product life and provides a better quality and safety, and

- Third, at the consumer level, who will have lower health risk, most durable and best technological featured products that will increase their level of satisfaction.

The studies that take part of this report have mainly focused on the first and more heterogeneous link of the food chain, the dairy sheep herd due to, from our point of view, the quality at the primary source is going to determine the quality of the rest of the chain and of the final product.

Taking into account variables that define milk quality, total bacterial count (viable mesophilic flora) is a very unspecific parameter which provides no information on other variables of microbiological interest also present in milk. Further studies and quantification of different bacterial groups in sheep tank milk is of great interest. Regarding quality and according to Robinson (2005) we can classify microorganisms present in milk stored in cooling tanks and silos as:

- Spoilage microorganisms, associated with technological or commercial quality: those that multiply in milk and cause degradation of proteins, carbohydrates and / or lipids decreasing the commercial value of milk or dairy products. In this group we can find psychrotrophic bacteria producers of degrading enzymes or butyric spores.
- Indicators and pathogenic microorganisms, related to the hygienic quality: respectively, those whose count is useful to evaluate environmental conditions and, if present in high concentrations, alert us to the possible presence of pathogenic bacteria and / or spoilers; and those disease-causing agents for man and/or animals. As indicators; aerobic mesophilic flora, thermodurics, psychrotrophic bacteria, coliforms and *Escherichichia coli*. Some important pathogens in sheep milk are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Mycoplasma agalactiae* or *Listeria monocytogenes* among others.

These organisms and bacterial groups come from the udder (*Str. agalactiae*, *M. agalactiae*, *S. aureus*) and from environmental sources; such as faeces (enterococci, coliforms and *E. coli*), straw (bacilli and clostridia) and milking equipment and facilities (mainly psychrotrophic and thermoduric bacteria). Consequently their study is of particular interest to the design of improved herd management strategies and to the quality of the milk itself. However, most of them have been neither quantified and characterized in bulk tank sheep milk nor related with other conventional variables such as total cell count or total bacterial count.

Additionally, special attention must be paid to the study and characterization of two types of microorganisms of great significance in sheep. On one hand, *Myc. agalactiae* as the causative agent of contagious agalactia in Castilla y León, an enzootic area, whose previous studies showed high genetic and mutational variability at certain points of its chromosome used to evade host immune response and be able to infect flocks for long time (Tola *et al.*, 1997; Flitman-Tene *et al.*, 2000). On the other hand, there is little information available about the Gram Positive Catalase Negative Cocci (GPCNC) group in dairy sheep, which includes several nosocomial human pathogens (Ruoff, 2002) and for which animal herds have been described as a reservoir (Bates *et al.*, 1994)

More specifically, in the case of *Myc. agalactiae*, the molecular characterization based on VNTR (Variable Number Tandem Repeats) and MLST (Multilocus Sequence Typing) analysis, and the study of its antigenic variability and its antibiotic sensitivity could provide an interesting approach to population variability mechanisms and antibiotic susceptibility in order to design more effective control strategies for contagious agalactia infections in affected areas. In the case of GPCNC, more accurate species identification, study of the relationship between their counts in bulk tank milk with other bacterial groups and the phenotypic and molecular characterization of antibiotic resistances and virulence mechanisms are of remarkable interest taking into account the potential pathogenicity for humans of most species of this bacterial group.

Despite the information available, the microbiological criteria applicable to small ruminants´ milk in current Regulation (EC) 853/2004 refer only to mesophilic flora total count, with limits set by 500,000 cfu / mL if the milk is not going to be heat treated and 1,500,000 cfu / mL if it is to be heat treated before use. Furthermore, contrary to cow's milk, there is no somatic cell count limit for small ruminants, measure that reflects mammary herd health.

Recently, the Spanish regulation 752/2011 (basic control legislation for the sector of raw sheep and goat´s milk), tries to perform quality control implemented in cattle in small ruminants as well, traditionally more deficient. However, it only indicates the obligation to monthly report the mobile geometric mean of somatic cell count without setting a limit proposal in it. This law also includes the use of azidiol as a preservative in sheep´s and goat´s milk samples. The effect of this preservative use on bacterial and somatic cell counts and the adequate storage period for sheep milk samples analysis have not been previously studied, as in the case of other preservation systems such as freezing. The use of azidiol brings up a methodological concern related to quality payment schemes determinations and analytical research. In this sense, some studies have demonstrated a significant effect of azidiol and different storage systems on sheep (Gonzalo *et al.*, 2004) and goat milk (Sanchez *et al.*, 2005) somatic cell counts. As a result, a study defining the optimal storage and preservation conditions for sheep milk microbiological variables in order to establish valid sampling criteria and methodologies is advisable.

Consequently the following needs came out:

1. Delimiting methodological criteria for sampling, preservation and storage of sheep tank milk for the analytical determination of different bacterial groups,
2. Defining relations among present bacterial groups and other bulk tank milk variables, as well as studying the systematic factors that influence their variation in milk and,
3. Characterizing the most prevalent bacteria related to human and/or animal health.

We set the following objectives based on the above needs:

1. Standardization of preservation and storage conditions of ewe silo milk using a system of aliquots that enables definition of optimal analytical conditions of the variables studied.
2. Study of the relationship among specific bacterial groups in sheep tank milk and some other sanitary and hygienic variables, such as total bacteriological and somatic cell counts, and the effect of environmental (i.e. season) or management (i.e. milking type or antimicrobial dry therapy) variation factors of interest in sheep flocks.
3. The creation of a collection of pathogenic species present in sheep milk of interest in human (streptococci, enterococci, staphylococci) and animal medicine (*Myc agalactiae*, *Str. agalactiae*, *S. aureus*), and food microbiology, for their characterization on virulence and antimicrobial resistance, molecular profiling, and epidemiological monitoring. Specifically:
    - 3.1. Isolation and characterization of catalase-negative gram-positive cocci in sheep bulk tank milk.
    - 3.2. Isolation and characterization of strains of *Mycoplasma agalactiae* isolated in bulk tank milk in a contagious agalactia endemic area (Castilla y León).

The study of these objectives will extend the current theoretical and interpretive framework of ewe's milk bacteriological quality in origin. Not only it optimizes monitoring systems for sheep milk quality and safety improvement; but also implements new useful tools for decision making.

The mentioned objectives have resulted in the publication of the following peer-reviewed works included in this report:

1. Influence of storage and preservation on microbiological quality of silo ovine milk.
   de Garnica ML, Santos JA, Gonzalo C. J Dairy Sci. 2011 Apr; 94(4):1922-7. doi: 10.3168/jds.2010-3787.

2. Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk.
   de Garnica ML, Linage B, Carriedo JA, De La Fuente LF, García-Jimeno MC, Santos JA, Gonzalo C. J Dairy Sci. 2013 Feb;96(2):1021-9. doi: 10.3168/jds.2012-5915. Epub 2012 Nov 29.

3. Presence of the *vanC1* gene in a vancomycin-resistant *Enterococcus faecalis* strain isolated from ewe bulk tank milk.
   de Garnica ML, Valdezate S, Gonzalo C, Saez-Nieto JA.
   J Med Microbiol. 2013 Mar;62(Pt 3):494-5. doi: 10.1099/jmm.0.052274-0. Epub 2012 Nov 15.

4. Diversity of Gram-positive catalase-negative cocci in sheep bulk tank milk by comparative 16S rDNA sequence analysis.
   de Garnica ML, Saez-Nieto JA, Gonzalez R, Santos JA, Gonzalo C.
   Submitted for publication.

5. Isolation, molecular characterization and antimicrobial susceptibilities of isolates of Mycoplasma agalactiae from bulk tank milk in an endemic area of Spain.
   de Garnica ML, Rosales RS, Gonzalo C, Santos JA, Nicholas RA. J Appl Microbiol. 2013 Feb 27. doi: 10.1111/jam.12176.

6. Multilocus sequence typing of *Mycoplasma agalactiae*.
   McAuliffe L, Gosney F, Hlusek M, **de Garnica** ML, Spergser J, Kargl M, Rosengarten R, Ayling RD, Nicholas RA, Ellis RJ. J Med Microbiol. 2011 Jun;60(Pt 6):803-11. doi: 10.1099/jmm.0.028159-0. Epub 2011 Mar 3.

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Koop, G., M. Nielen, and T. Van Werven. 2009. Bulk milk somatic cell counts are related to bulk milk total bacterial counts and several herd-level risk factors in dairy goats. J Dairy Sci. 92:4355-4364.

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Capítulo 1

INFLUENCIA DEL ALMACENAMIENTO Y LA CONSERVACIÓN SOBRE LA CALIDAD MICROBIOLÓGICA DE LA LECHE DE OVEJA ALMACENADA EN SILO

INTRODUCCIÓN

La legislación europea en su Reglamento (CE) 853/2004 únicamente se refiere al recuento de flora aerobia mesófila como criterio de calidad higiénica de la leche, estableciendo como límites recuentos ≤500.000 ufc/mL en el caso de leche cruda, y recuentos ≤1.500.000 ufc/mL si dicha leche va a ser sometida a tratamiento térmico. Esta normativa no hace, sin embargo, referencia a otros estándares microbiológicos que podrían ser de gran interés en la determinación de la calidad y seguridad de la leche, así como de su vida útil y opciones de comercialización, tales como el recuento de bacterias termodúricas, psicrotrofas, coliformes, *Escherichia coli* o patógenos mamarios (por ejemplo, *Staphylococcus aureus* o *Streptococcus agalactiae*). En todos los casos, cobra especial relevancia, tanto para el ganadero como para la industria, el conocimiento de la influencia del almacenamiento y conservación sobre esta calidad microbiológica de la leche de oveja con el fin de estandarizar los protocolos de muestreo, garantizar la precisión de los resultados analíticos y optimizar las condiciones de almacenamiento y preservación. El azidiol (AZ) es un conservante ampliamente utilizado en muestras de leche que van a ser sometidas a diferentes controles físico-químicos y microbiológicos (Real Decreto 752/2011), pero su efecto sobre las poblaciones bacterianas en leche de oveja no ha sido todavía delimitado. De la misma manera, no se conoce el efecto de la congelación sobre la recuperación microbiana en muestras de leche de oveja. A pesar de que la legislación vigente no contempla los distintos grupos bacterianos ni especies de interés tecnológico o sanitario presentes en la leche, resulta necesario definir el comportamiento de estos grupos y especies frente a diferentes condiciones de almacenamiento de las muestras como son la refrigeración, la congelación y la adición de conservantes como el azidiol. Para ello la leche almacenada en silos de centros lácteos es especialmente apta para este tipo de estudios ya que procede de la mezcla de un gran número de rebaños, lo que hace posible la coexistencia de gran variedad de especies y grupos microbianos al mismo tiempo.

El siguiente estudio fue diseñado con el propósito de determinar los efectos de las condiciones de almacenamiento y conservación mencionadas anteriormente sobre los recuentos de mesófilos, termodúricos, psicrotrofos y coliformes y sobre las especies *Escherichia coli*, *Staphylococcus aureus* y *Streptococcus agalactiae* en leche de silo destinada a la fabricación de productos lácteos.

MATERIALES Y MÉTODOS

Durante el periodo comprendido entre los meses de Noviembre de 2009 y Febrero de 2010 se tomaron asépticamente según las recomendaciones de la APHA (American

Public Health Association) para el examen de muestras de leche (White *et al.*, 1992) un total de 10 muestras de leche de silo correspondientes a 10 rutas diferentes de recogida del Consorcio de Promoción del Ovino (CPO), Villalpando, Zamora, España. La leche se mantuvo a una temperatura ≤ 6 ºC en los tanques de refrigeración de las explotaciones durante un periodo de 48 horas postordeño previo a la recogida, manteniéndose las muestras de leche de silo (500 mL) a 4ºC hasta su análisis laboratorial realizado nada más llegar al laboratorio de análisis microbiológico en el Departamento de Higiene y Tecnología de los Alimentos de la Universidad de León. Todas las muestras fueron negativas a la presencia de antibióticos β-lactámicos y tetraciclinas tras su análisis en el centro de recogida del CPO por medio del test rápido de cribado Rosa Charm® (Charm Sciences, MA, USA). A partir de la muestra original homogeneizada se obtuvieron 13 submuestras de 40 mL destinadas a las diferentes determinaciones clasificadas de la siguiente manera en función de diferentes condiciones de almacenamiento y conservación:

- Refrigeración (R): 5 alícuotas sin conservante mantenidas a 4 ºC durante 2, 24, 48, 72 y 96 h respectivamente.
- Conservación con AZ y refrigeración (RAZ): 5 alícuotas con conservante (AZ) mantenidas a 4 ºC durante 2, 24, 48, 72 y 96 h respectivamente. La concentración final de AZ (Panreac, Barcelona, España) en cada muestra fue de 3.3 μL/mL (según Real Decreto 1728/2007).
- Congelación (C): 3 alícuotas sin conservante mantenidas a -20 ºC durante 7, 15 y 30 días respectivamente.

Para la determinación de los recuentos de los grupos bacterianos mesófilos, termodúricos, psicrotrofos y coliformes y de las especies *Escherichia coli*, *Staphylococcus aureus* y *Streptococcus agalactiae*, las muestra de leche fueron sometidas a diluciones seriadas en un rango de 10^{-1} a 10^{-5} y al análisis microbiológico específico para los distintos grupos y especies según la metodología descrita en la Tabla1. Todos los recuentos obtenidos se expresaron en ufc/mL.

La composición en grasa, proteína, sólidos totales y el recuento de células somáticas (RCS) de todas las muestras de leche de silo se determinaron con el equipo COMBIFOSS 6000 FC (Foss Electric, Hillerϕd, Dinamarca) sometido a controles de calidad intercomparativos. Los valores medios ± DS para la grasa, proteína, sólidos totales y RCS fueron: 6.92 ± 0.49%, 5.41 ± 0.19%, 18.03 ± 0.60% and 998 ± 51 x 10^3 células/mL.

El análisis estadístico de los recuentos se llevó a cabo mediante el procedimiento GLM del SAS (SAS Institute, 1998), de acuerdo con el siguiente modelo matemático: $Y_{ijk} = M_i + S_j + A_{k:j} + e_{ijk}$ donde, Y_{ijk} fue la variable dependiente (log ufc/mL) de los grupos y especies bacterianos estudiados (mesófilos, termodúricos, psicrotrofos, coliformes, *E. coli*, *S. aureus* y *Str. agalactiae*), *Mi* fue el efecto del silo (10 niveles), S_j fue el efecto almacenamiento (3 niveles; R, RAZ y C), $A_{k:j}$ fue el efecto de la edad de la muestra dentro del tipo de almacenamiento (2, 24, 48, 72 y 96 h post-recogida para las muestras refrigeradas, conservadas y no-conservadas, y 7, 15 y 30 días para las muestras congeladas) y e_{ijk} fue el error residual.

Tabla 1. Resumen metodología análisis microbiológico de las distintas especies y grupos microbianos.

GRUPO /ESPECIE	MEDIO DE CULTIVO	TEMPERATURA DE INCUBACIÓN (ºC)	TIEMPO DE INCUBACIÓN (h)	PRUEBAS COMPLEMENTARIAS	INTERPRETACIÓN Y EXPRESIÓN DE RESULTADOS SEGÚN
Mesófilos	PCA[1, a]	30±1	24-48	No	APHA
Termodúricos	PCA[1]	30±1	24-48	No	APHA
Psicrotrofos	PCA[1]	7±0.5	7-10 días	No	APHA
Coliformes / *E. coli*	Petrifilm® [2] Coliforms/ *E. coli*	37±0.5	24-48	No	Instrucciones del fabricante.
S. aureus	BP-RPF [3, c]	37±0.5	24-48	No	Instrucciones del fabricante.
Str. agalactiae	EMM [b] con sangre desfibrinada de oveja (5-7%)[1]	37±0.5	24-48	CAMP test, catalasa, oxidasa y PCR (Martinez *et al.*, 2001)	Instrucciones del fabricante.

1. Oxoid Limited, Cambridge, UK. [a]:Plate Count Agar, [b]: Edwards Medium Modified.
2. 3M, MN, USA. Según instrucciones del fabricante.
3. Becton and Dickinson, NJ, USA. Según norma UNE-EN ISO 6888-2:1999. [c]: Baird-Parker-Rabbit Plasma Fibrinogen.

Un segundo modelo estadístico fue utilizado únicamente para las muestras refrigeradas a 4ºC (R), siguiendo el mismo procedimiento GLM del SAS con el fin de estudiar el efecto de la interacción conservación x edad de la muestra, como un factor del modelo estadístico. Este modelo fue: $Y_{ijk} = M_i + P_j + A_k + e_{ijk}$ donde, Y_{ijk} fue la variable dependiente (log ufc/mL), M_i fue el efecto silo (10 niveles), P_j fue el efecto conservación (2 niveles; R y RAZ), A_k fue el efecto de la edad de la muestra (2, 24, 48, 72 y 96 h post-recogida), PAjk fue el efecto de la interacción conservación x edad de la muestras, y e_{ijk} fue el error residual.

Las bacterias psicrotrofas fueron el grupo más numeroso (6.40 log ufc/mL), seguidas por la flora mesófila (5.36 log ufc/mL), los coliformes (3.45 log ufc/mL) y las bacterias termodúricas (2.41 log ufc/mL). Estos recuentos fueron consistentes con los referidos en leche de oveja en algún estudio previo (Sanjuan *et al.*, 2003), aunque notablemente superiores a los encontrados en leche de vaca (Jayarao *et al.*, 2004). Las menores producciones lecheras individuales de las ovejas y las inferiores condiciones higiénicas de manejo y ordeño existentes en los rebaños de pequeños rumiantes comparados con las granjas de ganado vacuno de leche podrían explicar estas diferencias entre especies. No obstante, la media geométrica de la flora mesófila (229 x 10^3 ufc/mL) se encuentra por debajo del límite inferior señalado por el Reglamento (CE) 853/2004 (≤ 500 x10^3 ufc/mL).

RESULTADOS Y DISCUSIÓN

Los resultados del análisis estadístico de las variables microbiológicas se muestran en la Tabla 2.

Tabla 2. Estadística descriptiva de las variables estudiadas en leche de silo.

Microorganismos	n	Media[1]	MG[2]	SD	Mínimo	Máximo	CV,%
Mesófilos	130	5.36	229	0.73	4.00	7.25	13.59
Termodúricos	130	2.41	0.25	0.59	1.00	4.02	24.82
Psicrotrofos	130	6.40	2511	0.91	4.51	8.08	14.34
Coliformes	130	3.45	2.81	1.26	1.30	6.64	36.67
E. coli	130	2.38	0.24	1.05	0.70	4.66	44.10
Str. agalactiae	130	3.67	4.67	0.55	2.48	5.18	15.03
S. aureus	130	2.63	0.42	0.67	0.70	3.90	25.67

[1]Logaritmo de ufc/mL.
[2]MG: Media geométrica x 10^3 ufc/mL.

En relación con las especies concretas, el recuento de *E. coli* (2.38 log ufc/mL) supuso el 8.5 % del total de coliformes, porcentaje similar al 7.4% encontrado en leche ovina de silo por Consentino y Palmas (1997), pero superior al 1.5% obtenido por Sanjuan *et al.*, (2003) en leche ovina de tanque. Los recuentos de patógenos causantes de mamitis contagiosa, *S. aureus* (2.63 log ufc/mL) y *Str. agalactiae* (3.67 log ufc/mL) fueron elevados e indicativos de infecciones mamarias y altos RCS en los rebaños ovinos, lo cual sugiere la necesidad de incrementar los programas de control de mamitis con el fin de reducir la prevalencia de mamitis contagiosas en los rebaños (Linage y Gonzalo, 2008; Gonzalo *et al.*, 2010).

Los efectos del silo ($P < 0.001$) y las condiciones de almacenamiento ($P < 0.01$ a $P < 0.001$) contribuyeron significativamente a las variaciones de los recuentos de todas las variables estudiadas. El efecto de la edad de la muestra de leche dentro del almacenamiento fue también significativo ($P < 0.001$) para las bacterias mesófilas, psicrotrofas, y coliformes. El efecto silo y las condiciones de almacenamiento influyeron significativamente en la variación de todas las variables estudiadas, mientras que el tiempo de almacenamiento sólo resultó significativo en el caso de los mesófilos, los psicrotrofos y los coliformes, pero no para el resto de grupos o especies bacterianos ($P > 0.05$).

El efecto de las 3 condiciones de almacenamiento estudiadas (R, RAZ y C) sobre la concentración de todas las variables se muestran en la Tabla 3.

Tabla 3. Medias de mínimos cuadrados (log ufc/mL ± ES) y significación estadística de las variables microbiológicas de la leche ovina de silo influenciada por el almacenamiento. ES: error estándar.

Grupos/Especies	Leche cruda refrigerada (R)	Leche refrigerada con conservante (RAZ)	Leche congelada a –20ºC (C)	*F*
Mesófilos	5.91 ± 0.05[a]	5.04 ± 0.05[b]	4.97 ± 0.07[b]	86.54***
Termodúricos	2.61 ± 0.06[a]	2.36 ± 0.06[b]	2.18 ± 0.08[b]	9.03***
Psicrotrofos	7.04 ± 0.08[a]	6.07 ± 0.08[b]	5.89 ± 0.10[b]	53.32***
Coliformes	4.58 ± 0.10[a]	2.90 ± 0.10[b]	2.49 ± 0.12[c]	109.13***
E. coli	2.67 ± 0.07[a]	2.62 ± 0.07[a]	1.51 ± 0.09[b]	57.51***
Str. agalactiae	3.84 ± 0.06[a]	3.73 ± 0.06[a]	3.31 ± 0.07[b]	17.37***
S. aureus	2.75 ± 0.06[a]	2.68 ± 0.06[a]	2.36 ± 0.08[b]	6.98**

***$P < 0.001$. **$P < 0.01$.

[a,b,c] Las medias dentro de una misma fila con diferente superíndice difieren ($P < 0.05$).

La leche R mostró recuentos más altos que la leche RAZ en todos los grupos microbianos ($P < 0.05$), no así en las especies concretas *E. coli*, *Str. agalactiae* y *S. aureus*, cuya concentración no difirió en condiciones de refrigeración La leche C obtuvo recuentos menores ($P < 0.05$) en los grupos y especies microbianos que la leche R, aunque mesófilos, termodúricos y psicrotrofos mostraron recuentos similares ($P > 0.05$) a los de la leche RAZ. Tras la descongelación, *E. coli*, *Str. agalactiae* y *S. aureus* mostraron una concentración significativamente menor que en las condiciones R y RAZ (Taba 3). El efecto de la congelación sobre el recuento de patógenos mamarios ha sido previamente estudiada con distinto resultado. Algunos autores postulan que el número de aislados aumenta debido a la ruptura de agregados bacterianos, macrófagos y neutrófilos derivada de la congelación, la cual liberaría los patógenos fagocitados (Villanueva *et al.*, 1991; Godden *et al.* 2002); mientras que otros autores (Schukken *et al.*, 1989) no encuentran un efecto significativo sobre los recuentos de estreptococos o *S. aureus* tras 16 semanas de congelación. No existen datos disponibles sobre el efecto de la congelación y el tiempo de almacenamiento en muestras procedentes de silos, sin embargo basándonos en los resultados del presente estudio existe una clara disminución del recuento de patógenos mamarios en leche de silo conservada a -20 ºC, posiblemente vinculada a una pérdida de viabilidad de tales organismos.

Tabla 4. Medias de mínimos cuadrados (log ufc/mL) y significación estadística de la leche ovina de silo almacenada a 4ºC influenciada por la conservación y la edad de almacenamiento. ES=error estándar.

Grupos/Especies	**R**					**RAZ**					***F***
	2h	24h	48h	72h	96h	2h	24h	48h	72h	96h	
Mesófilos[1]	5.13[a]	5.47[b]	5.86[c]	6.33[d]	6.78[e]	5.03[a]	4.97[a]	5.08[a]	4.93[a]	5.18[a]	14.74***
Termodúricos[2]	2.47	2.34	2.56	2.72	2.97	2.34	2.29	2.45	2.31	2.40	1.34[NS]
Psicrotrofos[3]	6.03[a]	7.02[b]	7.06[b]	7.35[b]	7.77[c]	6.05[a]	5.99[a]	6.02[a]	6.09[a]	6.19[a]	6.77***
Coliformes[4]	3.44[a]	4.17[b]	4.72[b]	5.17[c]	5.39[d]	3.15[a]	2.91[a]	2.90[a]	2.59[e]	2.95[a]	9.22***
E. coli[5]	2.79	2.43	2.62	2.69	2.80	2.69	2.62	2.62	2.63	2.53	1.04[NS]
Str. agalactiae[6]	3.64	3.64	3.91	3.86	4.13	3.77	3.64	3.74	3.73	3.76	1.03[NS]
S. aureus[7]	2.69	2.62	2.92	2.72	2.80	2.64	2.95	2.81	2.58	2.39	2.25[NS]

[a-,f,] Las medias dentro de una misma fila difieren ($P < 0.05$).
NS: No significativo ($P > 0.05$). ***$P < 0.001$. R: refrigeración. RAZ: refrigeración+azidiol.
[1]ES = 0.11. [2]ES = 0.14. [3]ES = 0.16. [4]ES = 0.22. [5]ES = 0.12. [6]ES = 0.13. [7]ES=0.13.

El estudio del efecto de la interacción de la conservación x la edad de la leche en las muestras refrigeradas (Tabla 4) reveló diferencias estadísticas solamente para las bacterias mesófilas, psicrotrofas y coliformes, cuyos recuentos se elevaron significativamente a lo largo del tiempo en la leche no conservada. Los recuentos más elevados fueron para la flora psicrotrofa, siendo ya las bacterias predominantes en el momento del muestreo. Estos resultados fueron consistentes con los obtenidos por Sanjuan *et al.* (2003), quienes evidenciaron un significativo incremento de la concentración de *Pseudomonas* spp. desde el momento del ordeño (4.07 log ufc/mL) hasta 96 h postordeño (7.21 log ufc/mL) en leche ovina de tanque refrigerada a 6ºC. Por tanto, la flora psicrotrofa de la leche llega a hacerse predominante unas pocas horas después del ordeño. Se trata de un relevante hallazgo teniendo probada su relación con una alta incidencia de acciones degradantes (por ejemplo, proteólisis y lipolisis vinculadas a enzimas exógenas de *Pseudomonas* spp.) sobre los componentes de la leche y del queso, lo cual aconseja una recogida y procesado de la leche en el menor periodo de tiempo posible con el fin de prevenir una pérdida significativa de la calidad tecnológica de la misma.

La leche RAZ mantuvo la misma concentración inicial de la leche R a lo largo del tiempo (Tabla 4), por lo que este procedimiento de conservación y almacenamiento podría ser usado por los laboratorios lecheros o por los esquemas de pago por calidad para determinar la concentración inicial de los recuentos bacterianos en leche de silo y de

tanque, particularmente en el caso de las bacterias mesófilas, psicrotrofas y coliformes. Estos resultados son relevantes dada la compatibilidad del AZ con otros análisis como el RCS o el análisis de componentes lácteos (grasa, proteína, etc…), por lo que una única muestra de leche conservada con AZ podría ser suficiente para la determinación de la composición físico-química, RCS y recuentos bacterianos (mesófilos, psicrotrofos y coliformes) en la leche ovina de silo o tanque.

Con relación a la congelación, el efecto de la edad de la muestra fue significativa sobre los recuentos de los grupos estudiados, excepto para los coliformes y *E. coli* cuyos recuentos disminuyeron ($P < 0.05$) desde los días 7 (2.84 y 1.94 log ufc/mL) a 30 (2.25 y 1.18 log ufc/mL) post-congelación, de forma compatible con los resultados encontrados en la leche de vaca (Pankey *et al.* 1987, Schukken *et al.* 1989).

CONCLUSIONES

El almacenamiento de la leche cruda sin conservante a 4 ºC muestra un incremento significativo de los recuentos bacterianos de mesófilos, psicrotrofos y coliformes a lo largo del tiempo, lo cual hace aconsejable el procesado en un plazo lo más corto posible para evitar alteraciones de la leche y sus productos. El resto de variables microbiológicas estudiadas (termodúricos, *E. coli*, *Str. agalactiae* y *S. aureus*) mantuvieron, sin embargo, recuentos invariables a lo largo del tiempo.

El azidiol ha demostrado ser un conservante adecuado para mantener los valores microbiológicos iniciales en todos los grupos y especies estudiados, y particularmente en el caso de mesófilos, psicrotrofos y coliformes a lo largo de 96h de almacenamiento en refrigeración.

La congelación afectó significativamente la viabilidad de coliformes, *E. coli*; *Str. agalactiae y S. aureus.*

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Short communication: Influence of storage and preservation on microbiological quality of silo ovine milk

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ABSTRACT

This study was designed to analyze the effects of the storage and preservation conditions on counts of mesophilic, thermoduric, psychotrophic, coliform, *Escherichia coli*, *Streptococcus agalactiae*, and *Staphylococcus aureus* organisms in silo ovine milk. A total of 910 analytical determinations were conducted from aliquots of 10 silo ovine milks. The conditions tested were unpreserved and azidiol-preserved milk stored at 4°C, and unpreserved milk stored at −20°C. Milk aged 2, 24, 48, 72, and 96 h post-collection for refrigerated aliquots, and 7, 15, and 30 d post-collection for frozen aliquots. The factors silo and storage conditions significantly contributed to variation of all microbiological variables, although milk age effect within storage was only significant for mesophilic, psychrotrophic, and coliform bacteria counts. In refrigerated raw milk, mesophile, psychrotroph, and coliform counts significantly increased over 96 h post-collection, whereas the other groups and bacteria species tested maintained their initial concentration. In all cases, azidiol preservation maintained the initial bacterial concentration in raw sheep milk under refrigeration throughout 96 h. Thus, azidiol was a suitable preservative for microbiological studies in sheep milk. Smallest counts were registered for frozen samples, particularly for coliforms, *E. coli*, *Strep. agalactiae* and *Staph. aureus*. Estimates of mesophilic, thermoduric and psychrotrophic organisms showed similar values on both azidiol-preserved and frozen milk samples. Coliforms and *E. coli* counts significantly decrease over time after freezing. Consequently, freezing at −20°C could also be appropriate for analysis of mesophilic, thermoduric, and psychrotrophic bacterial groups, but not for coliforms or mammary pathogens.

Key words: silo milk, bacterial culture, dairy sheep, milk microbiology

Short Communication

Silo and tank milk are both contaminated by bacteria from different sources, such as flora and pathogens present in beds, milking facilities, wash water, milking systems, udders, teats and teat canals, or mastitic milk. Some of these bacteria are pasteurization-resistant or are able to grow at low temperatures. These characteristics may hinder industrial dairy processing. Some of these species may also be pathogens for humans. Despite this, only aerobic mesophile count determination has been the target of various legal limits or quality payment schemes proposed by different countries. Thus, Regulation (EC) 853/2004 (European Commission, 2004) lays down mesophilic flora limit criterion for milk from other species than cows as ≤500,000 cfu/mL, when the final destination of milk does not include heat treatment; or 1,500,000 cfu/mL for heat-treated milk before processing. However, this policy makes no reference to other microbiological criteria, so no regulation exists on other bacterial standards of microbiological quality of sheep milk for many sheep milk-producing countries (e.g., Spain). In this context, other bacterial groups and species studied, such as thermoduric and psychrotrophic flora, coliforms, and *Escherichia coli* or mastitis-causing pathogens would be of great interest for ovine milk hygiene, safety, quality, and marketing. In all cases, knowledge of the influence of storage and preservation on sheep milk microbiological quality is important both to the farmer and the dairy industry to standardize sampling protocols, to ensure accuracy in test results and to optimize milk storage conditions.

Azidiol (**AZ**) is a widely used preservative to keep milk samples for several tests in dairy laboratories, although its effects on the viability of major bacterial groups and pathogens in sheep milk need to be well established when such samples are going to be used for microbiological purposes. In this sense, other preservatives (e.g., bronopol) significantly decreased the viability of milk bacterial species and groups (Shepherd et al., 1988; Amores et al., 2010) so AZ effect on sheep milk microbiology should be investigated. Similarly, milk freezing could be of remarkable interest in micro-

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biological studies on sheep milk and in mastitis-control plans due to its potential to decrease processing and collection costs and extend the analytical operation thereof. Thus, studies on goat milk (Sánchez et al., 2003; Amores et al., 2010) show that freezing may be used as a storage method for the study of some bacterial species or groups but not for others. Nonetheless, freezing effect on the viability of major bacterial groups and species present in sheep milk has not yet been studied.

The knowledge of preservation and storage effects on recovering bacterial groups and bacterial pathogens over time would allow the optimum analytical conditions for ovine milk to be defined. This knowledge is needed to implement adequate operational strategies and sampling protocols for practical analysis and research in this species. Silo milk is especially suitable for this purpose as it consists of large mixtures of milk from a large number of herds, which makes the study of different bacterial groups and pathogens possible at the same time. Additionally, such large mixes of milk are the raw material of all dairy products produced by the industry so its microbiological quality has a great economical, technological, hygienic, and marketing interest.

The aim of this study was to determine the effects of the most common storage and preservation conditions on the following microbiological quality variables: aerobic mesophilic, psychrotrophic, thermoduric, coliforms, *E. coli*, *Streptococcus agalactiae,* and *Staphylococcus aureus* bacteria.

Between November 2009 and February 2010, a total of 10 samples of silo sheep milk were collected at a milk plant, which includes milk from 10 different tank milk collection routes, from a total of 400 dairy sheep herds. According to standards recommended by the American Public Health Association (White et al., 1992), samples (500 mL) were aseptically collected in sterile containers from each of the silos immediately after milk tankers were unloaded. For this experiment, milk stored in each silo corresponded to a single milk tanker from a single collection route. Milk collection frequency in farms was always 48 h, during which the milk was kept at a temperature lower than 6°C in cooling tanks in the farms. Milk collection was carried out at the same time in each flock. Silo milk temperature was 4°C, maintaining that temperature until the bacteriological analysis, which was carried out immediately after arrival in the laboratory in the Department of Food Hygiene and Technology, University of León, Spain. Bulk tank milk of all flocks was periodically checked for antimicrobial detection by Eclipse-100ov screening test (ZEU-Immunotec, Zaragoza, Spain; Montero et al., 2005) in the Dairy Interprofessional Laboratory of Castilla-León region (Spain). In addition, before unloading in silos, tanker milks were always checked for β-lactams and tetracycline drugs by Rosa Charm rapid screening test (Charm Sciences, Inc., Lawrence, MA). Negative results were always obtained during the experiment.

The initial homogenized sample was divided into 13 aliquots of 40 mL each: 5 aliquots of unpreserved milk were kept refrigerated at 4°C, 5 aliquots of milk were preserved with AZ (Panreac Quimica S.A., Castellar del Vallès, Barcelona, Spain) and kept refrigerated at 4°C, and 3 aliquots of unpreserved milk were kept frozen at −20°C. Azidiol concentration in preserved samples was always 3.3 μL/mL (i.e., 133 μL/40 mL). Azidiol composition was 75 mg of chloramphenicol, 1 mL of ethanol, 1.8 g of sodium azide, 4.5 g of trisodium citrate·$5H_2O$, and 35 mg of bromophenol blue in 100 mL of distilled water. Bacteriological analysis of refrigerated aliquots was carried out at 2, 24, 48, 72, and 96 h post-collection. Frozen aliquots were defrosted at 4°C overnight and analyzed at 7, 15, and 30 d post-collection.

Total aerobic plate count determination was performed following the standards recommended by the American Public Health Association (APHA) for milk and dairy products (White et al., 1992). The total number of viable bacterial cells was determined by the SPC method. Milk samples were subjected to serial dilution in the 10^{-1} to 10^{-5} range and inoculated into plate count agar (PCA; Oxoid Limited, Cambridge, UK) petri plates. The inoculated plates were incubated at 30 ± 1°C for 48 h. Thermoduric count was carried out by the SPC method after laboratory pasteurization at 62.8 ± 0.5°C for 30 min following APHA recommendations (White et al., 1992). Psychrotrophic bacteria count was also performed by SPC, plates being incubated at 7°C between 7 and 10 d (White et al., 1992). The enumeration of coliforms and *E. coli* was carried out using 3M Petrifilm *E. coli*/coliform count plates (3M, St. Paul, Minnesota) according to the manufacturer instructions. In all cases plates were inoculated with 1 mL of milk sample dilution in the range of 10^{-1} and 10^{-3}. Plates were incubated at 37 ± 0.5°C for 24 to 48 h. Enumeration of each group consisted of considering as confirmed coliforms red and blue colonies with associated gas bubbles. Confirmed *E. coli* were considered as blue colonies with associated gas bubbles. Results were expressed as cfu/mL. Regulation UNE-EN ISO 6888–2:1999/ Amd 1:2003 (ISO, 2003) was used as the reference method for *Staph. aureus* enumeration. Finally, Edwards Medium Modified (Oxoid Limited, Cambridge, UK) supplied with 5 to 7% defibrinated sheep blood (Oxoid Limited) was used for *Strep. agalactiae* detection and enumeration (Zadoks et al., 2004). Incubation was at 35°C for 48 h. Hemolytic and nonhemolytic, esculin-negative blue colonies were suspected of being *Strep. agalactiae*. These colonies were tested for confirmation

Table 1. Descriptive statistics for microbiological variables studied in silo ovine milk

Organism	n	Mean[1]	GM[2]	SD	Minimum	Maximum	CV, %
Mesophiles	130	5.36	229	0.73	4.00	7.25	13.59
Thermoduric	130	2.41	0.25	0.59	1.00	4.02	24.82
Psychrotrophic	130	6.40	2,511	0.91	4.51	8.08	14.34
Coliforms	130	3.45	2.81	1.26	1.30	6.64	36.67
Escherichia coli	130	2.38	0.24	1.05	0.70	4.66	44.10
Streptococcus agalactiae	130	3.67	4.67	0.55	2.48	5.18	15.03
Staphylococcus aureus	130	2.63	0.42	0.67	0.70	3.90	25.67

[1]Logarithm of cfu/mL.
[2]Geometric mean $\times 10^3$ cfu/mL.

by the Christie, Atkins, and Munch-Petersen (CAMP) test, catalase and oxidase tests, and detection of *Strep. agalactiae* by PCR (Martinez et al., 2001) to ensure the results.

The milk composition and SCC of each silo sample was determined with COMBIFOSS 6000 FC (Foss Electric, Hillerød, Denmark), subjected to quality controls and inter-comparative trials. Mean values ± SD for fat, total protein, and total solids content and SCC were: 6.92 ± 0.49%, 5.41 ± 0.19%, 18.03 ± 0.60%, and 998 ± 51 $\times 10^3$ cells/mL.

The statistical analysis was carried out by the GLM procedure of SAS (SAS Institute, 1998). The model used was: $Y_{ijk} = M_i + S_j + A_{k:j} + e_{ijk}$, where Y_{ijk} was the dependent variable logarithm of cfu/mL of bacterial groups and species studied (mesophilic, thermoduric, psychotropic, coliform, *E. coli*, *Strep. agalactiae*, and *Staph. aureus* bacteria); M_i was the silo effect (10 levels); S_j was the storage effect (3 levels: unpreserved milk stored at 4°C, AZ-preserved milk stored at 4°C, and unpreserved milk stored at −20°C); $A_{k:j}$ was the age effect within storage (2, 24, 48, 72, and 96 h post-collection for unpreserved and AZ-preserved refrigerated milk, and 7, 15, and 30 d for frozen milk); and e_{ijk} was the residual error. A second mathematical model was only used for samples stored at 4°C, following the same GLM procedure of SAS with the objective of studying the effect of preservation × age interaction, as a factor of the statistical model. This model was: $Y_{ijk} = M_i + P_j + A_k + PA_{jk} + e_{ijk}$, where M_i was the silo effect (10 levels); P_j was the preservation effect (2 levels: unpreserved and AZ-preserved milk); A_k was the milk age effect (2, 24, 48, 72, and 96 h post-collection); and PA_{jk} was the preservation × age interaction effect.

The statistics of the microbiological variables studied are shown in Table 1. Psychrotrophic bacteria were the most numerous bacterial group (6.40 log cfu/mL) above mesophilic flora (5.36 log cfu/mL), coliform (3.45 log cfu/mL), and thermoduric flora (2.41 log cfu/mL). In general, these counts were consistent with those reported by Sanjuan et al. (2003) in bulk tank milk from sheep flocks, but higher than those found in cow milk (Jayarao et al., 2004). Lower individual milk production and the absence of teat washing before milking, as well as poorer facilities than those for cattle, could explain the higher bacterial counts found in ewe milk compared with cow milk. Nevertheless, the geometric mean of mesophilic flora found in silo milk for the storage and preservation conditions studied (229 $\times 10^3$ cfu/mL) was below the lower limit of 500,000 cfu/mL established by the European Union regulations for bulk tank milk from sheep and goat flocks.

Regarding bacterial species studied, *E. coli* count (2.38 log cfu/mL) represented 8.5% of the total coliform estimate, a similar percentage to 7.4% found in sheep silo milk by Cosentino and Palmas (1997), but higher than 1.5% obtained by Sanjuan et al. (2003) in ewe bulk tank milk. Counts of contagious mastitis pathogens *Strep. agalactiae* (3.67 log cfu/mL) and *Staph. aureus* (2.63 log cfu/mL) were elevated and indicative of mammary infections and high SCC from dairy sheep flocks. These results suggest the need to increase mastitis control programs in herds to decrease the prevalence of contagious mastitis in dairy sheep (Linage and Gonzalo, 2008; Gonzalo et al., 2010).

Effects of silo (F between 4.44 and 34.21; $P < 0.001$) and storage conditions (F between 6.98 and 109.13; $P < 0.01$ to $P < 0.001$) contributed significantly to log cfu/mL variations for all variables involved in this study. The effect of milk age within storage was also significant for mesophilic ($F = 12.69$; $P < 0.001$), psychrotrophic ($F = 5.39$; $P < 0.001$), and coliforms ($F = 5.79$; $P < 0.001$) bacteria, but not for the rest of groups and bacterial species ($P > 0.05$).

The effect of storage on the concentration of all variables is shown in Table 2. Non-preserved refrigerated milk showed higher counts ($P < 0.05$) than did AZ-preserved refrigerated milk for all bacterial groups, apart from bacterial species *E. coli*, *Strep. agalactiae* and *Staph. aureus*, whose concentration did not differ. Counts after freezing were lower ($P < 0.05$) than those obtained for non-preserved refrigerated milk, although

Table 2. Least squares means (log cfu/mL ± SE) of microbiological variables of silo ovine milk affected by storage, and statistical significance

Organism	Unpreserved milk, 4°C	Azidiol-preserved milk, 4°C	Unpreserved milk, −20°C	*F*-value
Mesophiles	5.91 ± 0.05[a]	5.04 ± 0.05[b]	4.97 ± 0.07[b]	86.54***
Thermoduric	2.61 ± 0.06[a]	2.36 ± 0.06[b]	2.18 ± 0.08[b]	9.03***
Psychrotrophic	7.04 ± 0.08[a]	6.07 ± 0.08[b]	5.89 ± 0.10[b]	53.32***
Coliforms	4.58 ± 0.10[a]	2.90 ± 0.10[b]	2.49 ± 0.12[c]	109.13***
Escherichia coli	2.67 ± 0.07[a]	2.62 ± 0.07[a]	1.51 ± 0.09[b]	57.51***
Streptococcus agalactiae	3.84 ± 0.06[a]	3.73 ± 0.06[a]	3.31 ± 0.07[b]	17.37***
Staphylococcus aureus	2.75 ± 0.06[a]	2.68 ± 0.06[a]	2.36 ± 0.08[b]	6.98**

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

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

mesophilic, psychrotrophic, and thermoduric bacteria showed similar counts ($P > 0.05$) to those from AZ-preserved refrigerated milk. After thawing, the counts of coliforms, *E. coli*, *Strep. agalactiae*, and *Staph. aureus* (2.49, 1.51, 3.31, and 2.36 log cfu/mL, respectively) were significantly lower than those obtained for non-preserved refrigerated milk (4.58, 2.67, 3.84, and 2.75 log cfu/mL, respectively) or for AZ-preserved refrigerated milk (2.90, 2.62, 3.73, and 2.68 log cfu/mL, respectively). Several authors (Villanueva et al., 1991; Godden et al., 2002) have investigated the effect of freezing on bacteriological culturing of *Staph. aureus* and other mastitis-causing pathogens from milk samples. It has been postulated that freezing process may rupture milk macrophages and neutrophils, releasing phagocytized bacteria, and also that freezing may disrupt bacterial cell aggregates. Thus, the number of cfu/mL should increase, thereby improving the sensitivity of microbiological culture. However, other authors did not find any significant effect of freezing on the number of positive samples of streptococci or *Staph. aureus* in mastitic cow milk after 16 wk (Schukken et al., 1989), and Godden et al., (2002) observed differences in freezing effect for pre-milking and post-milking samples from *Staph. aureus* mastitic quarters. No information concerning changes in concentration of these mammary pathogens for frozen silo milk has been published, but our results indicated a decrease of mammary pathogens concentration after freezing.

The study of preservation × age interaction in refrigerated milk samples (Table 3) revealed statistically significant differences only for groups of mesophilic, psychrotrophic, and coliforms bacteria, in which counts increased significantly over time in non-preserved milk. The highest concentration was for psychrotrophic flora even at the time of silo sampling. These results were consistent with those obtained by Sanjuan et al. (2003), which showed a significant increasing concentration of *Pseudomonas* spp. from the time of milking (4.07 log cfu/mL) until 96 h after milking (7.21 log cfu/mL) in ovine milk refrigerated at 6°C. Thus, the psychrotrophic flora of milk becomes predominant a few hours after milking. Because of the high concentration of psychrotrophic flora and its proven relationship with a high incidence of degradative actions (i.e., lipases of *Pseudomonas* spp.) on milk and cheese components, it is advisable to collect and process the milk (e.g., delivery, heat treatment) in the shortest time possible to prevent significant decline in milk and dairy product quality.

Azidiol-preservation and storage at 4°C kept over time the same initial counts of non-preserved milk (Table 3). Consequently, this procedure could be used by dairy laboratories (interprofessional, industry, research) or by quality payment schemes to determine the initial bacterial counts in silo or bulk tank milk, particularly in the case of mesophiles, psychrotrophic, and coliforms organisms. This is a relevant aspect, as AZ preservation is compatible with composition and SCC analysis in small ruminant milk (Gonzalo et al., 2004; Sánchez et al., 2005); thus, only 1 AZ-preserved milk sample could be sufficient for composition, SCC, and bacterial count analysis from bulk tank or silo milks.

In reference to freezing, the effect of sample age was not significant on the concentration of the microbial groups studied, except for coliforms and *E. coli* counts, which decreased ($P < 0.05$) from d 7 (2.84 and 1.94 log cfu/mL, respectively) until d 30 (2.25 and 1.18 log cfu/mL, respectively) post-freezing. In cow milk, evidence exists for a decrease both in the viability of *E. coli* at −20°C (Pankey et al., 1987) and in the diagnostic sensitivity of this organism after freezing (Schukken et al., 1989), which is consistent with our results.

In conclusion, unpreserved silo sheep milk stored at 4°C significantly increased the concentration of mesophiles, psychrotrophic, and coliform bacteria over time, which makes it advisable to rapidly process the milk stored in silos to avoid its rapid deterioration. The initial concentration of thermoduric, *E. coli*, *Strep. agalactiae* and *Staph. aureus* remained, however, invari-

Table 3. Least squares means (log cfu/mL) of microbiological variables of refrigerated silo ovine milk (4°C) affected by preservation and age, and statistical significance

	Unpreserved milk					Azidiol-preserved milk					
Organism	2 h	24 h	48 h	72 h	96 h	2 h	24 h	48 h	72 h	96 h	*F*-value
Mesophiles[1]	5.13^{a}	5.47^{b}	5.86^{c}	6.33^{d}	6.78^{e}	5.03^{a}	4.97^{a}	5.08^{a}	4.93^{a}	5.18^{ab}	14.74***
Thermoduric[2]	2.47	2.34	2.56	2.72	2.97	2.34	2.29	2.45	2.31	2.40	1.34^{NS}
Psychrotrophic[3]	6.03^{a}	7.02^{b}	7.06^{b}	7.35^{bc}	7.77^{c}	6.05^{a}	5.99^{a}	6.02^{a}	6.09^{a}	6.19^{a}	6.77***
Coliforms[4]	3.44^{a}	4.17^{b}	4.72^{bc}	5.17^{cd}	5.39^{d}	3.15^{ae}	2.91^{ae}	2.90^{ae}	2.59^{e}	2.95^{ae}	9.22***
Escherichia coli[5]	2.79	2.43	2.62	2.69	2.80	2.69	2.62	2.62	2.63	2.53	1.04^{NS}
Streptococcus agalactiae[6]	3.64	3.64	3.91	3.86	4.13	3.77	3.64	3.74	3.73	3.76	1.03^{NS}
Staphylococcus aureus[7]	2.69	2.62	2.92	2.72	2.80	2.64	2.95	2.81	2.58	2.39	2.25^{NS}

$^{a–e}$Means within a row with different superscripts differ ($P < 0.05$).

[1]SE = 0.11.

[2]SE = 0.14.

[3]SE = 0.16.

[4]SE = 0.22.

[5]SE = 0.12.

[6]SE = 0.13.

[7]SE = 0.13.

***$P < 0.001$.

able. Storage at 4°C of AZ-preserved sheep milk was a suitable method to maintain the initial concentration for all studied bacterial groups and species, particularly for mesophilic, psychrotrophic, and coliform organisms throughout 96 h. Freezing significantly decreased the viability of coliforms, *E. coli*, *Strep. agalactiae* and *Staph. aureus*.

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Capítulo 2

RELACIÓN ENTRE RECUENTOS BACTERIANOS ESPECÍFICOS Y LOS RECUENTOS DE BACTERIOLOGÍA TOTAL Y CÉLULAS SOMATICAS Y ANÁLISIS DE SUS FACTORES DE VARIACIÓN EN LECHE DE TANQUE DE REBAÑOS OVINOS

INTRODUCCIÓN

Los recuentos totales de bacteriología (RBTLT) y de células somáticas (RCSLT) de la leche de tanque son parámetros útiles pero no específicos en la monitorización de la calidad higiénica de la leche, el estado sanitario de los rebaños y la vida útil esperable de la leche y de sus productos lácteos derivados. En los laboratorios de análisis de leche estas variables se determinan de forma automatizada mediante equipos cuyo principio analítico se basa en la citometría de flujo, tales como el Bactoscan FC (Foss electric, Dinamarca) para el RBTLT y el Fossomatic (Foss electric, Dinamarca) para el RCSLT y cuya precisión y repetibilidad han sido demostrada por diversos autores (Tomáske *et al.*, 2006; Sierra *et al.*, 2009; Gonzalo *et al.*, 2004).

Como ya se ha mencionado anteriormente, los criterios microbiológicos actuales solo hacen referencia al recuento de flora mesófila y no de otros grupos microbianos tales como termodúricos, psicrotrofos, coliformes y cocos gram-positivos catalasa-negativos (CGPCN), los cuales podrían ser indicadores de gran interés relacionados con la higiene de la leche, la sanidad mamaria o la calidad comercial (Jayarao *et al.*, 2004), y que probablemente estén relacionados con el RBTLT y RCSLT, como indicadores principales de calidad higiénico-sanitaria del rebaño. En efecto, altos recuentos de flora termodúrica pueden ser causados por equipos sucios o inadecuadas prácticas de desinfección de los mismos, pudiendo dar lugar a alteraciones en la fabricación de quesos. El recuento de coliformes es un práctico indicador de higiene del ordeño y está a menudo relacionado con otras poblaciones bacterianas en leche de tanque (Jayarao *et al.*, 2004; Pantoja *et al.*, 2009). Los coliformes son también importantes patógenos mamarios (Hogan and Smith, 2003). Las bacterias psicrotrofas se incorporan a la leche a partir de animales sucios y equipos sucios y van a ser seleccionados por la refrigeración de la leche cruda. La población de cocos Gram positivos catalasa negativos (CGPCN) incluye grupos bacterianos significativos (por ejemplo, estreptococos y enterococos) relacionados con la contaminación fecal y las infecciones mamarias (Keefe, 1997; Zadoks *et al.*, 2004; Marogna *et al.*, 2010) que contaminan los equipos de ordeño y los tanques de almacenamiento de la leche (Gelsomino *et al.*, 2001). Los enterococos son también importantes en la maduración y desarrollo aromático de algunos quesos tradicionales del área Mediterránea, y pueden ser usados también como cultivos iniciadores o probióticos (Franz *et al.*, 2003). Adicionalmente, algunos CGPCN han sido descritos como patógenos emergentes de tipo oportunista o nosocomial en el hombre (Giraffa, 2002; Franz *et al.*, 2003).

A pesar de la importancia de los grupos bacterianos anteriormente señalados sobre la calidad de la leche, su determinación en los laboratorios lecheros es costosa y laboriosa,

puesto que no se han desarrollado aún procedimientos analíticos automatizados. Por tanto, el conocimiento de la relación entre estos grupos microbianos y los RBTLT y RCSLT podría ser de gran interés de cara a la monitorización de la calidad higiénica de la leche de tanque en el ovino lechero. Sin embargo no existe apenas información disponible sobre la variación y covariación de dichos parámetros microbianos (bacterias termodúricas, psicrotrofas, coliformes y CCGPCN) y los parámetros más comunes de monitorización de leche de tanque (RBTLT y RCSLT) determinados a gran escala. Un mayor conocimiento de estas asociaciones podría facilitar información valiosa para la mejora de la calidad higiénica de la leche, la sanidad mamaria o el manejo del rebaño.

Así, los objetivos de este experimento fueron:

- Analizar la relación entre los recuentos de las bacterias termodúricas, psicrotrofas, coliformes y de CGPCN con los recuentos automatizados RBTLT y RCSLT en la leche de tanque de rebaños ovinos.
- Identificar fuentes de variación de cada grupo bacteriano, tales como la raza, la estación del año, el sistema de ordeño, la terapia de secado y el volumen de producción de leche.

MATERIALES Y MÉTODOS

Entre Enero y Diciembre de 2011, un total de 751 muestras de leche de tanque fueron recogidas de 205 rebaños de ganado ovino lechero (172 de raza Assaf Española y 33 de raza Churra; 182 con ordeño mecánico y 23 con ordeño manual) pertenecientes a 2 cooperativas del Consorcio de Promoción del Ovino (CPO) en la Comunidad de Castilla y León, España. Las características y organización reproductiva de los rebaños ya ha sido previamente descrita (Gonzalo *et al.*, 2005).

La información recogida en los rebaños del CPO incluyó los siguientes factores de variación: rebaño, raza, estación, tipo de ordeño, producción lechera anual, número total de ovejas por rebaño y la antibioterapia de secado en cada rebaño. Esta terapia de secado fue siempre administrada bajo supervisión veterinaria y, cuando fue implementada, todos los animales fueron tratados al inicio del periodo seco (terapia completa o sistemática de secado). Los brotes clínicos de agalaxia contagiosa fueron también registrados, confirmándose por la presencia de *Mycoplasma agalactiae* en la leche de tanque. Auditorías anuales son realizadas en todos los rebaños del CPO y una base de datos de campo es continuamente actualizada en los rebaños e incorporada al sistema de trazabilidad del CPO. La producción lechera fue obtenida a partir del programa de facturación del CPO.

Muestreo, aislamiento y recuento

A lo largo del año 2011, se llevaron a cabo un total de 4 muestreos en cada rebaño, uno por estación. Finalmente fueron analizadas 751 muestras en 205 rebaños. Las 69 muestras restantes correspondieron mayoritariamente a rebaños que no estaban en lactación en alguno de los muestreos y, en algunos casos, a muestras no-válidas. Las muestras fueron recogidas justo antes de la descarga de la leche de tanque en los camiones

cisternas, 48 h después de la última descarga. La temperatura de la leche en el tanque de refrigeración fue siempre <6ºC. En cada muestreo se recogieron asépticamente 2 muestras de 50 mL, tras homogeneización de la leche de tanque, de acuerdo con los estándares recomendados por la APHA (White *et al.*, 1992). Una de las 2 muestras fue mantenida bajo condiciones R y la otra bajo condiciones RAZ, ya definidas en el capítulo anterior, siempre a 4ºC hasta el análisis correspondiente realizado nada más llegar al laboratorio de Higiene y Tecnología de los Alimentos (Área de Nutrición y Bromatología) de la Universidad de León. La metodología analítica de cada muestra se resume en la Tabla 1, la cual fue similar a la descrita en el capítulo anterior (de Garnica *et al.*, 2011), excepto para los CGPCN.

Tabla 1. Resumen de la metodología analítica de los distintos grupos microbianos, recuento bacteriológico total, recuento de células somáticas y test de cribado para la detección de residuos antibióticos.

Muestra	GRUPO /ESPECIE	MEDIO DE CULTIVO	TEMPERATURA DE INCUBACIÓN (ºC)	TIEMPO DE INCUBACIÓN (h)	INTERPRETACIÓN Y EXPRESIÓN DE RESULTADOS SEGÚN
R	**Termodúricos***	PCA[1,a]	30±1	24-48	APHA
	Psicrotrofos	PCA[1,a]	7±0.5	7-10 días	APHA
	Coliformes	Petrifilm® [2] Coliforms/*E. coli*	37±0.5	24-48	Instrucciones del fabricante.
	CGPCN	EMM [1,b] con sangre desfibrinada de oveja (5-7%)[1]	37±0.5	24-48	Instrucciones del fabricante.
RAZ	**RBTLT**	Bactoscan® 8000 (control de calidad y triales intercomparativos)			
	RCSLT	Fossomatic ® 5000 (control de calidad y triales intercomparativos)			
	Test residuo antibiótico	Rosa Charm® (Charm Science Inc. MA, USA)			

*Pasteurización previa en laboratorio 62-63 ºC/30 min.

CGPCN: Cocos Gram positivos catalasa negativos. R: refrigeración. RAZ: refrigeración + azidiol.

1. Oxoid Limited, Cambridge, UK. [a]:Plate Recuento de Agar, [b]: Edwards Medium Modified.
2. 3M, MN, USA. Según instrucciones del fabricante.

El cultivo y posible aislamiento de *Mycoplasma agalactiae* a partir de la leche de tanque de rebaños sospechosos de padecer un brote agalaxia contagiosa (incremento de mamitis clínicas, reducción significativa de la producción de leche, posible incremento del RCSLT, artritis de animales jóvenes) se hizo según la metodología descrita por Gonzalo *et al.* (2002).

Estudio estadístico

Las variables de la leche de tanque fueron normalizadas mediante transformación logarítmica $\log_{10}$ (1 + C) siendo C el recuento obtenido (ufc/mL o células/mL), y la normalidad fue investigada mediante los test de Shapiro-Wilk y Kolmogorov-Smirnov de acuerdo con el procedimiento UNIVARIATE del SAS (SAS Institute, 2009). A continuación se realizaron dos tipos de análisis estadísticos con el procedimiento MIXED del programa SAS (SAS Institute, 1998) con un efecto aleatorio (rebaño dentro del tipo de ordeño) para corregir las medidas repetidas dentro de los rebaños. Un primer tipo de modelos fue usado para analizar las relaciones entre los diferentes microrganismos específicos de la leche de tanque (considerados como covariables) y el RBTLT y RCSLT

(considerados como variables dependientes), y un segundo tipo de modelos fue realizado para analizar los factores de variación de los grupos bacterianos específicos (termodúricos, psicrotrofos, coliformes y CGPCN). Los factores de variación raza, estación, tipo de ordeño, terapia de secado y producción de leche fueron también incluidos como efectos fijos para ajustar la relación entre los recuentos bacterianos específicos y RBTLT y RCSLT, y para identificar las principales fuentes de variación de cada grupo bacteriano. En ambos tipos de modelo el efecto aleatorio fue absorbido en el proceso de análisis y sólo se mostraron las medias de mínimos cuadrados y la significación de los efectos fijos. La presencia de potenciales confundidores fue chequeada mediante la variación de la estimación-β tras la exclusión de una covariable; una variación mayor del 30% fue considerada como confundidora. Las correlaciones entre las variables de la leche de tanque fue determinada mediante los coeficientes de correlación de Spearman usando el procedimiento CORR del SAS (SAS Institute, 2009), y se chequeó igualmente la colinearidad para las 4 covariables bacterianas mediante el procedimiento REG (opción COLLIN) del SAS (SAS Institute, 2009).

El primer modelo usado fue:

$$Y_{ijklm} = \mu + B_i + M_j + A_k + b_1T_{ijklm} + b_2P_{ijklm} + b_3C_{ijklm} + b_4G_{ijklm} + b_5MILK_{ijklm} + F_{l(j)} + e_{ijklm}$$

donde, Y_{ijklm} fue la variable dependiente ($\log_{10}$ RBTLT), μ fue el intercepto; B_i fue el efecto fijo de la raza, M_j fue el efecto fijo de tipo de ordeño, A_k fue el efecto fijo de la antibioterapia de secado, $T_{ijklm,}$ $P_{ijklm,}$ C_{ijklm} y G_{ijklm} fueron los efectos fijos de los recuentos de termodúricos, psicrotrofos, coliformes y CGPCN, respectivamente, como covariables; $MILK_{ijklm}$, fue el efecto fijo de la producción anual de leche (L/oveja), también como covariable; b_1-b_5 fueron las pendientes de regresión correspondientes a las covariables; $F_{l(j)}$ fue el efecto aleatorio rebaño dentro del tipo de ordeño; y e_{ijklm} fue el efecto aleatorio residual. El efecto raza fue dividido en 2 niveles: Assaf española y Churra, y el efecto tipo de ordeño fue dividido en 3 niveles: ordeño manual, ordeño mecánico en sala con línea de leche cerrada en anillo y ordeño mecánico en sala con línea de leche de fondo ciego. Finalmente, la terapia de secado tuvo 2 niveles en función de si se llevó a cabo o no en el anterior secado. Se registraron 8 brotes de agalaxia contagiosa, pero este efecto no fue significativo y fue excluido del modelo, lo mismo que los efectos de la estación la interacción raza x tipo de ordeño y los términos cuadráticos de las covariables, que también fueron excluidos al no ser significativos.

La variable dependiente $\log_{10}$ RCSLT fue analizada usando un modelo mixto similar al anterior en el cual hemos incluido adicionalmente el efecto significativo de la estación y excluido el efecto no significativo del $\log_{10}$ de los recuentos en leche de tanque de las bacterias termodúricas, psicrotrofas y coliformes. La estación fue dividida en 4 niveles: invierno (meses de enero, febrero y marzo), primavera (meses de abril, mayo y junio), verano (meses de julio, agosto y septiembre) y otoño (meses de octubre, noviembre y diciembre).

Otros modelos mixtos fueron realizados para estudiar los factores de variación del $\log_{10}$ de los recuentos de bacterias termodúricas, psicrotrofas, coliformes y CGPCN de la

leche de tanque, como variables dependientes. Los efectos fijos analizados fueron la estación, el tipo de ordeño y la antibioterapia de secado con los mismos niveles anteriormente definidos. El rebaño dentro del tipo de ordeño fue considerado como aleatorio, al igual que en los modelos anteriores. Los efectos no significativos fueron excluidos de los modelos.

RESULTADOS Y DISCUSIÓN

Los estadísticos de las variables microbiológicas y de células somáticas estudiadas en la 4 estaciones de muestreo figuran en la Tabla 2. Los recuentos de bacterias psicrotrofas (5.70 log ufc/mL) fueron superiores a los de RBTLT (5.14 log ufc/mL), coliformes (3.83 log ufc/mL) , termodúricos (2.97 log ufc/mL) y CGPCN (2.95 log ufc/mL). En general, estos recuentos fueron consistentes con los reportados por otros autores en leche de oveja (Sanjuan *et al.*, 2003), pero superiores a los de leche de vaca (Jayarao *et al.*, 2004). Estas diferencias pueden ser explicadas por la menor producción y las más deficientes prácticas higiénicas y peores instalaciones en la oveja que en la vaca. No obstante, la media geométrica del RBTLT (136 x 10^3 ufc/mL) fue inferior al límite inferior de 500 x 10^3 ufc/mL establecido por la Unión Europea (Reglamento CE 853/2004) en leche de tanque de oveja y cabra.

Tabla 2. Estadística descriptiva de los recuentos bacteriológicos (total y diferencial) y de células somáticas de la leche de tanque, después de su transformación logarítmica (log_{10}), en cada estación de muestreo.

ESTACIÓN		RECUENTOS (log_{10} ufc/mL or log_{10} células/mL)					
		RBTLT[1]	Termodúricos	Psicrotrofos	Coliformes	CGPCN[2]	RCSLT[3]
Invierno (n = 196)	Media	5.23	2.93	6.36	4.57	2.84	6.03
	DS	0.42	0.77	1.07	1.43	2.02	0.21
	CV	7.99	26.45	16.88	31.34	71.11	3.59
Primavera (n= 191)	Media	5.11	2.96	5.58	3.69	2.78	6.01
	DS	0.38	0.72	1.18	1.44	1.91	0.21
	CV	7.55	24.29	21.21	38.97	68.67	3.45
Verano (n = 190)	Media	5.05	2.92	4.97	3.42	3.43	6.06
	DS	0.37	0.71	1.54	1.80	1.65	0.18
	CV	7.35	24.33	31.00	52.67	48.19	3.03
Otoño (n = 174)	Media	5.14	3.08	5.87	3.55	2.74	6.05
	DS	0.52	0.79	1.32	1.79	1.96	0.23
	CV	10.11	25.51	22.57	50.5	71.67	3.84
Valores totales	Min.	4.08	0	2.0	0	0	5.35
	Max.	6.70	5.0	9.0	9.0	6.2	6.70

[1]Recuento bacteriológico total.
[2]Cocos gram-positivos catalasa-negativos
[3]Recuento de células somáticas

Tabla 3. Test de significación de los efectos fijos de los recuentos de bacteriología total (RBTLT) y de células somáticas (RCSLT), coeficientes β del modelo mixto de regresión lineal, y coeficientes de correlación de Spearman (r) con las covariables de recuento bacteriano.

Fuente	Log_{10} RBTLT					Log_{10} RCSLT				
	gl	*F*	β	ES	r	df	*F*	β	ES	r
Intercepto			4.14	0.08				5.90	0.03	
Cov. Log_{10} recuento de termodúricos	1	13.27***	0.06	0.02	0.26					0.11
Cov. Log_{10} recuento de psicrotrofos	1	47.62***	0.07	0.01	0.46					0.13
Cov. Log_{10} recuento de coliformes	1	35.54***	0.05	0.01	0.41					0.11
Cov. Log_{10} recuento de CGPCN	1	13.93***	0.03	0.01	0.29	1	12.94***	0.01	0.00	0.21
Cov. Producción de leche anual por oveja	1	15.90***	-0.07[1]	0.00[1]	0.24	1	10.58**	-0.03[1]	0.00[1]	0.29
Raza	1	13.82***				1	6.07*			
Assaf			0.15	0.04				0.05	0.02	
Churra			0					0		
Tipo de ordeño	2	11.21***				2	34.44***			
Línea de leche cerrada en anillo			0.01	0.03				0.04	0.01	
Ordeño manual			0.21	0.05				0.20	0.02	
Línea de leche de extremo ciego			0					0		
Estación						3	3.89**			
Invierno								-0.03	0.01	
Otoño								0.01	0.01	
Primavera								-0.05	0.01	
Verano								0		
Terapia antibiótica de secado	1	32.87***				1	102.76***			
No			0.16	0.03				0.14	0.01	
Si			0					0		
Rebaño dentro del tipo de ordeño[2]	-	-				-	-			

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

[1]por 100 litros de leche.

[2]El rebaño fue considerado como el factor aleatorio absorbido en el modelo(nº de rebaños = 205).

Por otra parte, el RCSLT (6.04 log cel./mL ó 1093 x 10^3 cel./mL) es elevado y consistente con los recuentos encontrado en la leche de silo en el capítulo anterior (de Garnica *et al.*, 2011) y sugieren la necesidad de implementar programas de control de mamitis más efectivos. En los pequeños rumiantes, la Unión Europea no ha establecido aún ningún criterio relativo a esta variable de la leche de tanque.

Los tests de Shaphiro-Wilk y Kolmogorov-Smirnov evidenciaron que la distribución de las variables de la leche de tanque tras su transformación logarítmica difiere significativamente de la normalidad ($P < 0.05$ a $P < 0.001$), si bien la inspección visual de los histogramas y de las medidas de dispersión y centralidad nos permiten considerar las distribuciones de estas variables como aproximadamente normales. No obstante, sí que resultan posibles pequeños sesgos de infraestimación de los errores estándar y, consecuentemente, de sobreestimación de la significación para los efectos relativos y la comparación de medias estudiados. Ello no impide, sin embargo, que los modelos estadísticos propuestos puedan ser usados para valorar los efectos relativos de las variables en los modelos (Maas y Hox, 2004; Koop *et al.*, 2010).

La Tabla 3 muestra la significación de los factores que influencian la variación del RBTLT y RCSLT. El principal propósito del primer modelo mixto fue estudiar la significación de los diferentes recuentos bacterianos sobre el RBTLT y RCSLT; ya que los otros factores de variación han sido analizados previamente en investigaciones anteriores (Gonzalo *et al.*, 2005, 2006 y 2010). Todos los organismos estudiados como covariables para el RBTLT fueron estadísticamente significativos ($P < 0.001$), pero únicamente el recuento de los CGPCN fue también relevante ($P < 0.001$) para el RCSLT. Considerando la información incluida en la Tabla 3 relativa a los valores de F, los parámetros de regresión ($\beta \pm ES$) y los coeficientes de correlación de Spearman (r), las bacterias cuantitativamente más importantes relacionadas con el RBTLT fueron psicrotrofos y coliformes. La flora termodúrica y los CGPCN tuvieron también un efecto significativo sobre el RBTLT, si bien la importancia cuantitativa de estos grupos bacterianos fue menor. Adicionalmente, la colinearidad entre las 4 covariables bacterianas fue baja (por ejemplo, para la variable dependiente RBTLT, el factor de inflación de varianza estuvo entre 1.05 y 1.29). Una correlación moderada-alta ($r = 0.51$) fue encontrada entre las bacterias psicrotróficas y coliformes, aunque no es probable que se trate de variables potenciales confundidoras debido a que las variaciones de la estimación-β tras la exclusión de los recuentos de los psicrotrofos o coliformes fueron <28.7% en el modelo. Así, tal correlación podría ser explicada considerando las potenciales fuentes de contaminación bacteriana de la leche. En este sentido, unas prácticas de limpieza menos eficientes en los rebaños seleccionarían probablemente microorganismos menos resistentes y de rápido crecimiento (psicrotróficos), principalmente bacilos Gram-negativos (coliformes y *Pseudomonas* spp.) y algunos estreptococos en la leche (Wallace, 2008), lo cual es consistente con los resultados encontrados en el presente estudio. Globalmente, las asociaciones entre indicadores de calidad de leche de tanque en el ganado vacuno muestran correlaciones similares entre RBTLT y coliformes ($r = 0.41$) y termodúricos ($r = 0.17$) (Pantoja *et al.*, 2009). Las correlaciones entre los incrementos de RCSLT y otros organismos ambientales, tales como bacterias coliformes, termodúricas o psicrotrofas, fueron bajas (Tabla 3) y las pendientes de

la regresión no fueron significativas. Solamente los recuentos de CGPCN tuvieron un efecto significativo sobre el RCSLT. Algunas de estas especies han sido aisladas a partir de mamitis clínicas y subclínicas en los pequeños rumiantes (Bergonier *et al.*, 2003; Marogna *et al.*, 2010), lo cual sugiere que programas de control específicos para CGPCN podrían reducir también el RBTLT y RCSLT en los rebaños de ovino lechero. En conjunto, el RCSLT parece ser sólo un indicador parcial de la higiene de la producción de leche en origen, ya que no reflejó suficientemente el estatus higiénico de los rebaños de ovino lechero. Sin embargo, la correlación entre RCSLT y RBTLT fue moderada (r = 0.42) y similar a la obtenida por Koop *et al.* (2009, 2010). Estos autores sugieren que la infección mamaria es probablemente un factor importante que explicaría la correlación entre RCSLT y RBTLT en las cabras lecheras; por lo que los programas de mejora de la sanidad mamaria pueden tener un efecto positivo sobre ambas variables de leche de tanque. Esta observación es compatible con el efecto significativo ($P < 0.001$) de la terapia antibiótica de secado sobre el RBTLT encontrado en el presente estudio (Tabla 3) y en otros estudios anteriores en ovino lechero (Gonzalo *et al.*, 2010), en los cuales el RBTLT disminuyó tras la terapia de secado.

Finalmente, el recuento de coliformes no estuvo significativamente asociado al RCSLT, siendo su correlación muy baja (r=0.11), sugiriendo que este grupo no está asociado a infecciones mamarias ni a incrementos de RCSLT, sino a contaminación ambiental, lo cual viene avalado por las bajas prevalencias de infecciones mamarias por coliformes encontradas en los pequeños rumiantes (Gonzalo *et al.*, 2002; Contreras *et al.*, 2003).

En el presente estudio los casos clínicos de agalaxia contagiosa no contribuyeron a la variación del RBTLT ni del RCSLT, posiblemente debido a la implementación de un programa de control llevado a cabo durante muchos años en los rebaños del CPO. En el caso del RBTLT, estudios previos mostraron igualmente un efecto no significativo de este síndrome sobre el RBTLT en el ovino lechero (Gonzalo *et al.*, 2006)

En relación a los factores de variación que afectan a cada grupo bacteriano, la estación supuso un importante efecto ($P < 0.05$ a $P < 0.001$) que influyó sobre la variación de los recuentos de las bacterias termodúricas, psicrotrofas, coliformes y CGPCN (Tabla 4). Las mayores diferencias estacionales fueron observadas para los psicrotrofos, los cuales mostraron los valores más elevados en invierno (6.32 log ufc/mL) y los más bajos en verano (4.95 log ufc/mL). Así, la temperatura estacional selecciona probablemente para los organismos ambientales aislados en la leche. Los coliformes fueron también más elevados en invierno (4.55 log ufc/mL) que en otras estaciones (3.42 a 3.67 log ufc/mL), lo cual puede estar más íntimamente asociado a camas y ubres más sucias debido a una climatología más húmeda en el invierno y, por tanto, a variaciones estacionales de la higiene de los rebaños, más que a un efecto de la estación sobre la temperatura de la leche de tanque. Así, los RBTLT encontrados fueron mayores en invierno (5.23 log ufc/mL) que en verano (5.05 log ufc/mL), avalando la interpretación anterior. Otras prácticas de manejo estacionales, tales como el confinamiento *versus* el pastoreo podrían también influir sobre la presencia de psicrotrofos y coliformes en la leche de tanque. Los mayores recuentos de CGPCN se encontraron en verano (3.56 log ufc/mL) en comparación con otras estaciones

(2.87 a 2.93 log ufc/mL). Las elevadas temperaturas estivales y las reducidas producciones lecheras por oveja en esta época del año podrían ser un importante factor de riesgo para la diseminación de organismos contagiosos y ambientales pertenecientes a ese grupo bacteriano.

Tabla 4. Medias de mínimos cuadrados (± ES) de los recuentos de bacterias termodúricas, psicrotrofas, coliformes y cocos Gram-positivos catalasa-negativos (CGPCN) de la leche de tanque influenciados por el efecto estacional en ovino lechero.

Variable	Invierno	Primavera	Verano	Otoño	*F*
Log_{10} recuento termodúricos	2.97[a]	2.99[a]	2.97[a]	3.13[b]	2.45*
ES	0.06	0.06	0.06	0.06	
MG[1]	0.93	0.99	0.93	1.35	
Log_{10} recuento psicrotrofos	6.32[a]	5.53[b]	4.95[c]	5.85[d]	44.79***
ES	0.10	0.10	0.10	0.10	
MG[1]	2082	341	88	706	
Log_{10} recuento coliformes	4.55[a]	3.67[b]	3.42[b]	3.54[b]	23.70***
ES	0.13	0.13	0.13	0.14	
MG[1]	35.51	4.67	2.64	3.45	
Log_{10} recuento CGPCN	2.93[a]	2.87[a]	3.56[b]	2.89[a]	6.39***
ES	0.15	0.15	0.15	0.15	
MG[1]	0.86	0.74	3.62	0.79	

[a-d]Medias dentro de una fila con diferentes superíndices difieren $P < 0.05$.
[1]MG: Media geométrica (x 10^3 ufc/mL).
*P < 0.05. ***P < 0.001.

El efecto tipo de ordeño fue únicamente significativo para los CGPCN, tal como muestra la Tabla 5. Así, los recuentos de CGPCN fueron mayores para los rebaños con ordeño manual que para los rebaños con ordeño mecánico en sala. Junto con las pobres condiciones higiénicas del ordeño manual, la mano puede actuar de vector para la transmisión de patógenos ambientales (por ejemplo, *Enterococcus* spp.) y contagiosos (por ejemplo, *Streptococcus agalactiae*) incrementando sus recuentos en la leche. Dentro del ordeño mecánico no se encontraron diferencias en los recuentos de CGPCN en función del tipo de línea de leche.

Tabla 5. Medias de mínimos cuadrados (± ES) de los recuentos de los cocos Gram-positivos catalasa-negativos (CGPCN) de la leche de tanque en función del tipo de ordeño en los rebaños ovinos

Variable	Ordeño manual	Ordeño mecánico		*F*
		Anillo	Extremo ciego	
Log_{10} recuento	3.43[a]	2.88[b]	2.88[b]	3.45*
ES	0.24	0.10	0.15	
MG[1]	2.71	0.76	0.75	

[a-b]Medias dentro de una fila con diferentes superíndices difieren $P < 0.05$.
[1]MG: Media geométrica (x 10^3 ufc/mL).
*$P < 0.05$.

La terapia de secado fue también un importante efecto que disminuyó significativamente el RBTLT y RCSLT (Tabla 3) en consonancia con otros estudios en el ovino lechero (Gonzalo *et al.*, 2005, 2010), si bien esta práctica no fue efectiva para reducir los recuentos de CGPCN ($P > 0.05$). Es probable que este grupo bacteriano sea el resultado de contaminación de la leche de tanque (por ejemplo, contaminación fecal por enterococos), y no solamente a causa infecciones mamarias. Este hecho podría explicar los altos recuentos de CGPCN encontrados en los rebaños ordeñados a mano (Tabla 5), en los cuales unas pobres condiciones higiénicas favorecerían la contaminación de la leche y harían inefectiva la terapia de secado (Gonzalo *et al.*, 2009). La terapia antibiótica de secado fue, sin embargo, significativa para los recuentos de la flora psicrotrofa ($F = 6.6.5$; $P < 0.05$), cuyos valores fueron inferiores (5.49 log ufc/mL) en los rebaños que implementaron la terapia de secado que en los que no la hicieron (5.76 log ufc/mL). El hecho de que los recuentos de patógenos mamarios sean probablemente reducidos por la terapia de secado, sugiere que algunas cepas de patógenos son potencialmente bacterias psicrotróficas y pueden, por tanto, contribuir a incrementar los recuentos de flora psicrotrofa de la leche.

CONCLUSIONES

- El recuento bacteriológico total y de células somáticas de la leche de tanque de las explotaciones de ganado ovino lechero constituye una herramienta útil para la monitorización y la mejora continua de la calidad higiénica de la leche destinada a la producción de quesos.
- El recuento de las bacterias psicrotrofas, termodúricas y coliformes estuvo significativamente relacionado con el recuento bacteriológico total, mientras que el recuento de cocos Gram-positivos catalasa-negativos estuvo asociado tanto con el recuento bacteriológico total como de células somáticas en los rebaños de ovino lechero. Adicionalmente, estas 2 últimas variables estuvieron correlacionadas entre sí.
- La estación, terapia de secado y tipo de ordeño fueron importantes factores de variación de los recuentos diferenciales de la leche de tanque; los psicrotrofos y los

coliformes tuvieron elevados recuentos en invierno, mientras que de los cocos Gram-positivos catalasa-negativos fueron mayores en verano y en los rebaños ordeñados a mano.

- Serán necesarios ulteriores estudios que investiguen la influencia de todos los factores de riesgo asociados con el recuento bacteriológico total de la leche dentro de la propia granja y que permitan definir umbrales de discriminación efectivos para los recuentos específicos de la leche de tanque.

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Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk

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ABSTRACT

To analyze the relationship among the counts of different organisms and total bacterial count (BTTBC) and somatic cell count (BTSCC) as determined in dairy laboratories in ovine bulk tank milk, 751 bulk tank milk samples from 205 dairy sheep flocks belonging to Consortium for Ovine Promotion (CPO) were collected between January and December 2011. Four samplings were carried out in each flock, once per season, throughout 1 yr. Variables analyzed were bulk tank counts of thermoduric, psychrotrophic, coliform, and gram-positive catalase-negative cocci (GPCNC) bacterial groups. Thermoduric, psychrotrophic, and coliform species were significantly related to BTTBC, whereas GPCNC were correlated with both BTTBC and BTSCC variables. Highest counts were for psychrotroph and coliform groups, and a moderate to high correlation (r = 0.51) was found between both variables, indicating that poor cleaning practices in the flocks tend to select for less-resistant organisms, such as gram-negative rods. In addition, BTTBC correlated with BTSCC (r = 0.42). Some variation factors for specific bacterial counts, such as breed, season, milking type, dry therapy, and milk yield, were also analyzed. Flock information was collected from flock books, annual audits, and the CPO traceability system. Psychrotrophs and coliforms had elevated counts in winter, whereas GPCNC were higher in summer and in hand-milked flocks. Dry therapy contributed to the reduction in psychrotrophic bacteria; therefore, some strains of mammary pathogens could also be psychrotrophic bacteria. Results of this study would be helpful for troubleshooting milk quality problems and developing premium payment systems in dairy sheep.

Key words: bulk tank milk, dairy sheep, milk quality, milk organisms

INTRODUCTION

In dairy sheep, bulk tank total bacterial count (**BTTBC**) and bulk tank SCC (**BTSCC**) are important but nonspecific tools used by technicians and farmers to evaluate the efficiency of production processes, cleaning and sanitation practices, and flock mammary health, and to predict the keeping quality and shelf life of milk and dairy products. In milk testing and dairy interprofessional laboratories, BTSCC determination is automated by Fossomatic devices (Foss Electric, Hillerød, Denmark), and several researchers have investigated the global accuracy of rapid SCC counters in ovine milk (Gonzalo et al., 2004). Similarly, BTTBC is routinely determined using automated flow cytometry devices (i.e., Bactoscan FC, Foss Electric), and several researchers have explored the global accuracy of Bactoscan and the high correlation between Bactoscan and mesophilic bacterial counts in milk of small ruminants (Tomáska et al., 2006; Sierra et al., 2009).

Bulk tank milk is contaminated by bacteria from different sources such as flora and pathogens present in beds, milking facilities, wash water, milking system, udder, or mastitic milk. Some of these bacteria are resistant to pasteurization or are able to grow at low temperatures, and some indicate fecal contamination or mastitis. Despite this, only the aerobic mesophilic count (i.e., BTTBC) has been the target of various legal limits for ovine milk in the European Union. In this context, other bacterial groups such as thermoduric bacteria, psychrotrophic bacteria, coliforms, and gram-positive catalase-negative cocci (**GPCNC**) would be indicators of interest for milk hygiene, udder health, safety, quality, and marketing (Jayarao et al., 2004) and likely related to both BTTBC and BTSCC. Indeed, high counts of thermoduric species can be caused by unclean equipment or improper sanitizing practices, and these thermoduric bacteria may cause spoilage in pasteurized milk. Coliform count is a practical indicator of milking hygiene and is often correlated with the population of other bacteria in bulk tank milk (Jayarao et al., 2004; Pantoja et al., 2009); coliforms are also im-

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portant mastitis pathogens (Hogan and Smith, 2003). Psychrotrophic microorganisms enter the milk from soiled animals and dirty equipment and are selected by refrigeration of raw milk. Gram-positive catalase-negative cocci include significant groups such as streptococci and enterococci. Mastitis-causing streptococci are important contributors to BTTBC and BTSCC in dairy cattle (Keefe, 1997; Zadoks et al., 2004); thus, control of streptococci is important for improving the microbial and sanitary quality of raw milk. Enterococci are environmental organisms, mostly of fecal origin, that contaminate milking equipment and bulk storage tanks (Gelsomino et al., 2001) and cause mastitis and high SCC in dairy sheep (Marogna et al., 2010). Enterococci are also important for ripening and aroma development of certain traditional cheeses, especially those produced in the Mediterranean region, and they may be used as starter or probiotic cultures (Franz et al., 2003). In addition, some GPCNC are described as emergent opportunistic or nosocomial pathogens for humans (Giraffa, 2002; Franz et al., 2003).

In spite of the importance of the above-mentioned bacterial groups on milk quality, their determination in testing laboratories is expensive and laborious, and analytical automated procedures have not yet been developed. Therefore, the relationship among these bacterial groups and BTTBC and BTSCC is of great interest for monitoring the hygiene quality of bulk tank milk in dairy sheep. Nevertheless, little information is available about the microbial factors influencing variation and covariation of bulk tank milk variables, such as BTTBC and BTSCC, which can be determined on a large scale by automated procedures (Gonzalo et al., 2006), and no known studies have examined the variation of bulk tank bacterial groups (e.g., thermoduric, psychrotrophs, coliforms, or streptococci) and their relationship with BTTBC and BTSCC in dairy sheep. Wider knowledge of BTTBC and BTSCC and their associations with other bulk tank bacterial counts would enable decisions to be made on improving milk quality, farm management practices, and flock udder health.

The goals of this study were (1) to analyze the relationship among bulk tank thermoduric, psychrotrophic, coliform, and GPCNC microbial counts and automated BTTBC and BTSCC, and (2) to identify sources of variation in each bacterial group, such as breed, season, milking system, antibiotic dry therapy, and milk yield in dairy sheep.

MATERIALS AND METHODS

Flocks

Between January and December 2011, a total of 751 bulk tank milk samples were collected from 205 dairy sheep flocks (172 Spanish Assaf and 33 Churra; 182 machine milked and 23 hand milked) belonging to 2 cooperatives of the Consortium for Ovine Promotion (**CPO**) in the Castilla y León region of Spain. Average flock size was about 400 ewes, which is representative of the region; lambing is mostly concentrated in the late fall, winter, and spring, and the characteristic features of these flocks have been previously described (Gonzalo et al., 2005). Briefly, each herd is generally divided into 2 lots, the lambing periods of which alternate every 4 to 6 mo in the herd. Some herds undergo reproductive intensification to achieve 3 lambings every 2 yr, whereas in others, ewes have only 1 lambing per year. Teat washing was not done in any flocks before milking, as this is not a typical practice in dairy sheep.

The information recorded in CPO flocks included the following variation factors: flock, breed, season, milking type (hand or parlor machine milking), annual milk yield, total number of ewes per flock, and antibiotic dry therapy practice in each flock. Dry therapy is administered under veterinary supervision and, when implemented, all animals were treated at the beginning of the dry period (i.e., complete dry therapy). Clinical outbreaks of contagious agalactia were also reported and *Mycoplasma agalactiae* was isolated in bulk tank milk. Annual audits are performed in the CPO flocks and a field database is continuously maintained with above-mentioned information. The CPO veterinary services are centralized and information concerning animal health and antibiotic treatment along with new animals and falls are recorded in the each flock book. Milk yield was recorded from receipt in the CPO traceability system.

Sampling

Throughout the year, 4 samplings were carried out in each flock, once per season. In total, 751 bulk tank samples were analyzed; the 69 missing samples corresponded mostly to flocks that were not in lactation at sampling season and, in a few cases, to nonuseful samples. In each sampling, 2 samples of 50 mL were aseptically collected in sterile containers after milk homogenization according to standards recommended by the American Public Health Association (White et al., 1992). One of the 2 samples was preserved with azidiol (3.3 μL/mL, Panreac Quimica S.A., Castellar del Valles, Barcelona, Spain) according to de Garnica et al. (2011). Sampling was always done immediately before loading the bulk tank milk into the tankers. Milk tanker collection frequency in flocks was 48 h, and milk was kept at <6°C in cooling tanks on the farms in between collections. Milk samples were kept at 4°C until bacteriological analysis, which was carried

out immediately after arrival in the laboratory in the Department of Food Hygiene and Technology, University of León, Spain. Variables analyzed in the unpreserved milk aliquot were counts of thermoduric, psychrotrophic, coliform, and GPCNC groups. Antibiotic residue screening, BTTBC, and BTSCC variables were analyzed in the preserved aliquot in the milk testing laboratory of the National Center for Animal Breeding and Reproduction (León, Spain).

Analytical Determinations

As described previously (de Garnica et al., 2011), thermoduric count was carried out by SPC method after laboratory pasteurization at 62.8 ± 0.5°C for 30 min, following American Public Health Association recommendations (White et al., 1992). Psychrotrophic bacteria were also enumerated by SPC, and plates were incubated at 7°C for between 7 and 10 d (White et al., 1992). The enumeration of coliforms was carried out using 3M Petrifilm *E. coli*/Coliform Count Plates (3M, St. Paul, MN) according to the manufacturer's instructions. Plates were incubated at 37 ± 0.5°C for 24 to 48 h. Confirmed coliforms were considered as both red and blue colonies with associated gas bubbles; results were expressed in colony-forming units per milliliter. Finally, Edwards Medium Modified (Oxoid Ltd., Cambridge, UK) supplied with 5 to 7% defibrinated sheep blood (Oxoid Ltd.) was used for isolation and enumeration of GPCNC, and plates were incubated at 35°C for 48 h.

Culturing and possible isolation of *Mycoplasma agalactiae* from bulk tank milk was performed only when clinical outbreaks were suspected (e.g., increase in clinical mastitis, significant reduction in milk production, possible BTSCC increase, arthritis in young animals), according to the methodology previously described (Gonzalo et al., 2002).

Antibiotic residues in milk were always checked for β-lactams and tetracycline drugs by the Rosa Charm screening test (Charm Sciences Inc., Lawrence, MA). All samples tested negative for β-lactams and tetracyclines during the experiment. Total bacterial count was determined by using a Bactoscan 8000 instrument (Foss Electric) and BTSCC was analyzed in a Fossomatic 5000 (Foss Electric); both counts were subjected to quality controls (with ovine standards to 4,000 × 10^3 cfu/mL or cells/mL) and intercomparative trials. Analytical determinations of BTTBC and BTSCC were always performed within 36 h of bulk tank milk collection.

Statistical Analyses

Bulk tank variables were normalized by $\log_{10}$-transformation, and normality was investigated by Shapiro-Wilk and Kolmogorov-Smirnov test statistics according to the UNIVARIATE procedure of SAS (SAS Institute, 2009).

Statistical analyses were carried out using univariate mixed models according the MIXED procedure of SAS (SAS Institute, 2009). Mixed models were built with a random effect to correct for repeated measures within flocks. The variables analyzed were bulk tank thermoduric, psychrotrophic, coliform, and GPCNC counts, BTTBC, and BTSCC. Two different types of models were constructed; the first model was used to analyze the relationship among bulk tank organisms (considered covariables) and BTTBC and BTSCC (considered dependent variables), and the second model was performed to analyze the factors influencing the variation of thermoduric, psychrotrophic, coliform, and GPCNC bulk tank counts, considered as dependent variables. These variables were always transformed as log (1 + C), where C was the count obtained (cfu/mL) for each sample and organism. Factors of variation, such as breed, season, milking type, dry therapy, and milk yield, were also included as fixed effects to adjust the relationship between specific bacterial counts and BTTBC and BTSCC, and to identify the main sources of variation within each bacterial group. To identify the statistically significant effects, previous statistical analyses were performed using univariate mixed models. In all mixed models used, the flock random factor was absorbed in the analysis and only the significance of the fixed effect was shown. Least squares means and test of significance were obtained for the fixed effects. Presence of potential confounding was checked through the β-estimate variation after exclusion of a covariable; a variation higher than 30% was considered as confounding. Correlation between bulk tank variables was determined by Spearman correlation coefficients using the CORR procedure of SAS (SAS Institute, 2009), and collinearity was checked for 4 bacterial covariables by the REG procedure (COLLIN option) of SAS (SAS Institute, 2009).

The first model used was

$$Y_{ijklm} = \mu + B_i + M_j + A_k + b_1T_{ijklm} + b_2P_{ijklm} + b_3C_{ijklm} + b_4G_{ijklm} + b_5MILK_{ijklm} + F_{l(j)} + e_{ijklm},$$

where Y_{ijklmn} was the dependent variable $\log_{10}$ BTTBC; μ was the intercept; B_i was the fixed effect of breed; M_j was the fixed effect of milking type; A_k was the fixed effect of antibiotic dry therapy; T_{ijklm}, P_{ijklm}, C_{ijklm}, and G_{ijklm} were the fixed effects of $\log_{10}$ bulk tank counts of thermoduric, psychrotrophic, coliform, and GPCNC organisms, respectively, as covariables; $MILK_{ijklm}$ was the fixed effect of annual milk yield (L) per ewe, also

considered as covariable; b_1 to b_5 were the regression slopes corresponding to covariables; $F_{l(j)}$ was the random effect of flock within milking type; and e_{ijklm} was the random residual effect. The breed effect was divided into 2 levels: Spanish Assaf and Churra, and the type of milking effect was divided into 3 levels: hand, parlor machine milking with looped milkline, and parlor machine milking with dead-ended milkline. Finally, dry therapy was divided into 2 levels depending on whether, or not, it was carried out in each flock during the previous drying-off. Contagious agalactia outbreaks were recorded in 8 flocks, but this effect was not significant and was excluded from the statistical models. In addition, season, breed × milking type interaction, and covariable quadratic terms were not statistically significant and were excluded from the model.

The dependent variable $\log_{10}$ BTSCC was analyzed using a similar mixed model in which we additionally included the significant effect of season and excluded the nonsignificant effect of $\log_{10}$ bulk tank counts of thermoduric, psychrotrophic, and coliform organisms. Season was divided into 4 levels: winter (mo 1, 2, and 3), spring (mo 4, 5, and 6), summer (mo 7, 8, and 9), and fall (mo 10, 11, and 12).

Other mixed models were performed to study the factors influencing the variation of $\log_{10}$ bulk tank counts of thermoduric, psychrotrophic, coliform, and GPCNC as dependent variables. The fixed effects analyzed were season, milking type, and antibiotic dry therapy, with the same levels as previously defined. Flock within milking type was considered random, as in previous models. Nonsignificant effects were excluded from the models

RESULTS AND DISCUSSION

The statistics of the microbiological variables studied in the 4 sampling seasons are shown in Table 1. Psychrotrophic bacteria were the most numerous bacterial group (5.70 log cfu/mL) above BTTBC (5.14 log cfu/mL), coliforms (3.82 log cfu/mL), thermoduric bacteria (2.97 log cfu/mL), and GPCNC (2.95 log cfu/mL) counts. In general, these counts were consistent with those reported by Sanjuán et al. (2003) in bulk tank milk from sheep flocks, but higher than those found in cow milk (Jayarao et al., 2004). Lower individual milk production, the absence of teat washing before milking, and poorer facilities than those for cattle could explain the higher bacterial counts found in ewe milk compared with cow milk. Nevertheless, the geometric mean of BTTBC (136×10^3 cfu/mL) was below the limit of 500,000 cfu/mL established by the European Union (2004; Regulation EC 853/2004) for bulk tank milk from sheep and goat flocks; in addition, the high mean values for BTSCC (6.04 $\log_{10}$ cells/mL or $1{,}093 \times 10^3$ cells/mL) suggest the need to increase mastitis control programs in dairy sheep flocks, although the European Union has yet to regulate bulk tank SCC in ewe and goat milk. Regardless, BTTBC and BTSCC values found in this study were similar to those of previous observational studies (Gonzalo et al., 2010) in the same geographical area. Annual milk yield was 183 ± 91.11 L/ewe.

According to the Shapiro-Wilk and Kolmogorov-Smirnov tests, the distribution of bulk tank milk variables after $\log_{10}$-transformation was significantly different from normal ($P < 0.05$ to $P < 0.001$), although visual inspection of the histograms and measures of central tendency and dispersion allowed us to consider the distribution of these variables as approximately normal. However, a small downward bias of standard errors and, consequently, overestimated significance for relative effects and mean comparisons is possible. Nevertheless, the model can be used to assess the relative effect of the variables within the model (Maas and Hox, 2004; Koop et al., 2010).

Table 2 shows the significance of factors affecting BTTBC and BTSCC variation. The main purpose of the mixed model was to study the significance of different bacterial counts on BTTBC and BTSCC; the other factors of variation have been analyzed previously (Gonzalo et al., 2005, 2006, 2010). All organisms studied as covariables for BTTBC were statistically significant ($P < 0.001$), but only GPCNC count was also relevant ($P < 0.001$) for BTSCC. Considering the *F*-values along with the regression parameters (β ± SE) and Spearman correlation coefficients (r) shown in Table 2, the quantitatively most important organisms correlated with BTTBC were psychrotrophic and coliform bacteria. Thermoduric and GPCNC bacteria also had significant effects on BTTBC, although the quantitative importance of these bacterial groups was lesser. In addition, collinearity among the 4 bacterial covariables was low (e.g., for dependent variable BTTBC, the variance inflation factor was between 1.05 and 1.29). A moderate to high correlation (r = 0.51) was found between psychrotrophic and coliform bacteria; however, these variables are probably not potential confounding variables because β-estimate variations after exclusion of psychrotrophic or coliform counts were <28.7% in the model. These correlations might be explained by considering potential sources of bacterial species found in milk. Less-efficient cleaning practices in the flocks probably selects for the faster growing, less resistant organisms (psychrotrophic), principally gram-negative rods (coliforms and *Pseudomonas* spp.) and some streptococci in the milk (Wallace, 2008), which is consistent with results obtained in the present study. Globally,

Table 1. Separate descriptive statistics for bulk tank bacterial counts, total bacterial counts, and SCC variables (after $\log_{10}$ transformation) in each sampling season

	Count ($\log_{10}$ cfu/mL or $\log_{10}$ cells/mL)					
Sampling season	Total bacteria	Thermoduric	Psychrotrophic	Coliform	GPCNC[1]	SCC
Winter (n = 196)						
Mean	5.23	2.93	6.36	4.57	2.84	6.03
SD	0.42	0.77	1.07	1.43	2.02	0.21
CV	7.99	26.45	16.88	31.34	71.11	3.59
Spring (n = 191)						
Mean	5.11	2.96	5.58	3.69	2.78	6.01
SD	0.38	0.72	1.18	1.44	1.91	0.21
CV	7.55	24.29	21.21	38.97	68.67	3.45
Summer (n = 190)						
Mean	5.05	2.92	4.97	3.42	3.43	6.06
SD	0.37	0.71	1.54	1.80	1.65	0.18
CV	7.35	24.33	31.00	52.67	48.19	3.03
Fall (n = 174)						
Mean	5.14	3.08	5.87	3.55	2.74	6.05
SD	0.52	0.79	1.32	1.79	1.96	0.23
CV	10.11	25.51	22.57	50.5	71.67	3.84
Minimum value[2]	4.08	0	2.0	0	0	5.35
Maximum value[2]	6.70	5.0	9.0	9.0	6.2	6.70

[1]GPCNC = gram-positive catalase-negative cocci.

[2]Minimum and maximum values refer to total data (n = 751).

the associations among bulk milk quality indicators in dairy cattle show a similar correlation between BTTBC and coliforms (r = 0.41) and laboratory pasteurization count (r = 0.17; Pantoja et al., 2009). Correlations between increases in BTSCC and other environmental organisms, such as coliform, thermoduric, or psychrotrophic bacteria, were low (Table 2), and slopes in the regression were not significant. Only GPCNC count had a significant effect on BTSCC; some GPCNC species have been isolated from clinical or subclinical mastitis in small ruminants (Bergonier et al., 2003; Marogna et al., 2010), suggesting that specific mastitis control programs for this bacterial group would reduce BTTBC and BTSCC in dairy sheep flocks. As a whole, BTSCC seems to be a partial indicator of the hygiene status of primary milk production, as it did not sufficiently reflect the hygiene status of producing dairy sheep flocks. However, the correlation between BTSCC and BTTBC was moderate (r = 0.42) and similar to that obtained by Koop et al. (2009, 2010). Koop et al. (2009) suggest that IMI is probably an important factor driving the correlation between BTSCC and BTTBC in dairy goats; thus, programs to improve udder health may have a positive effect on both BTSCC and BTTBC. That observation is compatible with the significant effect ($P < 0.001$) of antibiotic dry therapy on BTTBC found in the current study (Table 2) and other studies (Gonzalo et al., 2010) in dairy sheep flocks, in which BTTBC decreased after dry therapy.

Finally, coliform counts were not associated with BTSCC increases in the present study. In dairy cattle, mammary glands experimentally infected with *Escherichia coli* have been demonstrated to shed from 10^5 to 10^8 cfu/mL during short periods (Erskine and Bartlett, 1993; Van Werven et al., 1997). However, previous studies show that IMI by coliforms are rare in small ruminants (Gonzalo et al., 2002; Contreras et al., 2003), so the source of coliforms in bulk tank milk would be attributed to environmental contamination instead of mammary infection. These facts could explain the low correlation in dairy sheep (r = 0.11; Table 2) between BTSCC and coliform count.

In the current study, clinical outbreaks of contagious agalactia did not contribute to BTTBC and BTSCC variation. A surveillance program to prevent this infectious disease has been implemented for many years in all CPO flocks in the Castilla y León region of Spain. This program, based on health measures, border movement control, epidemiological monitoring, and annual or biannual flock vaccination, probably explains the limited effect of contagious agalactia on BTSCC. In the case of BTTBC, previous studies also showed a nonsignificant influence of this disease on BTTBC in dairy sheep (Gonzalo et al., 2006).

Regarding factors of variation for each bacteria group, season was an important effect ($P < 0.05$ to $P < 0.001$) influencing the variation of thermoduric, psychrotrophic, coliform, and GPCNC bacterial counts (Table 3). Greater seasonal differences were observed for psychrotrophs, which showed the highest values in winter (6.32 log cfu/mL) and lowest in summer (4.95 log cfu/mL); thus, seasonal temperature likely selects

Table 2. Test of significance of fixed effects of bulk tank total bacterial count (BTTBC) and bulk tank SCC (BTSCC) and β-coefficients from linear mixed regression model, and Spearman coefficients of correlation (r) with bacterial count covariables (Cov.)

	Log_{10} BTTBC					Log_{10} BTSCC				
Source	df	*F*-value	β	SE	r	df	*F*-value	β	SE	r
Intercept			4.14	0.08				5.90	0.03	
Cov. Log_{10} thermoduric count	1	13.27***	0.06	0.02	0.26					0.11
Cov. Log_{10} psychrotrophic count	1	47.62***	0.07	0.01	0.46					0.13
Cov. Log_{10} coliform count	1	35.54***	0.05	0.01	0.41					0.11
Cov. Log_{10} GPCNC[1] count	1	13.93***	0.03	0.01	0.29	1	12.94***	0.01	0.00	0.21
Cov. annual milk yield per ewe	1	15.90***	−0.07[2]	0.00[2]	0.24	1	10.58**	−0.03[2]	0.00[2]	0.29
Breed	1	13.82***				1	6.07*			
Assaf			0.15	0.04				0.05	0.02	
Churra			0					0		
Milking type	2	11.21***				2	34.44***			
Looped milkline			0.01	0.03				0.04	0.01	
Hand milking			0.21	0.05				0.20	0.02	
End-ended milkline			0					0		
Season						3	3.89**			
Winter								−0.03	0.01	
Fall								0.01	0.01	
Spring								−0.05	0.01	
Summer								0		
Antibiotic dry therapy	1	32.87***				1	102.76***			
No			0.16	0.03				0.14	0.01	
Yes			0					0		
Flock within milking type[3]	—	—				—	—			

[1]GPCNC = gram-positive catalase-negative cocci.

[2]Per 100 L of milk.

[3]Flock was considered as the random factor absorbed in the model; number of levels = 205.

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

Table 3. Least squares means of bulk tank thermoduric, psychrotrophic, coliform, and gram-positive catalase-negative cocci (GPCNC) counts by seasonal effect in dairy sheep

Variable	Winter	Spring	Summer	Fall	*F*-value
Log_{10} thermoduric count	2.97[a]	2.99[a]	2.97[a]	3.13[b]	2.45*
SE	0.06	0.06	0.06	0.06	
GM[1]	0.928	0.993	0.925	1.345	
Log_{10} psychrotrophic count	6.32[a]	5.53[b]	4.95[c]	5.85[d]	44.79***
SE	0.10	0.10	0.10	0.10	
GM	2,081.613	341.036	88.024	705.992	
Log_{10} coliform count	4.55[a]	3.67[b]	3.42[b]	3.54[b]	23.70***
SE	0.13	0.13	0.13	0.14	
GM	35.514	4.667	2.644	3.445	
Log_{10} GPCNC count	2.93[a]	2.87[a]	3.56[b]	2.89[a]	6.39***
SE	0.15	0.15	0.15	0.15	
GM	0.859	0.737	3.615	0.786	

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]Geometric mean $\times 10^3$ cfu/mL.

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

for environmental organisms. Coliforms were also higher in winter (4.55 log cfu/mL) than in other seasons (3.42 to 3.67 log cfu/mL), which can be associated with more dirty beds and udders due to the wetter weather. For instance, in winter, psychrotrophic bacteria, along with psychrotrophic strains of coliforms, could multiply rapidly in milk held at low temperatures, particularly when the hygiene of beds and udders is deficient. This effect was probably linked to variation in seasonal hygiene in the flocks more than to an effect of season on milk temperature in bulk tanks. Seasonal differences in bulk tank milk temperature were not compatible with seasonal values found for BTTBC, which were also highest in winter (5.23 log cfu/mL) and lowest in summer (5.05 log cfu/mL). Indeed, BTTBC data from this study indicate that milk storage systems efficiently hold the milk temperature within regulatory limits, so seasonal differences in bulk tank milk temperature should be small. Seasonal management practices (i.e., confinement versus grazing) would also influence the presence of psychrotrophs and coliforms in milk. The high concentration of psychrotrophic and coliform flora increased in winter, and its proven relationship with a high incidence of degradative actions on milk and cheese components warrants optimizing milking hygiene practices to prevent significant decline in milk and dairy product quality (de Garnica et al., 2011). The highest counts of GPCNC were found in summer (3.56 log cfu/mL) compared with the other seasons (2.87 to 2.93 log cfu/mL). Elevated temperatures and reduced milk yield per ewe would be important risk factors to dissemination of environment and contagious organisms belonging to this bacterial group.

Milking type effect was significant only for GPCNC, as shown in Table 4. Counts of GPCNC were higher for hand milking than for parlor machine milking. Along with the poor hygienic conditions of hand milking, the hand can act as a vector for transmission of environmental (e.g., *Enterococcus* spp.) and contagious (e.g., *Streptococcus agalactiae*) pathogens, increasing their counts in milk. Within machine milking, differences in GPCNC counts in parlors with looped milkline compared with dead-ended milkline (Table 4) were not significant.

Dry therapy was an important effect that significantly decreased BTTBC and BTSCC (Table 2), in agreement with other studies in dairy sheep (Gonzalo et al., 2005, 2010), but this practice was not effective to reduce GPCNC counts ($P > 0.05$). It is likely that GPCNC arise from bulk tank milk contamination (e.g., enterococci fecal contamination), not only from IMI. This fact could explain the high GPCNC counts found for hand-milked flocks (Table 4), in which the poor hygiene conditions favored milk contamination and made dry therapy ineffective (Gonzalo et al., 2009). The antibiotic dry therapy effect was, however, significant for psychrotrophic counts ($F = 6.65$, $P < 0.05$); counts (5.49 log cfu/mL) were lower when dry therapy was implemented than when this practice was not used (5.76 cfu/mL). Because mastitis pathogens are probably reduced by dry therapy, this result suggests that some strains of pathogens are potential psychrotrophic bacteria and may therefore contribute to the psychrotrophic count.

CONCLUSIONS

The BTTBC and BTSCC variables determined in dairy laboratories are very useful tools for continuous monitoring and improvement of milk hygiene quality in dairy sheep. Psychrotrophic, coliform, and thermoduric bacterial groups were significantly related to BTTBC,

Table 4. Least squares means of bulk tank gram-positive catalase-negative cocci counts by milking effect in dairy sheep

Variable	Hand milking	Machine milking: Looped milkline	Machine milking: Dead-ended milkline	*F*-value
Log_{10} count	3.43[a]	2.88[b]	2.88[b]	3.45*
SE	0.24	0.10	0.15	
GM[1]	2.710	0.761	0.753	

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Geometric mean $\times 10^3$ cfu/mL.

*$P < 0.05$.

whereas GPCNC was associated with both BTTBC and BTSCC variables in dairy sheep flocks. In addition, BTTBC correlated with BTSCC. Season, dry therapy, and milking type were important sources of variation for bulk tank milk differential counts; psychrotrophs and coliforms had elevated counts in winter, whereas GPCNC counts were higher in summer and in hand-milked flocks. Further studies are necessary to investigate on-farm risk factors associated with BTTBC in dairy sheep and to define effective discrimination thresholds for specific bacterial counts in bulk tank milk.

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Capítulo 3

COCOS GRAM POSITIVOS CATALASA NEGATIVOS EN LECHE DE OVEJA Y HALLAZGO DEL GEN *vanC1* EN UNA CEPA DE *ENTEROCOCCUS FAECALIS* VANCOMICINA RESISTENTE EN LECHE OVINA DE TANQUE.

INTRODUCCIÓN

Los cocos Gram-positivos catalasa-negativos (CGPCN) son un extenso grupo de géneros y especies de diferente importancia clínica y tecnológica en la leche de oveja y los productos lácteos. Pertenecen a las bacterias ácido lácticas (BAL) de bajo contenido en G+C y al filo *Firmicutes*. Los géneros más representativos son *Streptococcus*, *Enterococcus* y *Lactococcus;* aunque en la actualidad se han descrito no menos de 14 géneros dentro de este grupo taxonómico. La identificación precisa de los géneros y especies de CGPCN no es siempre posible en los laboratorios, ya que el diagnóstico de estos organismos en leche se realiza tradicionalmente basándose en pruebas fenotípicas y bioquímicas, las cuales son insuficientes para poder discernir entre especies que posean el mismo perfil fenotípico (Sawant *et al.*, 2002; Fortin *et al.* 2003, Zadocks *et al.*, 2011).

El desarrollo de técnicas de identificación basadas en la comparación de secuencias del 16S ADNr en bases de datos públicas ha permitido una identificación a nivel de género y especie mucho más rápida y precisa, además de su clasificación filogenética, especialmente en muestras de leche y otro tipo de ambientes con una amplia microbiota (Facklam y Elliott, 1995; Giraffa 2007).

Considerando la escasa documentación disponible sobre la diversidad CGPCN en leche de oveja, el objetivo de este estudio fue determinar la variabilidad de este grupo usando técnicas de secuenciación de la subunidad menor del ADNr intentando aportar una visión más objetiva y normalmente no requerida por los laboratorios de diagnóstico de mamitis o la autoridad competente.

La importancia de una identificación adecuada y el conocimiento de la diversidad de CGPCN en leche de oveja destinada a producción de queso reside en la dualidad de estos organismos, ya que entre ellos existen especies que pueden poseer un carácter patógeno para el hombre o los animales, o bien, ser usados como cultivos iniciadores o probióticos en productos lácteos.

Entre las especies de doble carácter, patógeno y beneficioso, una de las que mayor interés, preocupación y estudios genera es *Enterococcus faecalis*, debido fundamentalmente al incremento de casos de infección en humanos a nivel mundial. Ante la prevalencia observada de *E. faecalis* en leche de oveja se decidió realizar un screening de resistencia a glicopéptidos en los aislados de esta especie. La resistencia a un amplio espectro de antimicrobianos, principalmente a β-lactámicos, aminoglicósidos y glicopéptidos (vancomicina y teicoplanina), es una característica bien conocida en las especies del género *Enterococcus*. Los mecanismos de resistencia se pueden clasificar en adquiridos o intrínsecos y están asociados normalmente a una especie concreta o a un grupo de especies. Debido a

la dificultad para combatir las infecciones, en particular en pacientes con un sistema inmune deprimido, la asociación de aminoglicósidos con β-lactámicos o vancomicina constituye la terapia de elección en caso de infección grave debido a la acción de éstos dos últimos sobre la pared celular bacteriana (Teixeira *et al*, 2005). En 1988 la resistencia a la vancomicina se describió por primera vez en cepas de enterococos (Leclercq *et al*., 1988; Uttley *et al*., 1989) y en la actualidad se sospecha que la cadena alimentaria puede ser una fuente de Enterococcos Vancomicina Resistentes (EVR) (Robredo *et al*., 2000). Hasta el momento se han descrito 8 fenotipos diferentes de resistencia inducible a la vancomicina y un tipo no inducible (VanC) (Teixeira *et al*, 2005; Boyd *et al*., 2008). Los fenotipos más comúnmente descritos son VanA, VanB y VanC y están codificados respectivamente por los genes *vanA, vanB* y *vanC*. Los tipos VanA y VanB estan ligados a *Enterococcus faecalis* y *Enterococcus faecium* y confieren a las cepas portadoras altos niveles de resistencia a la vancomicina, en ambos casos, y de alta resistencia a la teicoplanina en el caso de VanA, mientras que las cepas VanB son sensibles a este último fármaco (Teixeira *et al*, 2005). El fenotipo VanC posee tres genotipos diferentes asociados, como marcadores de especie, a *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2*) y *E. flavescens* (*vanC3*). Estos tres genes son presumiblemente no transferibles debido a su localización cromosómica y confieren un nivel medio de resistencia a la vancomicina (CMI 4-32 mg/L) y sensibilidad a la teicoplanina (MIC ≤ 8 mg/L) de acuerdo con los puntos de corte del CLSI 2012 (National Committee for Clinical Laboratory Standards).

MATERIALES Y MÉTODOS

El estudio se llevó a cabo sobre un total de 192 cepas de CGPCN aisladas en Edwards Medium Modified (Oxoid Limited, Cambridge, UK) suplementado con 5 a 7 % de sangre desfibrinada de oveja (Oxoid Limited, Cambridge, UK), entre Octubre de 2010 y Diciembre de 2012. Colonias hemolíticas y no hemolíticas se sometieron al test de la catalasa. Los aislados se recultivaron en Agar Columbia con 5% de sangre desfibrinada de oveja a 37 ºC en atmósfera anaerobia. Se extrajo el ADN añadiendo 300 μL de cloroformo puro (Merk, NJ, USA) a un volumen igual de suspensión celular en agua pura. Tras una agitación las muestras se incubaron en microtubos de 1.5 mL a 80 ºC durante 20 min y posteriormente congelados a -20 ºC lo siguientes 20 min. El sobrenadante acuoso obtenido después de una centrifugación a 12000*g* se usó como ADN patrón. La amplificación del 16S rDNA fue llevada a cabo con los cebadores E8f and rP2 descritos por Wood *et al.* (1998). Los productos de PCR se purificaron con ExoSAP-IT (Isogen Life Science B.V. De Meern, Holanda) según las instrucciones del fabricante. La secuenciación de los amplicones purificados se llevó a cabo en el servicio de secuenciación del Centro Nacional de Microbiología (ISCIII, Majadahonda, España) usando el protocolo Big-Dye Terminator® Cycle Sequencing Kit v. 3.1 l (Applied Biosystems, Foster City, CA, US) y los oligonucleótidos de secuenciación E786F y U1115R (Baker *et al*., 2003). Las secuencias obtenidas se analizaron con el software Lasergene SeqMan II (DNASTAR Inc., WI, USA) y se compararon con la base de datos de secuencias del NCBI Gen Bank mediante la herramienta BLAST (Basic Local Alignment Search Tool, www.ncbi.nlm.nhi.gov) con el fin de identificar las especies.

El análisis de las secuencias de los aislados incluyó un alineamiento por el método Clustal W, la estimación de distancias por el algoritmo de Jukes-Cantor y la construcción de un árbol filogenético UPGMA con ayuda del programa Geneious Pro software v 4.8.5 (Biomatters Ltd., Auckland, New Zealand). Las secuencias menores de 1300 pb y 97% de similitud (considerado como umbral de especie) con su pariente más cercano en la herramienta BLAST fueron eliminadas del estudio. Las cepas de referencia usadas para la construcción del árbol y sus números de acceso en la base de datos Genbank (http://www.ncbi.nlm.nih.gov/genbank/) figuran en la Tabla 1.

Las cepas de *E. faecalis* obtenidas en este estudio fueron sometidas a un *screening* de resistencia a glicopéptidos por Multiplex PCR (Dutka-Malen *et al.*, 1995). Los amplicones obtenidos fueron purificados con ExoSAP-IT y comparados con secuencias disponibles en la herramienta BLAST. Con el fin de comprobar fenotípicamente la resistencia a glicopéptidos y a otros antimicrobianos de interés clínico se testaron 21 cepas de representativas de 15 explotaciones distribuidas en distintas localizaciones de Castilla y de 6 silos de almacenamiento del Consorcio de Promoción del Ovino (CPO) por medio de Etest (AB BIODISK) y/o Placas Microscan PM28 (Siemens). Para completar la caracterización se comprobó la presencia de una serie de genes de virulencia por multiplex PCR (Vankerckhoven *et al.*, 2004): sustancia agregante (*asa1*), gelatinasa (*gelE*), activador de citolisina (*cylA*), proteína de superficie enterocócica (*esp*) y hialuronidasa (*hyl*) y a un análisis MLST (Multylocus Sequence Typing) según el protocolo descrito en http://efaecalis.mlst.net/.

RESULTADOS Y DISCUSIÓN

La relación de especies y géneros, su número de aislados y la proporción respecto del total se muestran en la Tabla1. Las secuencias obtenidas se registraron en GenBank (números de acceso KC699044 - KC699235). La longitud media de las secuencias parciales de ADNr 16S fue 1368 ±180.2 bp (SD). La comparación de secuencias en la herramienta BLAST resultó en la identificación de 23 especies pertenecientes a 5 géneros: *Enterococcus* (10 especies), *Streptococcus* (9 especies), *Lactococcus* (2 especies), *Trichococcus* (1 especie) and *Aerococcus* (1 especie). El árbol con las posiciones filogenéticas relativas de las secuencias obtenidas entre sí y respecto a las cepas de referencia se muestra en la Figura 1. Las especies con porcentajes de similitud con su taxón más próximo mayores al 97 % fueron agrupadas en el árbol UPGMA de acuerdo a sus linajes ribosómicos. La consistencia del análisis filogenético fue avalado por altos valores de *bootstrap*.

La relevancia de las especies y grupos aislados de CGPCN en leche de grandes mezclas se resume en la Tabla 2.

El *screening* de resistencia a glicopéptidos por Multiplex PCR de las 54 cepas identificadas en el estudio anterior y otras tres cepas identificadas como *E. faecalis* que no cumplieron los requisitos (longitud de la secuencia y 97 % similitud) destacó la presencia de un único aislado que portaba el gen *vanC1*(cepa CNM 460_12). El amplicón *vanC1* purificado con ExoSAP-IT fue secuenciado y comparado con las secuencias disponibles en la base de datos de la herramienta BLAST corroborando su identidad al 100% con el gen "*Enterococcus gallinarum* strain eS464 *vanC1* gene" (número de acceso Genbank EU151772) y otras cepas portadoras del gen *vanC1*. Ante el hallazgo de una cepa de *E. faecalis* portadora

de un gen marcador de otra especie se registró la secuencia del amplicón obtenido en el NCBI Genbank y se le asignó el consiguiente número de acceso JX435778. La caracterización fenotípica de resistencia a glicopéptidos y a otros antimicrobianos de interés clínico con Microscan® PM28 plates reveló sensibilidad a ampicilina, macrólidos, aminoglicósidos y fluoroquinolonas, al contrario que otros EVR, normalmente de naturaleza multirresistente. Etest confirmó la resistencia a la vancomicina (CMI=12 mg/L) y la susceptibilidad a la teicoplanina (CMI=1 mg/L), según los criterios del CLSI. La amplificación de genes de virulencia enterocócicos por multiplex PCR dio lugar al siguiente perfil: *asa1*+, *gelE*+ y *cylA*+. El análisis MLST según el protocolo descrito en http://efaecalis.mlst.net/ que reveló el siguiente perfil alélico: ST-168 (*gdh*-20, *gyd*-1, *psts*-7, *gki*-25, *aroE*-23, *xpt*-2, *yqil*-2), compartiendo grupo con 15 cepas españolas de *E. faecalis* de origen vegetal y dos cepas europeas de origen clínico.

La expresión fenotífica de la resistencia a glicopéptidos estudiada con tiras de vancomicina Etest mostró en todos los *E. faecalis* una CMI ≥ 4 mgL^{-1} (Rango de CMI =4-12 mgL^{-1}) que es el punto de corte establecido por los organismos internacionales de referencia CLSI* y EUCAST** en 2012. Por otra parte, y de acuerdo con las mismas referencias todas las cepas de *E.faecalis* fueron sensibles a la teicoplanina (Rango de CMI = 0.5-2 mgL^{-1}, CMI puntos de corte = ≤8* and ≤2** mgL^{-1}).

Además, todos los *E. faecalis* portaron al menos un factor de virulencia: 3 cepas fueron *esp*+, 18 cepas *asa1*+, 19 cepas *gelE*+ y 6 cepas *cylA*+. Ninguna portaba el gen *hyl*+. Los perfiles de virulencia presentes fueron: *gelE*+ (3 cepas, 14,3%); *asa1*+/*gelE*+ (9 cepas, 42.9%); *esp*+/ *asa1*+/*gelE*+(1 strain, 7.4 %) and *asa1*+/*gelE*+/*cylA*+ (6 cepas, 28.6%). Los perfiles de sensibilidad a glicopéptidos y de virulencia se muestran en la Tabla 3.

Todas las especies enterocócicas aisladas en leche de tanque en este trabajo se consideran especies infecciosas para el hombre, excepto *E. italicus* y *E. viikkiensis* -este último aislado por primera vez en leche de oveja- y deben ser tomadas en consideración debido a que los EVR procedentes de leche y otras fuentes alimentarias pueden ser un reservorio de genes de resistencia transmisibles al hombre (Franz *et al.* 1999; Robredo *et al.*, 2000; Witte, 2000).

Según los datos bibliográficos disponibles, este es el primer hallazgo, tanto en cepas de origen humano como animal, de una cepa de *E. faecalis vanC1* fenotipo VanC. Un trabajo previo realizado en Alemania en el año 2012 ha puesto de manifiesto la presencia de dos cepas de *E. faecalis* portadoras de este gen pero que, en cambio, eran fenotípicamenete sensibles a la vancomicina (MIC = 1 mg/L) (Schwaiger *et al.*, 2012).

Este hallazgo puede ser considerado de gran importancia ya que el gen *vanC1* es utilizado como herramienta en la identificación de la especie *E. gallinarum* (Dutka-Malen *et al.*, 1995; Schwaiger *et al.*, 2012). Debido a que el uso de glicopéptidos en medicina veterinaria no está permitido en España, podemos presumir que la presencia del gen *vanC1* se debe a la transferencia horizontal entre *E. gallinarum* y *E. faecalis*. Este fenómeno puede ser atribuido a la presencia del factor de virulencia *asa1,* ya que confiere a las cepas que lo portan capacidad de intercambio genético (Vankerckhoven *et al.,* 2004).

Ambos hallazgos en cepas de origen ovino y porcino hacen necesario que se reconsideren los protocolos de monitorización e identificación de resistencias a glicopéptidos intrínsecas y adquiridas en *Enterococcus* spp.

CONCLUSIONES

Este trabajo reveló la presencia en leche de oveja de una amplia diversidad de CGPCN cultivables normalmente identificados erróneamente por métodos fenotípicos comunes. Es probable que estas especies hayan sido encontradas previamente en leche ovina pero que se hayan asociado con otros taxones del mismo género o de otros. El uso de técnicas moleculares para la identificación de este extenso grupo taxonómico puede ser de interés tanto para los productores como para la industria láctea, dando lugar a una visión más aproximada a la realidad de CGPCN presentes en sus productos y en relación a programas de control de calidad, control de mamitis, seguridad alimentaria o desarrollo de nuevos productos. Por otra parte, la leche de oveja producida en los países de la cuenca mediterránea, entre ellos España, se usa fundamentalmente para la producción de quesos y productos lácteos a partir de leche cruda o pasteurizada que pueden constituir una fuente de flora comensal potencialmente patógena y/o resistente a antibióticos. La ampliación del estudio sobre factores de virulencia y resistencia a antimicrobianos en CGPCN debe tenerse en cuenta en el futuro.

En el caso concreto de la presencia de *E. faecalis* vancomicina resistentes hallados en leche de oveja destinada a productos lácteos la relevancia aumenta, ya que esta especie es el enterococo predominante en los quesos producidos en los países del área mediterránea con recuentos de 10^4 a 10^7 ufc/g, constituyendo una fuente de adquisición potencial para el ser humano, como ya han apuntado diversos autores.

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INFORMACIÓN COMPLEMENTARIA C1.

Listado completo de cepas aisladas, denominación, especie, porcentaje de identificación y número de acceso en el Genbank.

Tabla 1. Aislados de CGPCN en leche de oveja de acuerdo a si identificación por análisis de la secuencia del ADNr 16S. Porcentajes en el total de aislados. Cepas de referencia y su número de acceso a GenBank. N: número de cepas.

AISLADOS	N	%	CEPA DE REFERENCIA	Nº ACC. GenBank
Enterococcus	**133**	**69.3**		
E. faecalis	54	28.1	ATCC 19433	AB012212.1
E. italicus/ E. saccharominimus	48	25.0	DSM 15952	AJ582753/AJ626902
E. hirae	11	5.7	DSM 20160	Y17302
E. durans	10	5.2	DSM 20633	AJ276354
E. gallinarum	4	2.1	ATCC 49573	AF039900
E. faecium	2	1.0	LMG 11423	AJ301830
E. pseudoavium	1	0.5	ATCC 49372	AF061002
E. viikkiensis	1	0.5	IE3.2	HQ378515
E. dispar	1	0.5	ATCC 51266	AF061007
E. malodoratus	1	0.5	LMG 10747	AJ301835
Trichococcus	**1**	**0.5**		
T. pasteurii	1	0.5	DSM 2381	X87150
Aerococcus	**1**	**0.5**		
A. viridans	1	0.5	ATCC 11563	M58797
Lactococcus	**14**	**7.2**		
L. lactis subsp. *lactis*	12	6.2	NCDO 604T	AB100803.1
L. garvieae	2	1.0	NIZO2415T	EU091459
Streptococcus	**43**	**22.4**		
Str. parauberis	14	7.3	DSM 6631	AY584477
Str. uberis	7	3.6	JCM 5709	AB023573
Str. agalactiae	7	3,6	JCM 5671	AB023574
Str. bovis/Str. equinus	4	2.1	ATCC 33317	M58835
Str. macedonicus	4	2.1	LAB617	Z94012
Str. infantarius subsp. *infantarius*	4	2.1	HDP 90056	AF177729
Str. salivarius	1	0.5	ATCC 7073	AY188352
Str. gallolyticus subsp. *gallolyticus*	1	0.5	ACM 3611	X94337
Str. lutetiensis	1	0.5	NEM 782	AJ297215
Total	192	**100**		

Tabla 2. Cuadro resumen de la importancia de los grupos y especies de CGPCN aislados en leche de oveja en Medicina Humana, Animal y Microbiología de los Alimentos.

Importancia en	Medicina Humana	Medicina Animal	Microbiología de los alimentos	REFERENCIAS
Enterococcus				
Grupo "faecalis"	Infección nosocomial. Multirresistencia a antibióticos.	Mamitis en rumiantes y perros.	Probiótico. Altos recuentos en quesos tradicionales de la cuenca mediterránea. Agente de maduración en quesos. Producción de bacteriocinas (enterocinas).	Ariznabarreta *et al.*, 2002 Franz *et al.*, 2003 y 2011 Vankerckhoven *et al.*, 2004
Grupo "faecium "	Infección nosocomial. Multirresistencia a antibióticos.	Mamitis en rumiantes.	Probiótico. Agente de maduración en quesos. Producción de bacteriocinas (enterocinas).	Nieto-Arribas *et al.*, 2011 Prakash *et al.*, 2005
Grupo "italicus"	ND*	ND	Altos recuentos en quesos tradicionales de la cuenca mediterránea. Aislado en leche y productos lácteos.	Fortina *et al.*, 2008 Fornasari *et al.*, 2008
Streptococcus				
Grupo "pyogenes"	GBS**: sepsis en neonatos y adultos inmunocomprometidos. *Str. uberis* y *Str. parauberis* ND.	Mamitis contagiosa en rumiantes.	ND	Hassan *et al.*, 2001 Bergonier *et al.*, 2003 Zadocks *et al.*, 2011
Grupo "bovis"	Asociado a infección nosocomial y patologías del colon.	Mamitis en pequeños rumiantes y vacuno.	Microbiota de leche y productos lácteos de todo el mundo.	Facklam 2002 McDougall *et al.*, 2002 Schlegel *et al.*, 2003 Jans *et al.*, 2011
Grupo "salivarius"	Sepsis y mamitis.	ND	Considerado como probiótico y cultivo iniciador debido a su cercanía con *Str. thermophilus*.	Facklam 2002 Contreras y Rodriguez, 2011
Lactococcus				
L. garvieae y L. lactis	Septicemia y endocarditis.	Mamitis en ganado vacuno. Lactococosis en peces.	*L. lactis* es probiótico y starter GRAS*** en productos lácteos. Recientemente se pone en duda su seguridad.	Facklam y Elliot, 1995 EFSA Journal 2012
Aerococcus y Trichococcus				
Aerococcus viridans	Mamitis e infecciones oportunistas.	Flora propia de la ubre. Aumento del recuento bacteriológico en tanque.	Flora termotolerante en leche y productos lácteos.	Devriese *et al.*, 1999 Verdier-Metz *et al.*, 2012
Trichococcus pasteurii	ND	ND	Flora psicrófila alterante en productos lácteos.	Hantsis-Zacharov y Halpern, 2007

*ND: no documentado.

** Grupo B de Streptococcus (*Str. agalactiae*).

***Generally Recognised as Safe. Especie reconocida como segura para su uso en alimentos.

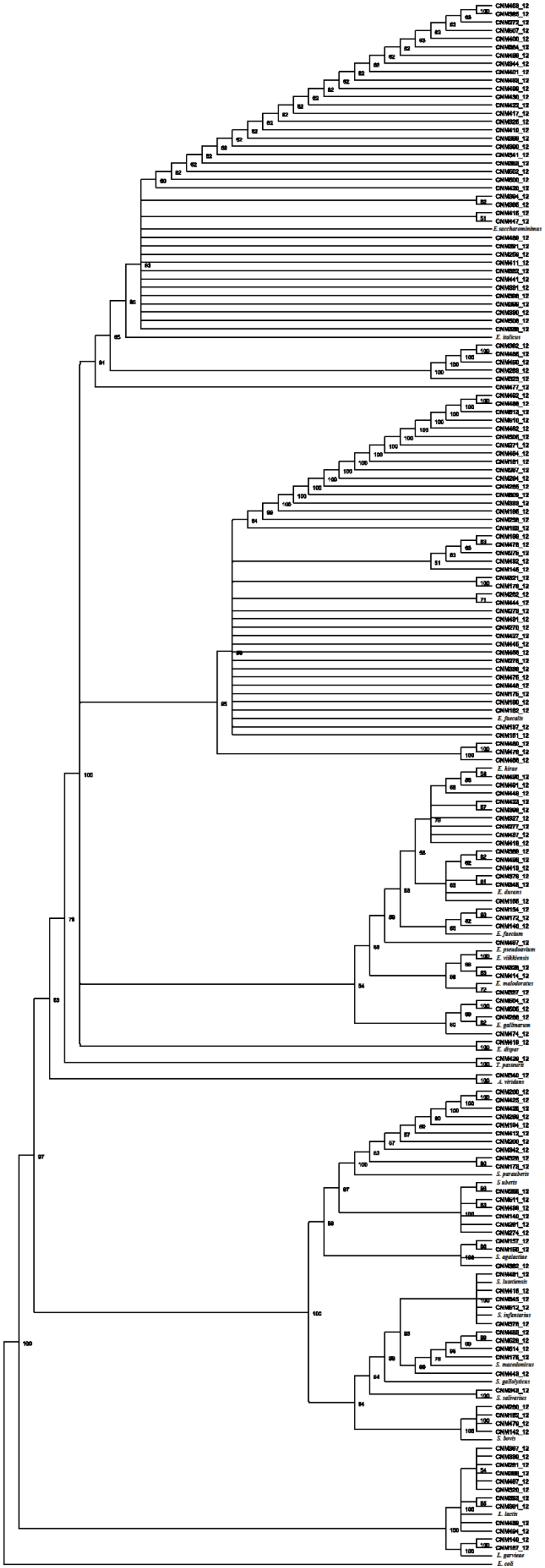
Tabla 3. Concentracion Minima Inhibidora (CMI) frente a glicopéptidos y perfil de virulencia de los aislados de *Enterococcus faecalis* procedentes de leche de oveja.

Cepa	CMI (mgL^{-1})		Perfil de virulencia				
Enterococcus faecalis	Vancomicina	Teicoplanina	*esp*	*hyl*	*asa1*	*gelE*	*cylA*
CNM134_12	4	2	+		+		
CNM135_12	6	0.75			+	+	
CNM136_12	6	0.5	+		+	+	
CNM137_12	12	0.75	+		+		
CNM138_12	4	0.75				+	
CNM178_12	6	0.75				+	
CNM258_12	6	1.5			+	+	
CNM262_12	6	0.38			+	+	+
CNM264_12	8	1			+	+	
CNM265_12	12	1.5			+	+	
CNM267_12	8	1				+	
CNM270_12	4	0.5			+	+	+
CNM271_12	6	0.75			+	+	+
CNM273_12	6	1			+	+	+
CNM276_12	12	1			+	+	
CNM321_12	6	1			+	+	
CNM336_12	8	1			+	+	
CNM444_12	8	1.5			+	+	
CNM456_12	6	0.75			+	+	
CNM460_12	12	1			+	+	+
CNM475_12	12	0.75			+	+	+
Rango CMI	4-12	0.5-2					

CNM: Centro Nacional de Microbiología.

Abreviaturas: sustancia agregante (*asa1*), gelatinasa (*gelE*), activador de citolisina (*cylA*), proteína de superficie enterocócica (*esp*) y hialuronidasa (*hyl*).

Figura 1. Dendrograma mostrando el análisis filogenético de las de CGPCN especies aisladas en leche de oveja y sus cepas de referencia. *Escherichia coli* se usó como *outgroup*.



INFORMACIÓN COMPLEMENTARIA C1. Cocos Gram positivos Catalasa Negativos aislados en leche de oveja. Identificación, longitud de la secuencia del ADNr 16S, porcentaje de similitud y número de acceso asignado en GenBank.

Cepa/Identificación secuencia	% Similitud	Año	Longitud secuencia (pb)	GenBank Acceso no.	BLAST más cercano ADNr 16S
CNM134_12	99.1	2010	905	KC699044	*Enterococcus faecalis*
CNM135_12	98.0	2010	292	KC699045	*Enterococcus faecalis*
CNM136_12	99.3	2010	907	KC699046	*Enterococcus faecalis*
CNM137_12	100	2010	1457	KC699047	*Enterococcus faecalis*
CNM138_12	99.3	2010	1197	KC699048	*Enterococcus faecalis*
CNM139_12	99.7	2010	927	KC699049	*Enterococcus faecium*
CNM140_12	99.2	2010	1437	KC699050	*Streptococcus uberis*
CNM141_12	94.5	2010	221	KC699051	*Enterococcus faecalis*
CNM142_12	99.4	2010	1426	KC699052	*Streptococcus bovis*
CNM144_12	99.8	2010	988	KC699053	*Enterococcus durans*
CNM145_12	99.9	2010	1429	KC699054	*Enterococcus faecalis*
CNM146_12	99.4	2010	1395	KC699055	*Enterococcus durans*
CNM147_12	99.8	2010	1002	KC699056	*Lactococcus garvieae*
CNM148_12	95.1	2010	1428	KC699057	*Enterococcus faecalis*
CNM149_12	99.7	2010	1452	KC699058	*Lactococcus garvieae*
CNM150_12	99.7	2010	1388	KC699059	*Streptococcus agalactiae*
CNM151_12	99.1	2010	1297	KC699060	*Enterococcus faecalis*
CNM152_12	99.1	2010	1464	KC699061	*Streptococcus bovis*
CNM153_12	98.9	2010	530	KC699062	*Enterococcus faecalis*
CNM154_12	98.2	2010	1365	KC699063	*Enterococcus durans*
CNM155_12	99.7	2010	1380	KC699064	*Enterococcus durans*
CNM156_12	99.1	2010	1025	KC699065	*Streptococcus agalactiae*
CNM157_12	99.2	2010	1440	KC699066	*Streptococcus agalactiae*
CNM172_12	99.6	2010	1428	KC699067	*Enterococcus durans*
CNM173_12	98.9	2010	1433	KC699068	*Streptococcus parauberis*
CNM174_12	99.6	2010	1235	KC699069	*Streptococcus agalactiae*
CNM175_12	99.6	2010	1426	KC699070	*Enterococcus faecalis*
CNM176_12	99.8	2010	1359	KC699071	*Streptococcus macedonicus*
CNM177_12	99.7	2010	1186	KC699072	*Enterococcus faecalis*
CNM178_12	99.3	2010	1444	KC699073	*Enterococcus faecalis*
CNM179_12	99.3	2010	1186	KC699074	*Streptococcus agalactiae*
CNM180_12	99.8	2010	1438	KC699075	*Enterococcus faecalis*
CNM181_12	99.5	2010	1462	KC699076	*Enterococcus faecalis*
CNM182_12	100	2010	1476	KC699077	*Enterococcus faecalis*
CNM183_12	99.7	2010	1419	KC699078	*Enterococcus faecalis*
CNM184_12	99.9	2010	1461	KC699079	*Streptococcus parauberis*
CNM185_12	99.9	2010	1417	KC699080	*Enterococcus faecalis*
CNM186_12	99.4	2010	948	KC699081	*Enterococcus faecalis*
CNM187_12	99.4	2010	1472	KC699082	*Lactococcus garvieae*
CNM188_12	99.7	2010	1476	KC699083	*Enterococcus faecalis*
CNM200_12	99.7	2010	1373	KC699084	*Streptococcus parauberis*
CNM258_12	100	2011	1432	KC699085	*Enterococcus faecalis*
CNM259_12	100	2011	1372	KC699086	*Enterococcus italicus*
CNM260_12	99.6	2011	1424	KC699087	*Streptococcus parauberis*
CNM261_12	99.4	2011	1445	KC699088	*Streptococcus uberis*
CNM262_12	99.7	2011	1450	KC699089	*Enterococcus faecalis*
CNM263_12	99.9	2011	1414	KC699090	*Enterococcus italicus*
CNM264_12	99.7	2011	1424	KC699091	*Enterococcus faecalis*
CNM265_12	99.7	2011	1424	KC699092	*Enterococcus faecalis*
CNM266_12	99.6	2011	1423	KC699093	*Streptococcus uberis*
CNM267_12	99.0	2011	1426	KC699094	*Enterococcus faecalis*
CNM268_12	99.9	2011	1416	KC699095	*Enterococcus gallinarum*
CNM269_12	99.1	2011	1458	KC699096	*Streptococcus parauberis*
CNM270_12	99.9	2011	1425	KC699097	*Enterococcus faecalis*
CNM271_12	100	2011	1435	KC699098	*Enterococcus faecalis*
CNM272_12	99.7	2011	1425	KC699099	*Enterococcus italicus*
CNM273_12	99.4	2011	1450	KC699100	*Enterococcus faecalis*
CNM274_12	99.3	2011	1379	KC699101	*Streptococcus uberis*
CNM275_12	99.6	2011	1423	KC699102	*Enterococcus faecalis*
CNM276_12	99.9	2011	1426	KC699103	*Enterococcus faecalis*
CNM277_12	99.9	2011	1430	KC699104	*Enterococcus hirae*
CNM280_12	99.6	2011	1382	KC699105	*Streptococcus bovis*
CNM281_12	99.9	2011	1429	KC699106	*Lactococcus lactis subsp. lactis*
CNM320_12	99.6	2011	1424	KC699107	*Lactococcus lactis subsp. Lactis*
CNM321_12	100	2011	1460	KC699108	*Enterococcus faecalis*
CNM322_12	99.7	2011	868	KC699109	*Streptococcus parauberis*
CNM323_12	99.9	2011	1414	KC699110	*Enterococcus italicus*
CNM325_12	99.7	2011	1422	KC699111	*Enterococcus italicus*
CNM326_12	99.4	2011	1458	KC699112	*Streptococcus parauberis*
CNM327_12	99.9	2011	1426	KC699113	*Enterococcus hirae*
CNM328_12	99.6	2011	1425	KC699114	*Enterococcus viikkiensis*

CNM330_12	99.9	2011	1371	KC699115	*Enterococcus italicus*
CNM331_12	99.9	2011	1383	KC699116	*Enterococcus italicus*
CNM332_12	99.9	2011	1381	KC699117	*Enterococcus italicus*
CNM333_12	99.7	2011	1423	KC699118	*Enterococcus faecalis*
CNM336_12	99.7	2011	1435	KC699119	*Enterococcus faecalis*
CNM337_12	99.9	2011	1415	KC699120	*Enterococcus malodoratus*
CNM338_12	99.9	2011	1371	KC699121	*Enterococcus italicus*
CNM339_12	99.9	2011	1423	KC699122	*Lactococcus lactis subsp. lactis*
CNM340_12	99.7	2011	1427	KC699123	*Aerococcus viridans*
CNM341_12	99.0	2011	1426	KC699124	*Enterococcus italicus*
CNM342_12	99.7	2011	1463	KC699125	*Streptococcus parauberis*
CNM343_12	99.2	2011	1426	KC699126	*Streptococcus salivarius*
CNM344_12	100	2011	1413	KC699127	*Enterococcus italicus*
CNM345_12	99.3	2011	1426	KC699128	*Streptococcus infantarius subsp. infantarius*
CNM346_12	99.4	2011	1412	KC699129	*Enterococcus durans*
CNM378_12	99.6	2011	1416	KC699130	*Streptococcus infantarius subsp. infantarius*
CMN379_12	99.6	2011	1384	KC699131	*Enterococcus durans*
CNM380_12	98.6	2011	1260	KC699132	*Enterococcus hirae*
CNM381_12	99.8	2011	1426	KC699133	*Lactococcus lactis subsp. lactis*
CNM382_12	99.7	2011	1433	KC699134	*Streptococcus agalactiae*
CNM383_12	99.7	2011	1408	KC699135	*Enterococcus italicus*
CNM384_12	99.6	2011	1414	KC699136	*Enterococcus italicus*
CNM385_12	99.9	2011	1425	KC699137	*Enterococcus italicus*
CNM386_12	99.8	2011	1443	KC699138	*Enterococcus italicus*
CNM387_12	99.8	2011	1422	KC699139	*Lactococcus lactis subsp. lactis*
CNM388_12	99.6	2011	1411	KC699140	*Lactococcus lactis subsp. lactis*
CNM389_12	99.6	2011	1417	KC699141	*Enterococcus durans*
CNM390_12	99.7	2011	1424	KC699142	*Enterococcus italicus*
CNM391_12	99.2	2011	1414	KC699143	*Enterococcus italicus*
CNM392_12	99.9	2011	1383	KC699144	*Enterococcus italicus*
CNM393_12	99.1	2011	1421	KC699145	*Lactococcus lactis subsp. lactis*
CNM394_12	99.9	2011	1424	KC699146	*Enterococcus italicus*
CNM395_12	99.4	2011	1430	KC699147	*Enterococcus italicus*
CNM396_12	99.8	2011	1398	KC699148	*Enterococcus hirae*
CNM397_12	100	2011	1040	KC699149	*Enterococcus hirae*
CNM398_12	99.4	2011	1432	KC699150	*Enterococcus italicus*
CNM399_12	99.9	2011	1371	KC699151	*Enterococcus italicus*
CNM400_12	99.8	2011	1416	KC699152	*Enterococcus italicus*
CNM401_12	99.9	2011	1416	KC699153	*Enterococcus italicus*
CNM410_12	99.8	2011	1416	KC699154	*Enterococcus italicus*
CNM411_12	99.8	2011	1381	KC699155	*Enterococcus italicus*
CNM412_12	99.4	2011	1439	KC699156	*Streptococcus parauberis*
CNM413_12	99.1	2011	1429	KC699157	*Enterococcus durans*
CNM414_12	99.8	2011	1397	KC699158	*Enterococcus pseudoavium*
CNM415_12	99.7	2011	1417	KC699159	*Streptococcus infantarius*
CNM416_12	99.7	2011	1432	KC699160	*Enterococcus italicus*
CNM417_12	99.7	2011	1424	KC699161	*Enterococcus italicus*
CNM418_12	99.9	2011	1429	KC699162	*Enterococcus hirae*
CNM419_12	99.8	2011	1394	KC699163	*Enterococcus dispar*
CNM420_12	99.8	2011	1413	KC699164	*Enterococcus italicus*
CNM422_12	99.5	2011	1426	KC699165	*Enterococcus italicus*
CNM423_12	99.8	2011	1425	KC699166	*Enterococcus hirae*
CNM425_12	99.4	2011	1458	KC699167	*Streptococcus parauberis*
CNM426_12	99.2	2011	1457	KC699168	*Streptococcus parauberis*
CNM427_12	99.9	2011	1421	KC699169	*Enterococcus faecalis*
CNM429_12	99.5	2011	1435	KC699170	*Trichococcus pasteurii*
CNM430_12	99.8	2011	1423	KC699171	*Enterococcus italicus*
CNM431_12	100	2011	1425	KC699172	*Enterococcus faecalis*
CNM432_12	99.7	2011	1471	KC699173	*Enterococcus faecalis*
CNM437_12	99.7	2011	1430	KC699174	*Enterococcus hirae*
CNM438_12	99.7	2011	1299	KC699175	*Streptococcus uberis*
CNM439_12	99.5	2011	1519	KC699176	*Lactococcus lactis subsp. lactis*
CNM441_12	99.8	2011	1383	KC699177	*Enterococcus italicus*
CNM443_12	99.8	2011	1463	KC699178	*Streptococcus gallolyticus subsp. gallolyticus*
CNM444_12	99.6	2011	1457	KC699179	*Enterococcus faecalis*
CNM445_12	99.8	2011	1398	KC699180	*Enterococcus faecalis*
CNM447_12	99.1	2011	1388	KC699181	*Enterococcus italicus*
CNM448_12	99.8	2011	1422	KC699182	*Enterococcus faecalis*
CNM449_12	100	2011	1415	KC699183	*Enterococcus hirae*
CNM450_12	99.3	2011	1429	KC699184	*Enterococcus italicus*
CNM453_12	99.9	2011	1423	KC699185	*Enterococcus italicus*
CNM456_12	99.8	2011	1456	KC699186	*Enterococcus faecalis*
CNM459_12	95.5	2011	1082	KC699187	*Streptococcus agalactiae*
CNM460_12	98.8	2011	1388	KC699188	*Enterococcus faecalis*
CNM461_12	96.8	2011	1387	KC699189	*Enterococcus italicus*
CNM463_12	99.6	2011	1429	KC699190	*Enterococcus italicus*
CNM464_12	95.5	2011	1302	KC699191	*Enterococcus italicus*

CNM465_12	96.5	2011	1450	KC699192	*Streptococcus parauberis*
CNM466_12	98.7	2011	1419	KC699193	*Enterococcus faecalis*
CNM467_12	98.7	2011	1417	KC699194	*Enterococcus faecium*
CNM468_12	96.7	2011	1426	KC699195	*Enterococcus faecalis*
CNM471_12	99.4	2011	653	KC699196	*Streptococcus parauberis*
CNM472_12	99.2	2011	1001	KC699197	*Streptococcus parauberis*
CNM473_12	96.1	2011	1415	KC699198	*Streptococcus uberis*
CNM474_12	98.4	2011	1369	KC699199	*Enterococcus gallinarum*
CNM475_12	99.9	2011	1423	KC699200	*Enterococcus faecalis*
CNM476_12	98.8	2011	1452	KC699201	*Enterococcus faecalis*
CNM477_12	99.0	2011	1423	KC699202	*Enterococcus italicus*
CNM478_12	99.0	2011	1421	KC699203	*Enterococcus faecalis*
CNM479_12	99.4	2011	1434	KC699204	*Streptococcus bovis*
CNM480_12	99.6	2011	1268	KC699205	*Enterococcus italicus*
CNM481_12	99.6	2011	1387	KC699206	*Streptococcus infantarius subsp. infantarius*
CNM482_12	99.7	2011	1458	KC699207	*Enterococcus faecalis*
CNM483_12	99.7	2011	1453	KC699208	*Streptococcus macedonicus*
CNM484_12	99.7	2011	1433	KC699209	*Enterococcus faecalis*
CNM485_12	99.9	2011	1427	KC699210	*Enterococcus italicus*
CNM486_12	99.9	2011	1415	KC699211	*Enterococcus italicus*
CNM487_12	99.5	2011	1412	KC699212	*Lactococcus lactis subsp. lactis*
CNM488_12	99.8	2011	1421	KC699213	*Enterococcus faecalis*
CNM489_12	99.6	2011	1418	KC699214	*Enterococcus italicus*
CNM490_12	100	2011	1421	KC699215	*Enterococcus hirae*
CNM491_12	99.9	2011	1421	KC699216	*Enterococcus hirae*
CNM492_12	99.6	2011	1424	KC699217	*Enterococcus faecalis*
CNM494_12	99.9	2011	1423	KC699218	*Lactococcus lactis subsp. lactis*
CNM498_12	99.4	2011	1418	KC699219	*Enterococcus durans*
CNM499_12	99.9	2011	1414	KC699220	*Enterococcus italicus*
CNM500_12	100	2011	1422	KC699221	*Enterococcus italicus*
CNM501_12	100	2011	1259	KC699222	*Lactococcus lactis subsp. lactis*
CNM502_12	99.8	2011	1422	KC699223	*Enterococcus italicus*
CNM504_12	99.8	2011	1440	KC699224	*Enterococcus gallinarum*
CNM505_12	100	2011	1428	KC699225	*Enterococcus gallinarum*
CNM506_12	100	2011	1466	KC699226	*Enterococcus faecalis*
CNM507_12	99.6	2011	1424	KC699227	*Enterococcus italicus*
CNM508_12	100	2011	1371	KC699228	*Enterococcus italicus*
CNM509_12	99.8	2011	1424	KC699229	*Enterococcus faecalis*
CNM510_12	99.8	2011	1398	KC699230	*Enterococcus faecalis*
CNM511_12	99.4	2011	1453	KC699231	*Streptococcus uberis*
CNM512_12	99.7	2011	1464	KC699232	*Streptococcus lutetiensis*
CNM513_12	99.7	2011	1435	KC699233	*Enterococcus faecalis*
CNM514_12	99.8	2011	1453	KC699234	*Streptococcus macedonicus*
CNM528_12	99.7	2011	1434	KC699235	*Streptococcus macedonicus*

JMM Correspondence

Presence of the *vanC1* gene in a vancomycin-resistant *Enterococcus faecalis* strain isolated from ewe bulk tank milk

A broad spectrum of antimicrobial resistance, mainly to aminoglycosides, β-lactams and glycopeptides (vancomycin and teicoplanin) is a well-known characteristic of *Enterococcus* species. Resistance mechanisms can be classified as either acquired or intrinsic and are usually associated with a particular species or a given group of species. Due to the difficulty in eliminating systemic and local infections, especially in patients with an impaired immune system, a synergistic combination of a cell-wall-active antimicrobial (β-lactam or glycopeptide) plus an aminoglycoside constitutes a first-line choice for serious infections (Teixeira & Facklam, 2005). In 1988 vancomycin resistance was described for the first time in enterococcal strains (Leclercq *et al.*, 1988; Uttley *et al.*, 1989) and nowadays the food chain is suspected of being a source of vancomycin-resistant enterococci (VRE) (Robredo *et al.*, 2000; Bonten *et al.*, 2001). At present, one type of non-inducible (VanC) and eight different types of inducible glycopeptide resistance have been described (Teixeira & Facklam, 2005; Boyd *et al.*, 2008; Xu *et al.*, 2010; Lebreton *et al.*, 2011). The most common phenotypes are VanA, VanB and VanC. VanA and VanB (encoded by the *vanA* and *vanB* genes) are linked to *Enterococcus faecalis* and *Enterococcus faecium* strains, and are considered the most clinically relevant phenotypes, resulting in high-level resistance to both vancomycin and teicoplanin in the case of VanA strains, and a moderate to high level of vancomycin resistance and sensitivity to teicoplanin in the case of VanB enterococci (Teixeira & Facklam, 2005). The *vanC* intrinsic resistance genotype is associated with several enterococcal species: *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2*) and *E. flavescens* (*vanC3*). The chromosomal location of VanC genes makes them presumably non-transferable, conferring an intermediate resistance level to vancomycin (MIC 4–32 mg l^{-1}) and sensitivity to teicoplanin (MIC $\leqslant$8 mg l^{-1}), according to the Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints (CLSI, 2012). In the context of a study on the diversity of Gram-positive, catalase-negative cocci isolated from sheep's bulk tank milk destined for the fabrication of cheese, a total of 57 strains of *E. faecalis* was isolated. Laboratory isolation from milk was performed by inoculating modified Edward's medium (Oxoid) supplemented with 5 % defibrinated sheep blood, as per manufacturer's instructions. Colonies obtained were subcultured overnight on Columbia agar (Oxoid) in a 5 % CO_2 atmosphere at 37 °C. DNA from pure cultures was extracted with BackLight Cards (2B Blackbio S.L). Strain identity was confirmed by API Rapid ID 32 Strep (bioMérieux) and by 16S rRNA gene sequencing. Glycopeptide-resistance genotype screening by PCR (Dutka-Malen *et al.*, 1995) detected the presence of *vanC1* in a unique isolate, strain CNM_460/12. This *vanC1* amplicon was purified with ExoSAP-IT (Isogen Life Science), sequenced and compared with sequences in the National Center for Biotechnology Information database (Basic Local Alignment search tool, www.ncbi.nlm.nih.gov), which corroborated its similarity with the sequence of the *vanC1* gene from *E. gallinarum* strain eS464 (GenBank accession no. EU151772) and with those of other VanC1 strains. In order to determine phenotypic antimicrobial susceptibilities, the strain was subjected to Microscan PM28 plates (Siemens) and showed susceptibility to ampicillin, macrolides, aminoglycosides and fluoroquinolones, unlike clinical VRE strains. The Etest (AB bioMérieux) confirmed resistance to vancomycin (MIC=12 mg l^{-1}) and susceptibility to teicoplanin (MIC=1 mg l^{-1}), interpreted according to CLSI criteria (CLSI, 2012). The following enterococcal virulence factors were tested by multiplex PCR (Vankerckhoven *et al.*, 2004): aggregation substance (*asa1*), gelatinase (*gelE*), cytolysin activator (*cylA*), enterococcal surface protein (*esp*) and hyaluronidase (*hyl*), with positive results for the *asa1*, *gelE* and *cylA* genes. Multi-locus sequence typing analysis was performed as described in http://efaecalis.mlst.net/ and determined that this strain belongs to sequence type (ST)168 (allelic profile: *gdh*-20, *gyd*-1, *psts*-7, *gki*-25, *aroE*-23, *xpt*-2, *yqil*-2). This infrequent ST is shared by 15 other Spanish strains isolated from vegetables and two clinical isolates from France and the Netherlands. This is the first report, to the best of our knowledge, of a *vanC1* gene in *E. faecalis* ST168 with a vancomycin-resistant phenotype isolated from sheep bulk tank milk intended for dairy products. A previous report on diseased pigs in Germany detected the *vanC1* gene in two *E. faecalis* isolates; however, these strains were susceptible to vancomycin (MIC=1 mg l^{-1}) (Schwaiger *et al.*, 2012). This finding is significant, as the detection of the *vanC1* resistance gene is a useful tool for the identification of *E. gallinarum* (Dutka-Malen *et al.*, 1995; Schwaiger *et al.*, 2012). Because the veterinary use of glycopeptide compounds is not permitted in Spain, we can presume, as in the case of porcine isolates, that the presence of the *vanC1* gene in an *E. faecalis* isolate may be due to the horizontal transfer between *E. gallinarum* and *E. faecalis*. This phenomenon may be attributed to the *asa1* gene, as it facilitates conjugative exchange (Vankerckhoven *et al.*, 2004; Schwaiger *et al.*, 2012). These two recent occurrences of *vanC1* genotypes in *E. faecalis* animal isolates emphasize the need for screening for the presence of both acquired and intrinsic glycopeptide resistance genes. The important finding of vancomycin-resistant *E. faecalis* in sheep's milk intended for manufacturing dairy products is significant, as *E. faecalis* is the predominant enterococcal species in

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Mediterranean cheeses (enterococcal counts range from 10^4 to 10^7 c.f.u. g^{-1}) (Franz *et al.*, 2003; Nieto-Arribas *et al.*, 2011), which could potentially be transferred to humans. In addition, the ability of this species to acquire and transfer resistance genes suggest that its presence should be carefully monitored throughout the food chain.

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Abstract: Species diversity of 192 isolates of a collection of Gram Positive Catalase Negative cocci (GPCNC) from sheep bulk tank milk was investigated using 16S rDNA sequencing. Molecular approach enabled the identification of 23 species of GPCNC of sanitary and technological importance within Enterococcus, Streptococcus, Lactococcus, Aerococcus and Trichococcus genera. This is relevant since ovine dairy products migth be a source of commensal and potentially pathogenic or drug resistant GPCNC. Control strategies are needed in the food chain to optimize food safety and product development.

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León, June 11th 2013

Dear Sir,

We are submitting the manuscript entitled **"Diversity of Gram-positive catalase-negative cocci in sheep bulk tank milk by comparative 16S rDNA sequence analysis"** to be considered for publication in the International Dairy Journal as a short communication.

The paper has been prepared from a longer version previously submitted (INDA-D-13-00204), taking into account the suggestions made by the editorial office.

Yours sincerely,

Jesus A. Santos

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Diversity of Gram-positive catalase-negative cocci in sheep bulk tank milk by comparative 16S rDNA sequence analysis.

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ABSTRACT

Species diversity of 192 isolates of a collection of Gram Positive Catalase Negative cocci (GPCNC) from sheep bulk tank milk was investigated using 16S rDNA sequencing. Molecular approach enabled the identification of 23 species of GPCNC of sanitary and technological importance within *Enterococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Trichococcus* genera. This is relevant since ovine dairy products migth be a source of commensal and potentially pathogenic or drug resistant GPCNC. Control strategies are needed in the food chain to optimize food safety and product development.

1. Introduction

Bacterial population of raw milk comes from different sources as the interior and the skin of the udder, incorrect milking practices, storage and milking equipment and

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the environment of the farm or the parlor. Current EU regulations on small ruminants' raw milk microbiological content (EC Regulation no. 853/2004) implement aerobic mesophilic counts without taking into account specific bacterial identification. Gram positive catalase negative cocci (GPCNC) are a group of genera and species with clinical and technological importance in milk and milk products. The most representative genera of this group are *Streptococcus*, *Enterococcus* and *Lactococcus* and not less than other 14 genera have been described (Ruoff, 2002). Several species of GPCNC have been pointed as primary or secondary mammary, opportunistic, emerging and/or nosocomial pathogens, and even as probiotics or maturation and ripening agents in dairy products (Franz, Stiles, Schleifer& Holzapfel, 2003). GPCNC species identification is likely to be unreliable because traditional diagnosis is based on phenotypic characteristics and biochemical reactions that are not sufficient for an accurate identification since some of them are so closely related that show the same pheno-profile (Sawant, Pillai & Jayarao 2002). The development of 16S rDNA sequence databases has enabled a rapid and more precise identification and classification of genera and species, especially in milk and other samples with a wide and variable microbiota (Facklam 2002)

Considering the above background and the little information available on GPCNC diversity in sheep milk, the aim of this study was to assess the variability of this group using 16S rDNA analysis in order to provide an accurate view on streptococci and streptococci-like organisms present in bulk tank sheep milk. The dual interest of knowing the diversity of GPCNC in sheep milk lies in the importance of the species of this group as harmful human pathogens and beneficial technological starter and probiotic cultures.

2. Materials and methods

2.1. Microorganisms

The study has been conducted on a collection of 192 isolates of GPCNC obtained from samples of silo and bulk tank sheep milk by conventional microbiological methods in previous works (de Garnica, Santos & Gonzalo, 2011; de Garnica, et al., 2012). The strains were recovered from frozen cultures by incubating in BHI broth (Oxoid, Basingstoke, UK) for 24 h at 37 ºC.

2.2. Molecular identification

DNA from a loop of a fresh overnight culture was extracted as previously described (de Garnica, Valdezate, Saez-Nieto & Gonzalo, 2013). The amplification of 16S rDNA gene was performed with primers E8f (5´-AGA GTT TGA TCC TGG CTC AG-3´) and rP2-R (5´-ACG GCT ACC TTG TTA CGA CTT-3´) and 5 µL of DNA template. Sequencing of amplicons was carried out with sequencing primers E786F and U1115R (Baker, Smith & Cowan, 2003). 16S rDNA sequences were assembled using Lasergene SeqMan II Pro software (DNASTAR Inc., WI, USA) and compared against sequences registered in GenBank database by BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis of the 16S rDNA sequences included distance estimation with Jukes-Cantor algorithm and the construction of a tree with the UPGMA method in Geneious Pro software v 4.8.5 (Biomatters Ltd., Auckland, New Zealand). Sequences under 1300 bp length were removed from the phylogenetic study. Reference strains used for the tree construction and their Genbank accession numbers are shown in Table 1.

2.3. Phenotypic characterization

Highly similar sequence matches were resolved supplementing the 16S rDNA analysis with the API Rapid ID 32 Strep (bioMérieux, Marcy l'Etoile, France) and Biolog GP microplate identification systems (Biolog, Hayward, CA, USA) and/or the latex agglutination Lancefield group test Pastorex Strep (Bio-Rad, Hercules, CA, USA).

3. Results and discussion

The identification to species level of the GPCNC collection obtained in bulk tank sheep milk was based on the analysis of the 192 sequences obtained (accession numbers KC699044 - KC699235). The BLAST comparison resulted in identification of 23 species belonging to five genera: *Enterococcus* (10 species, 133 isolates), *Streptococcus* (9 species, 36 isolates), *Lactococcus* (2 species, 14 isolates), *Trichococcus* (1 species, 1 isolate) and *Aerococcus* (1 species, 1 isolate). Genera and species identified and reference strains used are listed in Table 1. The tree showing phylogenetic relative positions of isolates and related type strains is shown in Figure 1. The consistency of the analysis was supported by high bootstrap values.

According to the current classification of the genus *Enterococcus* (Devriese, Pot & Collins, 2008; Fornasari Rossetti, Remagni & Giraffa, 2008), species identified among our isolates were as follows: one species of the *"*faecalis*"* group (*E. faecalis,* 54 strains, 28.1 %), one of the *"*italicus*"* group (*E. italicus,* 48 strains, 25.0 %), one species of the "casseliflavus" group (*E. gallinarum*, 4 strains, 2.1 %), three of the "faecium" group (*E. hirae* ,11 strains, 5.7 %; *E. durans*, 10 strains, 5.2 % and *E. faecium*, 2 strains, 1.0 %), other three species of the "avium" group (*E. pseudoavium*, *E. viikkiensis*, *E. malodoratus*, 1 strain and 0.5% each) and a unique strain (0.5 %) of *E. dispar* that traditionally forms a separate lineage.

E. faecalis was the predominant species, in correspondence with other works on microbial diversity of sheep and goat raw milk and cheeses in Spain and close Mediterranean countries (Franz et al., 2003). This is of interest since *E. faecalis* is one of the most prevalent enterococcal pathogens in human nosocomial opportunistic infections

The *Enterococcus faecium* group (*E. durans*, *E. faecium* and *E. hirae*) has been previously described in sheep milk (Ariznabarreta, Gonzalo & San Primitivo, 2002**)**. *E. faecium* group occur in human and farm animal faeces including sheep (Franz et al., 2011) and may play a role as probiotics and ripening agents in dairy products (Franz et al., 2011). Other benefit attributed to these species is the bacteriocin (enterocin) production against other important pathogens as *Listeria* spp., *S. aureus* or *Clostridium* spp. (Franz et al., 2003). There are limited data available on *E. italicus* presence in milk and dairy products, presumably due to identification difficulties, although quantification in Italian cheeses demonstrated a considerable amount per gram (Fornasari et al., 2008). *E. viikkiensis*, a newly described species (Rahkila et al., 2011), was confirmed by 16S analysis and positive D Lancefield reaction for the first time in milk to the best of our knowledge.

Remarkably, all the enterococcal species identified in milk in this work, except for *E. italicus* and *E. viikkiensis*, are considered as infectious for humans.

According to Kilian (2005), the streptococci isolates obtained in this work are divided into three clusters. The pyogenic group was predominant (24 strains, 61.5% of streptococci) with three species identified (*S. parauberis* (14 strains, 7.3%), *S. uberis* (7 strains, 3.6%) and *S. agalactiae* (7 strains, 3.6%)), followed by the "bovis" group (14 strains, 35.9% of streptococci, *S. bovis/S. equinus* (4 strains, 2.1%), *S. macedonicus* (4

117 strains, 2.1%), *S. infantarius* subsp. *infantarius* (4 strains, 2.1%), *S. gallolyticus* subsp.
118 *gallolyticus* (1 strain, 0.5%) and *S. lutetiensis* (1 strain, 0.5%)) and a single
119 representative of "salivarius" group (*S. salivarius*, 1 strain, 2.6 % of streptococci).

120 Streptococci are considered commensal potentially pathogenic or pathogenic
121 bacteria (Hardie & Wiley 1997). *S. uberis* and *S. parauberis* are not documented as
122 human infection agents, whereas *S. agalactiae* (group B streptococcus or GBS) is
123 associated with neonatal and immunocompromised adult severe sepsis. Though GBS
124 zoonosis has been discarded in cattle (Zadoks, Middleton, McDougall, Katholm&
125 Schukken 2011), no comparative studies between human and ovine strains have been
126 performed. *S. uberis* and *S. agalactiae* were previously reported by other authors in
127 sheep bulk tank milk (Ariznabarreta et al., 2002; de Garnica et al., 2011), however we
128 have found no available data for *S. parauberis* in dairy sheep farms probably due to its
129 phenotypic similarity with *S. uberis*.

130 Species of *Streptococcus* belonging to *S. bovis* group are causative agents of
131 infection in humans and animals. Bacteriaemia, endocarditis and colon malignancies
132 attributed to "bovis" group are widely documented in human medicine (Kilian, 2005).
133 Species of this cluster identified in this work have been reported as microflora of milk
134 and/or dairy products all over the world (Jans, Lacroix& Meile, 2012).

135 The only isolate of the "salivarius" group found was *S. salivarius*. This species
136 causes nosocomial infection and mastitis in humans (Contreras & Rodríguez, 2011)
137 however it has been considered a suitable probiotic and starter culture in fermented food
138 as it is closely related to *Streptococcus thermophilus*, a generally recognized as safe
139 microorganism (EFSA, 2012).

140 The remaining isolates were identified as *Lactoccocus lactis* subsp. *lactis* (11
141 strains), *Lactococcus garviae* (3 strains), *Aerococcus viridans* (1 strain) and
142 *Trichococcus pasteurii* (1 strain).

143 Lactococcal species from milk isolated in this work are generally considered as
144 safe due to their low incidence in human infections (Casalta & Montel, 2008). The
145 European Food Safety Authority (EFSA) considers appropriate the use of *L. lactis*
146 subsp. *lactis* in fermented food but manifests a growing concern about its pathogenic
147 profile (EFSA ,2012).

148 *Aerococcus viridans*, another opportunistic human pathogen, has been reported
149 to increase bacterial counts in bulk tank cow milk (Zadoks,Gonzalez, Boor & Schukken,
150 2004). *A. viridans*, *E. durans*, *E. faecalis*, *E. faecium* and *S. macedonicus* has recently
151 pointed as thermotolerant bacteria in the dairy industry which could involve persistence
152 in pasteurized dairy products (Walsh, Meade, Mcgill & Fanning, 2012).

153 Little information is available on *Trichococcus pasteurii* presence in milk and/or
154 dairy products. Some authors have included *Trichococcus* genus within milk
155 psychrotrophic spoiler bacterial communities, together with *S. parauberis*, *A. viridans*,
156 *E. faecium* and *L. lactis*, other GPCNC we have identified in sheep bulk tank milk
157 (Hantsis-Zacharov & Halpern, 2007).

158

159 **4. Conclusions**

160 This study revealed a wide diversity of culturable GPCNC in sheep bulk tank
161 milk normally under or misidentified by common phenotypic methods. It is likely that
162 these species have been found in ovine milk before but have been associated with other

taxa within the same genus or others. The molecular approach to the identification of this large taxonomic group can be of help to both farmers and dairy industry in getting more accurate view of gram positive cocci population in sheep milk regarding quality control programs, food safety and new products development.

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236

237

238 **Table1**. GPCNC isolates in sheep milk according to genus and species identified by
239 16S rDNA analysis, percentage in total isolations, reference strains code and accession
240 number to GenBank database.

Isolates	N	%	Reference strain	GenBank Accession number
Enterococcus	**133**	**69.3**		
E. faecalis	54	28.1	ATCC 19433	AB012212.1
E. italicus/ E. saccharominimus	48	25.0	DSM 15952	AJ582753/AJ626902
E. hirae	11	5.7	DSM 20160	Y17302
E. durans	10	5.2	DSM 20633	AJ276354
E. gallinarum	4	2.1	ATCC 49573	AF039900
E. faecium	2	1.0	LMG 11423	AJ301830
E. pseudoavium	1	0.5	ATCC 49372	AF061002
E. viikkiensis	1	0.5	IE3.2	HQ378515
E. dispar	1	0.5	ATCC 51266	AF061007
E. malodoratus	1	0.5	LMG 10747	AJ301835
Trichococcus	**1**	**0.5**		
T. pasteurii	1	0.5	DSM 2381	X87150
Aerococcus	**1**	**0.5**		
A. viridans	1	0.5	ATCC 11563	M58797
Lactococcus	**14**	**7.2**		
L. lactis subsp. *lactis*	12	6.2	NCDO 604T	AB100803.1
L. garvieae	2	1	NIZO2415T	EU091459
Streptococcus	**43**	**22.4**		
S. parauberis	14	7.3	DSM 6631	AY584477
S. uberis	7	3.6	JCM 5709	AB023573
S. agalactiae	7	3.6	JCM 5671	AB023574
S. bovis/S. equinus	4	2.1	ATCC 33317	M58835
S. macedonicus	4	2.1	LAB617	Z94012
S. infantarius subsp. *infantarius*	4	2.1	HDP 90056	AF177729
S. salivarius	1	0.5	ATCC 7073	AY188352
S. gallolyticus subsp. *gallolyticus*	1	0.5	ACM 3611	X94337
S. lutetiensis	1	0.5	NEM 782	AJ297215
Total	192	**100**		

241 N: number of isolates.

242

243

244 **Figure caption:**

245 **Fig. 1**. Relative phylogenetic relations of GPCNC isolated from sheep bulk tank milk on
246 the basis of ClustalW sequence alignment and inferred by the Neighbor-Joining method.
247 Bootstrap values for a total of 100 replicates are shown in the tree nodes. *Escherichia*
248 *coli* ATCC 11775 (GenBank: X80725) was set as outgroup.

249

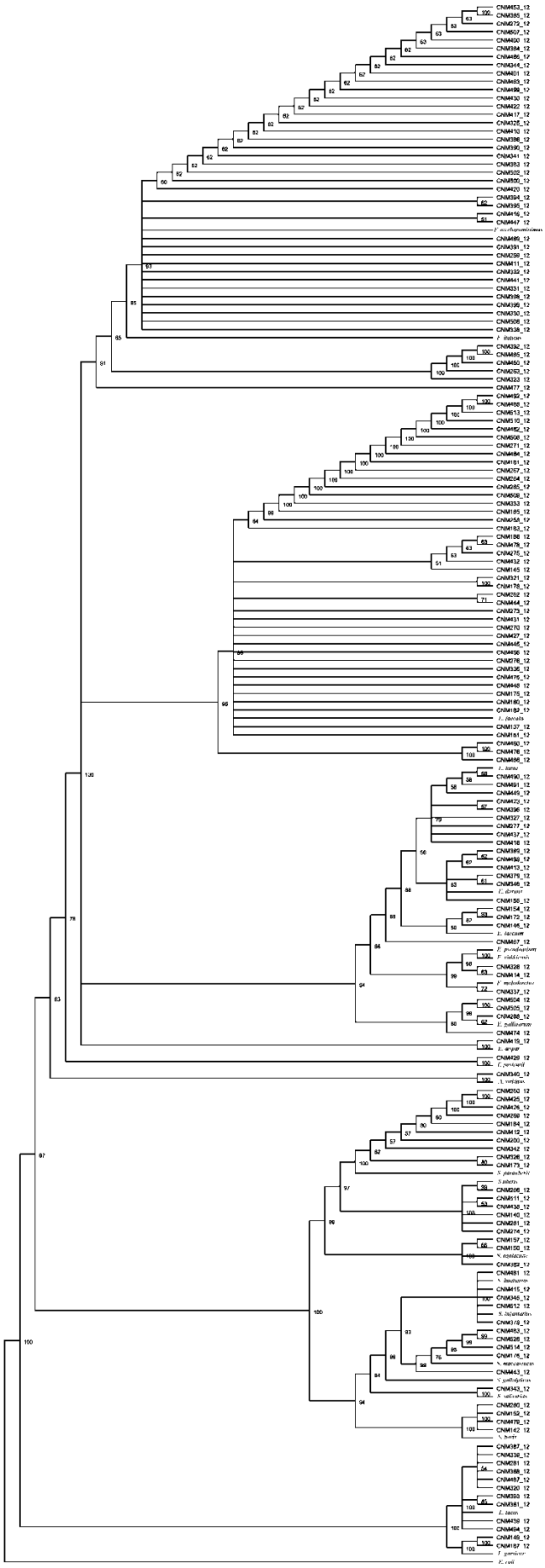


Figure 1

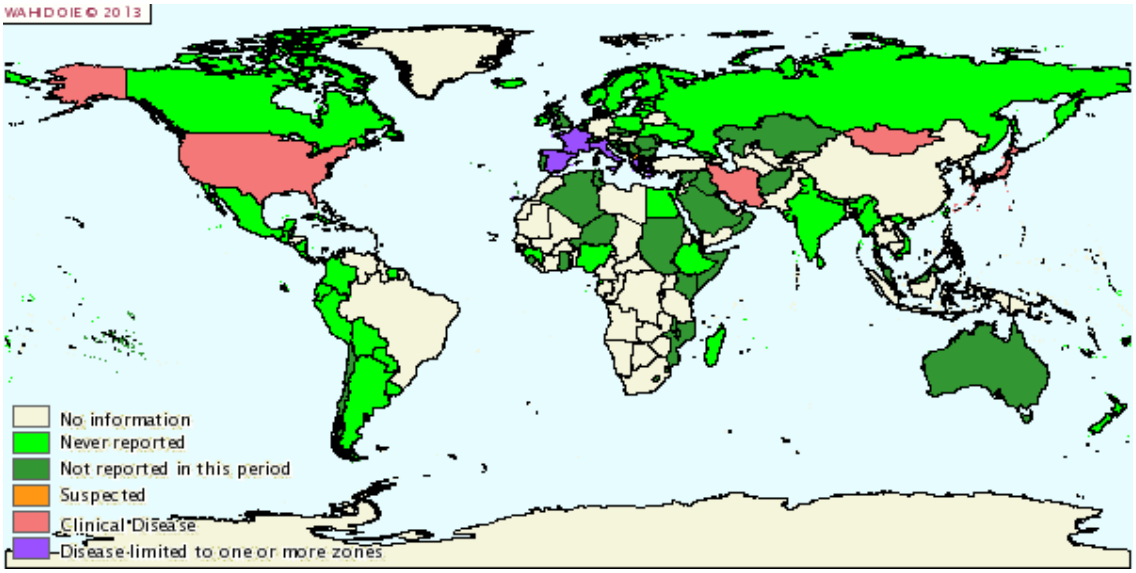
Capítulo 4

AGALAXIA CONTAGIOSA Y *MYCOPLASMA AGALACTIAE* EN UNA ZONA ENDÉMICA DE ESPAÑA

INTRODUCCIÓN

La Agalaxia Contagiosa (AC) es una enfermedad conocida como tal en Europa desde el siglo XIX. En nuestro país existen evidencias de su presencia en el siglo XVI en descripciones realizadas por los miembros del *Honorable Consejo de La Mesta*, los cuáles ya la consideraban una enfermedad contagiosa. La AC en España ha gozado de diversos nombres comunes tales como *ubrero, gota* o *mal seco*. En Italia, otro país de gran incidencia, el nombre tradicional utilizado desde hace siglos es *mal di sito* (mal del sitio), a consecuencia de su resistencia a abandonar las zonas donde se manifiesta. Actualmente se encuentra distribuida por los cinco continentes, siendo los países más afectados los pertenecientes a la Cuenca Mediterránea.

Figura 1. Mapa de distribución mundial de la AC en 2012. En España la enfermedad queda limitada a ciertas zonas. (WAHID, OIE. www.oie.int. Último acceso: 12/03/2013).



La AC afecta a las especies de pequeños rumiantes, siendo un síndrome cuyas dianas principales son la glándula mamaria, las articulaciones y los ojos. Está caracterizado por mastitis, artritis y queratoconjuntivitis –o *tríada clásica*-; ocasionando muy rara vez enfermedad respiratoria o aborto. En la mayoría de los casos los animales se recuperan rápidamente de los signos agudos de la infección, pero suelen evolucionar a una cronificación de la enfermedad con excreción del agente etiológico en leche y otros fluidos corporales, en algunas ocasiones durante años. Estos portadores asintomáticos transmiten la enfermedad a otros animales susceptibles por distintas vías de penetración, principalmente la vía oral, aunque también existe la posibilidad de una transmisión vía respiratoria, mamaria, ocular y muy excepcional y atípicamente genital. Así, la enfermedad

se propaga en un breve espacio de tiempo por la introducción de animales portadores en rebaños sanos y la diseminación del microorganismo por medio de los equipos de ordeño, las camas, los ganaderos, la alimentación y el agua de bebida contaminada con las secreciones.

Además de los síntomas clínicos, la importancia de la AC en el sector lácteo ovino y caprino reside en las pérdidas económicas que resultan de una caída de la producción de hasta un 30 %, incluso en ausencia de síntomas, debido a la eliminación de efectivos como resultado de la disminución o la ausencia de producción de leche o de lesiones fruto de la enfermedad, normalmente cambios irreversibles en la glándula mamaria y artritis (Bergonier *et al.*, 1997; Corrales *et al.*, 2007).

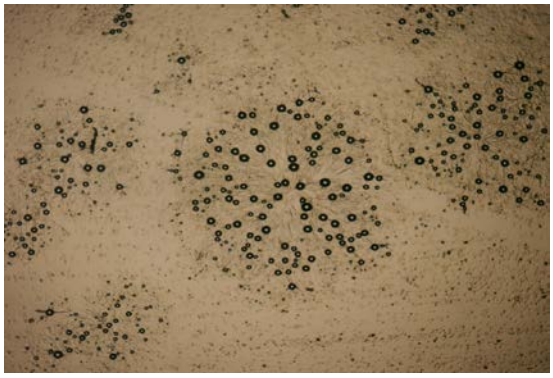
El agente etiológico principal es *Mycoplasma agalactiae (Myc. agalactiae)*, pero también *Mycoplasma mycoides* subsp. *capri (Myc. mycoides capri)* - anteriormente denominado *Myc mycoides* subsp. *mycoides LC -, Mycoplasma capricolum* subsp. *capricolum (Myc. capricolum capricolum)* y *Mycoplasma putrefaciens (Myc. putrefaciens)* se han aislado en distintos países en cabras con mamitis.

Myc. agalactiae afecta por igual a ovejas y cabras, considerándose el causante "clásico" de la enfermedad. Es la única especie descrita en casos de AC en ganado ovino. *Myc. mycoides capri* se asocia a síntomas más graves y a enfermedad respiratoria en ganado caprino. También en cabras de producción lechera se describen *Myc. capricolum capricolum* y *Myc. putrefaciens* aunque con menor frecuencia que los anteriores.

Los Mycoplasmas (Clase *Mollicutes*) son las células de vida libre más pequeñas conocidas, siendo capaces de atravesar filtros de 0.45 μm de poro, una característica muy útil en su aislamiento. Carecen de pared celular y poseen un genoma de muy pequeño tamaño (0.58-1.38 Mpb) con bajo contenido en G+C (23-40 mol %). El genoma de *Myc. agalactiae* fue secuenciado por completo en el año 2007, encontrándose entre los de menor tamaño de la clase *Mollicutes* con 877.438 pb. Estas características hacen que estos organismos dependan del hospedador para el aporte de ciertos nutrientes como aminoácidos, ácidos grasos y moléculas precursoras de los ácidos nucleicos necesarios para su multiplicación y supervivencia.

Una característica típica de las colonias de *Myc. agalactiae* es la morfología de *huevo frito*, y un fenómeno visible en medio sólido denominado formación de películas y cristales (*films and spots*), que lo diferencia de *Myc. mycoides capri* y *Myc. capricolum capricolum.*

Figura 2. Fotografía del fenómeno films and spots en la cepa de referencia PG2. Foto: R.S. Rosales y M.L. de Garnica



La aparición de la enfermedad en los rebaños se produce fundamentalmente por la entrada de portadores asintomáticos. Requieren medidas especiales de prevención los rebaños más susceptibles, a saber, los que nunca han padecido la enfermedad, ya que es en ellos donde los síntomas aparecen de manera más agresiva y en los que la afección causaría las pérdidas más significativas.

El tratamiento antibiótico recomendado incluye oxitetraciclina, eritromicina, espiramicina, tilosina, lincomicina, florfenicol, enrofloxacino y norfloxacino entre otros, el cuál mejora los síntomas de la enfermedad pero no impide que se sigan excretando micoplasmas en la leche. Estos fármacos tienen como principal microorganismo diana *Myc. mycoides capri* que aunque es un agente importante en cabra, se encontraría difícilmente en ganado ovino. La difícil erradicación de la enfermedad exige que los antibióticos de elección posean una baja concentración mínima inhibidora (CMI), permanencia en el tejido infectado y fácil difusión de la sangre al tejido mamario. Por lo tanto la necesidad de estudios de sensibilidad *in vitro* es de gran importancia a la hora de escoger tratamientos efectivos *in vivo*.

Además de la terapia antibiótica, en los rebaños estudiados se sigue una pauta de vacunación que consiste en una primera dosis al tercer o cuarto mes de vida, y una segunda dosis a las 2 ó 3 semanas de vida. En zonas endémicas se lleva a cabo una profilaxis vacunal semestral. En ningún caso se ha demostrado que la vacunación sea totalmente eficaz en la lucha contra la AC y suele proporcionar una inmunidad breve.

Para prevenir intercambios comerciales de riesgo, la legislación vigente prohíbe la compra-venta de animales procedentes de rebaños que no estén libres de Agalaxia Contagiosa en al menos, los 6 meses previos a la transacción comercial (Real Decreto 1941/2004, de 27 de septiembre, por el que se establecen las normas de policía sanitaria que regulan los intercambios intracomunitarios y las importaciones de terceros países de animales de las especies ovina y caprina). De la misma manera, la AC es considerada una enfermedad de declaración obligatoria por su importancia sanitaria y económica, y en caso de aparecer un brote, éste debe comunicarse al MAGRAMA por las vías de notificación establecidas para ello (Orden ARM/831/2009, de 27 de marzo, por la que se modifican los anexos I y II del Real Decreto 617/2007, de 16 de mayo, por el que se establece la lista de las enfermedades de los animales de declaración obligatoria y se regula su notificación). Además figura en la lista de enfermedades de las especies ovina y caprina de la Organización Mundial de Sanidad Animal (OIE), organismo al que se notifican los brotes de manera regular. En el ámbito autonómico, La Junta de Castilla y León recoge un *PROGRAMA DE CONTROL Y LUCHA CONTRA LA AGALAXIA CONTAGIOSA* que debe ser aplicado por la Asociación de Defensa Sanitaria (ADS) correspondiente (RESOLUCIÓN de 24 de mayo de 2007, del Director General de Producción Agropecuaria, por la que desarrollan los programas sanitarios a realizar por las Agrupaciones de Defensa Sanitaria Ganaderas, definidos en la Orden AYG/1131/2006, de 30 de junio).

A pesar de las medidas sanitarias y normativas tomadas en España y otros países europeos, incluyendo la declaración obligatoria, el control fronterizo, los programas de vigilancia continua, las pautas de vacunación o el tratamiento antibiótico, de acuerdo con los datos ofrecidos por la OIE, se registraron entre los años 2010 y 2011 un total de 924 brotes de AC en Castilla y León (919 ovino/5 otras especies) del total de 931 declarados en

el territorio nacional. Aparte del alto número, ya por sí mismo considerable, cabe destacar el aumento de brotes en comparación con los 17, 19 o 224 casos declarados en la misma región en los años 2007, 2008 y 2009 respectivamente.

El objetivo del estudio llevado a cabo en rebaños de Castilla y León, una región endémica de AC, fue estudiar la presencia de *Myc. agalactiae* en leche de ovino almacenada en tanques y silos de refrigeración y caracterizar los aislados obtenidos por medio de tests de susceptibilidad a antimicrobianos y tipificación molecular.

MATERIALES Y MÉTODOS

Un total de 45 muestras de leche de oveja fueron recogidas entre Noviembre de 2009 y Marzo de 2010 de las cuales 35 (50 mL) procedieron de explotaciones sospechosas de padecer o haber padecido AC y las 10 restantes (500 mL) de silos de almacenamiento de un centro lácteo donde llega leche procedente de más de 400 rebaños y distintas rutas de recogida de Castilla y León.

Para aislar *Myc. agalactiae* se inoculó tras su homogeneización 100-200 µL de leche en viales de 3 mL de caldo Eaton y se incubaron a 37 ºC/5-10% CO_2/ 24 h con agitación periódica. Se practicaron subsiguientes filtraciones (45µm de poro) e inoculaciones en el mismo caldo hasta observar la turbidez típica de este cultivo. Dicho caldo se sembró posteriormente en agar de Eaton durante 3-7 días hasta observar las colonias típicas en superficie con una lupa de aumento 40X. Una única colonia fue inoculada en caldo e incubada en las condiciones anteriormente mencionadas para la obtención del cultivo puro.

El resumen de métodos utilizados para la tipificación molecular y el perfil de resistencia a antimicrobianos se muestra en la siguiente tabla:

Tabla 1. Metodología de caracterización de los aislados de *Myc. agalactiae*.

DETERMINACIÓN	MÉTODO	REFERENCIA
Extracción de ADN	Genelute gDNA extraction kit (Sigma Aldrich, UK).	de Garnica *et al.*, 2013
Confirmación de aislados de *Myc. agalactiae* y pureza del cultivo	PCR-DGGE	McAuliffe *et al.*, 2003
Tipificación PFGE	PFGE	de Garnica *et al.*, 2013
Tipificación VNTR	VNTR analysis	McAuliffe *et al.*, 2011
Tipificación MLST	MLST analysis	McAuliffe *et al.*, 2011
Perfil proteico y antigénico	SDS-PAGE/IMMUNOBLOT	de Garnica *et al.*, 2013
Perfil resistencia a 13 antimicrobianos de uso veterinario*	Sensititre plates (Trek Diagnostics, UK)	de Garnica *et al.*, 2013

PCR-DGGE: Polymerase Chain Reaction –Denaturing Gradient Gel Electrophoresis,VNTR: Variable Number Tandem Repeat, MLST: MultiLocus Sequence Typing, SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

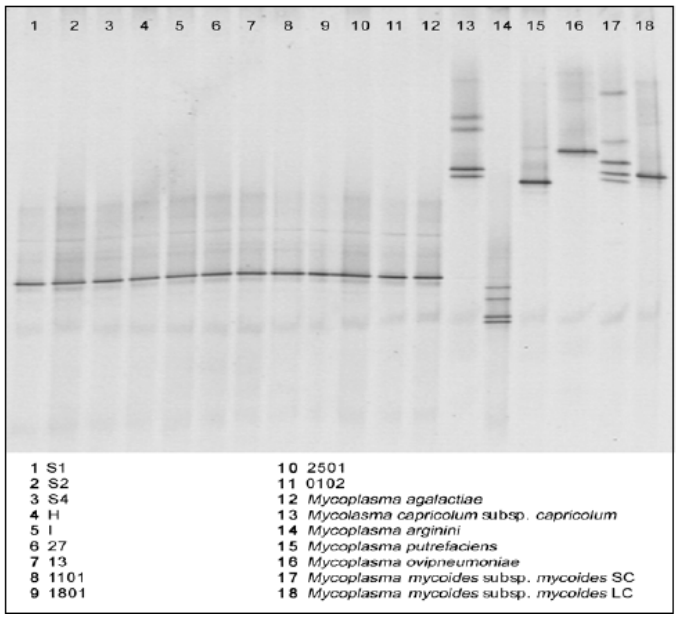
Los aislados de *Myc. agalactiae* de Castilla y León se incluyeron en un estudio de caracterización de 52 cepas por análisis MLST a nivel mundial (McAuliffe *et al.*, 2011).

RESULTADOS Y DISCUSIÓN

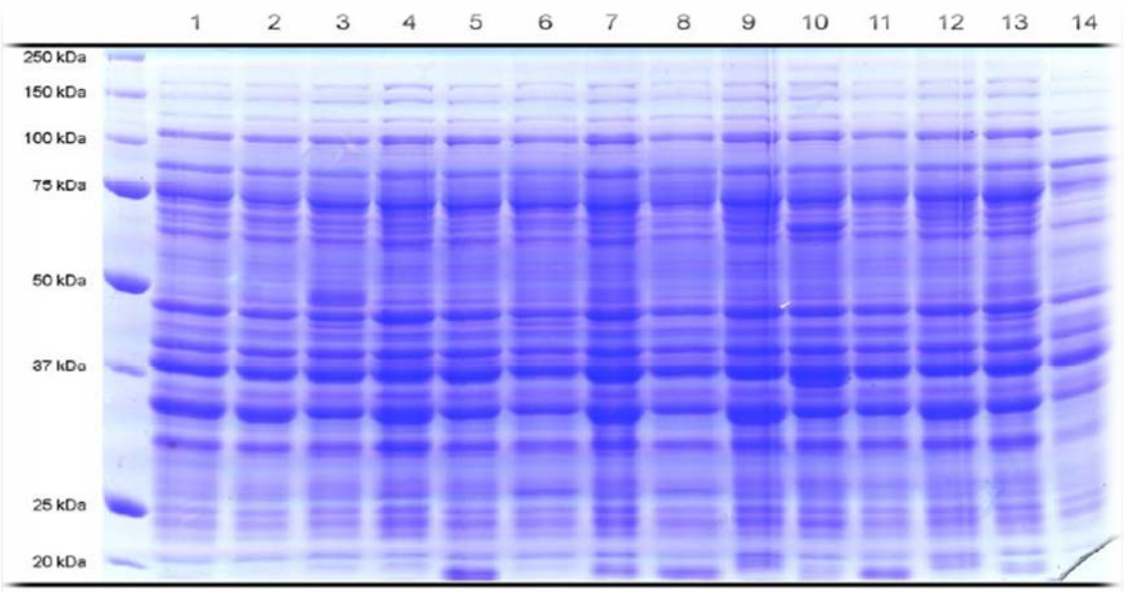
Se confirmaron por medio de PCR-DGGE (Figura 3) un total de 13 aislados puros de *Myc. agalactiae*, siendo positivas el 28.8 % de las muestras analizadas. La lista de cepas y sus características están disponibles en la Tabla 4.

Brevemente, todas las cepas mostraron un único perfil de PFGE para la enzima de restricción *Sma I* de aproximadamente 466, 174, 132, 91, 58 y 11 Kpb y un único perfil VNTR (1121) al que pertenecen la mayor parte de las cepas europeas. El perfil proteico obtenido por SDS-PAGE fue asimismo homogéneo y similar al de la cepa de referencia PG2, aunque una pequeña variabilidad en la intensidad de banda se observó en proteínas de bajo peso molecular (menor 20KDa) y a nivel de 50KDa en la cepa de tanque SP-S4 y a nivel 36 y 66 KDa en la cepa de silo SP-2501. De la misma manera, el análisis por immunoblot reveló un perfil antigénico homogéneo en las bandas de alto peso molecular, pero a diferencia de los resultados del análisis SDS-PAGE se observaron notables diferencias en ausencia/presencia de bandas y su intensidad a niveles inferiores a 30 KDa. Basándose en esas diferencias las cepas se clasificaron en 4 perfiles antigénicos: perfil 1 (6 cepas), perfil 2 (3 cepas), perfil 3 (2 cepas) y perfil 4 (2 cepas). La tipificación molecular por MLST mostró una variabilidad limitada, dividiendo los aislados en tres secuenciotipos (ST), todos pertenecientes al mismo complejo clonal (ST-5, 10 cepas; ST-16, 2 cepas y ST-17, 1 cepa).

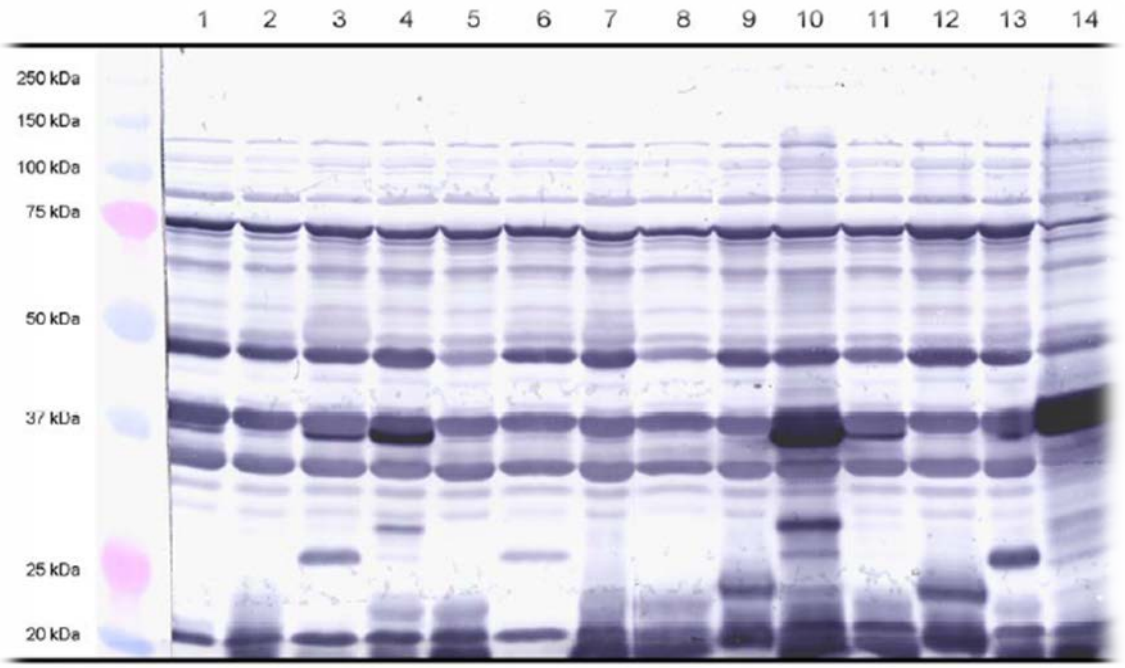
Figura 3. PCR-DGGE de los 13 aislados de *Mycoplasma agalactiae* en leche de tanque y silo, comparado con otras especies de mycoplasmas de rumiantes.



Figuras 4 y 5. Perfiles proteico y antigénico (SDS-PAGE e Immunoblot) a partir de células completas de los aislados de *Mycoplasma agalactiae* en comparación con la cepa de referencia PG2



1. S1
2. S2
3. S4
4. H
5. I
6. 27
7. 13
8. 1101
9. 1801
10. 2501
11. 0102
12. 23
13. E
14. *M. agalactiae* NCTC (PG 2)



1. S1
2. S2
3. S4
4. H
5. I
6. 27
7. 13
8. 1101
9. 1801
10. 2501
11. 0102
12. 23
13. E
14. *M. agalactiae* NCTC (PG 2)

Figura 6. Perfiles VNTR de cepas aisladas en todo el mundo incluidos los aislados de este estudio en el *cluster* 12 mayoritario europeo. Recuadrados los aislados de Castilla y León.

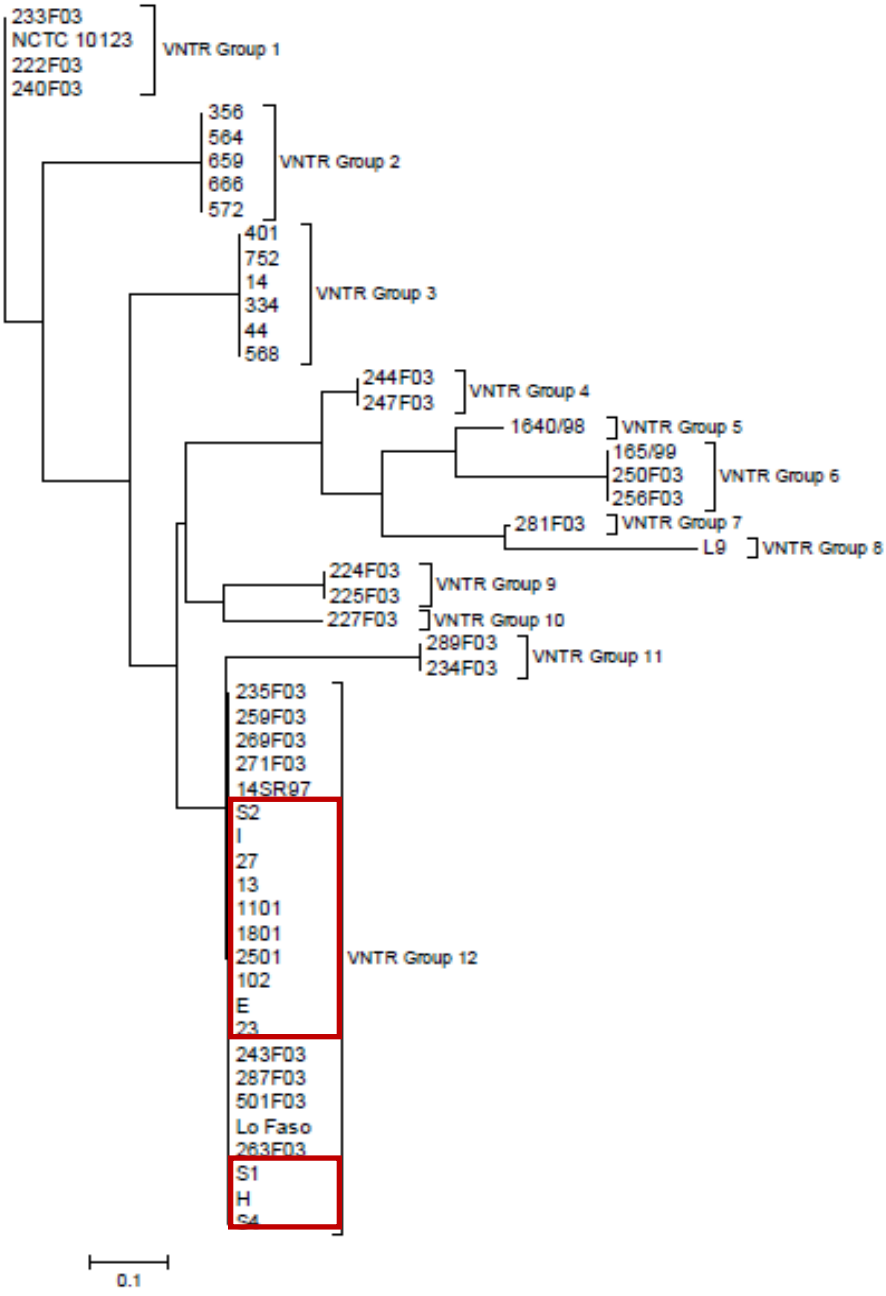
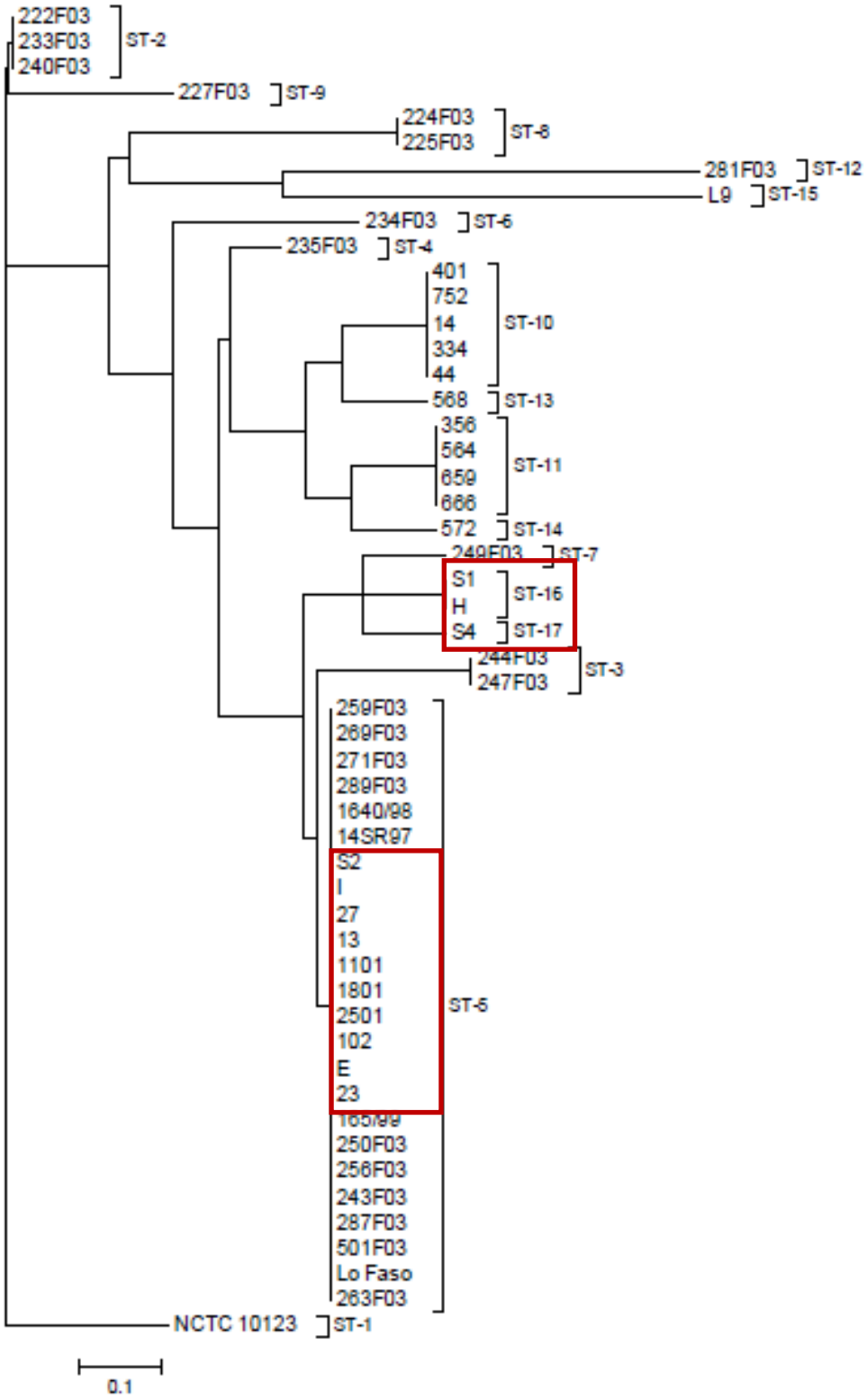


Figura 7. Perfiles MLST de cepas aisladas en todo el mundo. Recuadrados los aislados españoles de este trabajo**.**



La determinación de la CMI frente a 13 antimicrobianos de uso veterinario (Tablas 3 y 4) revelaron la efectividad *in vitro* de las quinolonas frente a *Myc. agalactiae*, incluidas dentro de los tratamientos habituales contra la AC y coincidiendo con datos similares aportados por Antunes *et al.* (2007) sobre la susceptibilidad de cepas de *Myc. agalactiae* aisladas en España. Aunque la clindamicina se mostró como el antimicrobiano más efectivo en todos los casos, no existen datos disponibles sobre su CMI y su uso en esta patología.

Tabla 2. Antibióticos utilizados en la determinación CMI agrupados en familias**.**

MACRÓLIDOS	TERACICLINAS	QUINOLONAS	LINCOSAMIDAS	AMINOGLISÓSIDOS	OTROS
Tilosina	Oxitetraciclina	Danofloxacino	Lincomicina	Espectinomicina	Cloranfenicol
Tilmicosina		Enrofloxacino		Clindamicina	Florfenicol
Eritromicina		Marbofloxacino			
Tulatromicina					

Tabla 3 Rango CMI, CMI_{90}, CMI_{50} (μg /mL) para 13 antimicrobianos de uso veterinario.

Antimicrobianos*	Rango CMI	CMI_{50}	CMI_{90}
TYL	0.5 - 2	1	2
TLM	0.5 - 8	1	8
ERY	8 - >32	>32	>32
TUL	1 - 8	4	8
OXY	1 - 8	8	8
DAN	0.25 - 0.5	0.25	0.5
ENR	<0.12 - 0.5	0.25	0.5
MAR	0.5 - 2	0.5	0.5
LIN	0.5 - 2	1	1
CLI	<0.12	<0.12	<0.12
SPT	2 - 8	2	8
CHL	8	8	8
FLO	2 - 8	8	8

CMI, concentración mínima inhibidora; TYL, tilosina; TLM, tilmicosina; ERY, eritromicina; TUL, tulatromicina; OXY, oxitetraciclina; DAN, danofloxacino; ENR, enrofloxacino; MAR, marbofloxacino; LIN, lincomicina; CLI, clindamicina; SPT, espectinomicina; CHL, cloranfenicol; and FLO, florfenicol.

Cabe destacar la resistencia a la eritromicina, que ha sido considerada durante mucho tiempo como una opción en el tratamiento frente a la AC. Aunque otros micoplasmas son susceptibles a dicho antibiótico, su uso en este caso no estaría indicado. Estos datos son consistentes también con los aportados en el trabajo de Antunes *et al.* (2007).

En contraposición a los resultados de Antunes *et al.* (2007), los aislados de este estudio mostraron valores muy superiores de CMI frente a la oxitetraciclina y ligeramente superiores en el caso de la tilosina, antibiótico de elección en el tratamiento frente a la enfermedad; mientras que los valores para la espectinomicina fueron coincidentes con los de dichos autores.

Se observó una respuesta variable (1-8 μg/mL) a la tulatromicina (Draxxin®), perteneciente a un grupo de macrólidos de nueva generación. Así, deberían tenerse en cuenta estos resultados para su uso en el tratamiento de la enfermedad en las provincias de León y Zamora (España).

Los valores de rango CMI, CMI_{50} y CMI_{90} determinados según las directrices de Hannan (2000) para microorganismos del género *Mycoplasma* se muestran en la Tabla 3.

CONCLUSIONES

Los aislados de *Myc. agalactiae* caracterizados en este trabajo provienen de un área endémica y comparten un perfil molecular de escasa variabilidad y similar al resto de aislados europeos caracterizados por distintos autores. La clindamicina y las quinolonas fueron los antibióticos más efectivos in vitro y podrían ser considerados como tratamientos estándar frente a AC. La tipificación por MLST demostró un mayor poder de discriminación que otros métodos moleculares en la caracterización de la población de cepas de *Myc. agalactiae* de Castilla y León en un estudio internacional con 52 cepas procedentes de numerosos países, revelando una inesperada variabilidad con la aparición de dos nuevos secuenciotipos (ST-16 y ST-17).

Tabla 4. Perfil completo de los aislados de *Myc. agalactiae* en leche de oveja.

Cepa	Fuente	Provincia	Perfil PFGE	Perfil MLST*	Perfil VNTR*	Perfil IMMUNOBLOT	Valores CMI (µg /mL)												
							TYL	TLM	LIN	CLI	ERY	CHL	FLO	SPT	OXY	DAN	ENR	MAR	TUL
SP-S1	Tanque	León	1	16	1121	1	1	1	1	<0.12	8	8	8	2	8	<0.12	<0.12	0.5	2
SP-S2	Tanque	León	1	5	1121	1	1	8	1	<0.12	>32	8	2	2	8	0.5	0.5	2	8
SP-S4	Tanque	León	1	17	1121	2	1	1	1	<0.12	>32	8	8	8	8	0.25	0.25	0.5	2
SP-13	Tanque	Zamora	1	5	1121	1	0.5	0.5	0.5	<0.12	>32	8	2	2	2	0.25	0.25	0.5	1
SP-23	Tanque	Zamora	1	5	1121	4	2	2	1	<0.12	>32	8	8	2	8	0.25	0.25	0.5	4
SP-27	Tanque	Zamora	1	5	1121	2	0.5	1	1	<0.12	>32	8	8	8	8	0.5	0.5	0.5	4
SP-0102	Silo	Zamora	1	5	1121	1	2	8	1	<0.12	>32	8	8	8	8	0.25	0.25	0.5	8
SP-1101	Silo	Zamora	1	5	1121	1	1	1	1	<0.12	>32	8	2	2	8	0.25	0.25	0.5	2
SP-1801	Silo	Zamora	1	5	1121	4	1	2	1	<0.12	>32	8	8	2	2	0.5	0.5	1	2
SP-2501	Silo	Zamora	1	5	1121	3	1	2	2	<0.12	>32	8	8	8	8	0.25	<0.12	0.5	8
SP-E	Silo	Zamora	1	5	1121	2	1	1	1	<0.12	>32	8	8	8	8	0.25	0.25	0.5	4
SP-H	Silo	Zamora	1	16	1121	3	0.5	0.5	0.5	<0.12	>32	2	2	2	1	0.25	<0.12	0.5	1
SP-I	Silo	Zamora	1	5	1121	1	1	1	1	<0.12	>32	8	8	2	2	0.25	0.25	0.5	4

PFGE, Pulse Field Gel Electrophoresis; VNTR, Variable Number Tandem Reapeat; MLST, Multi Locus Sequence Typing; CMI, concentración mínima inhibidora; TYL, tilosina; TLM, tilmicosina; ERY, eritromicina; TUL, tulatromicina; OXY, oxitetraciclina; DAN, danofloxacino; ENR, enrofloxacino; MAR, marbofloxacino; LIN, lincomicina; CLI, clindamicina; SPT, espectinomicina; CHL, cloranfenicol; and FLO, florfenicol.

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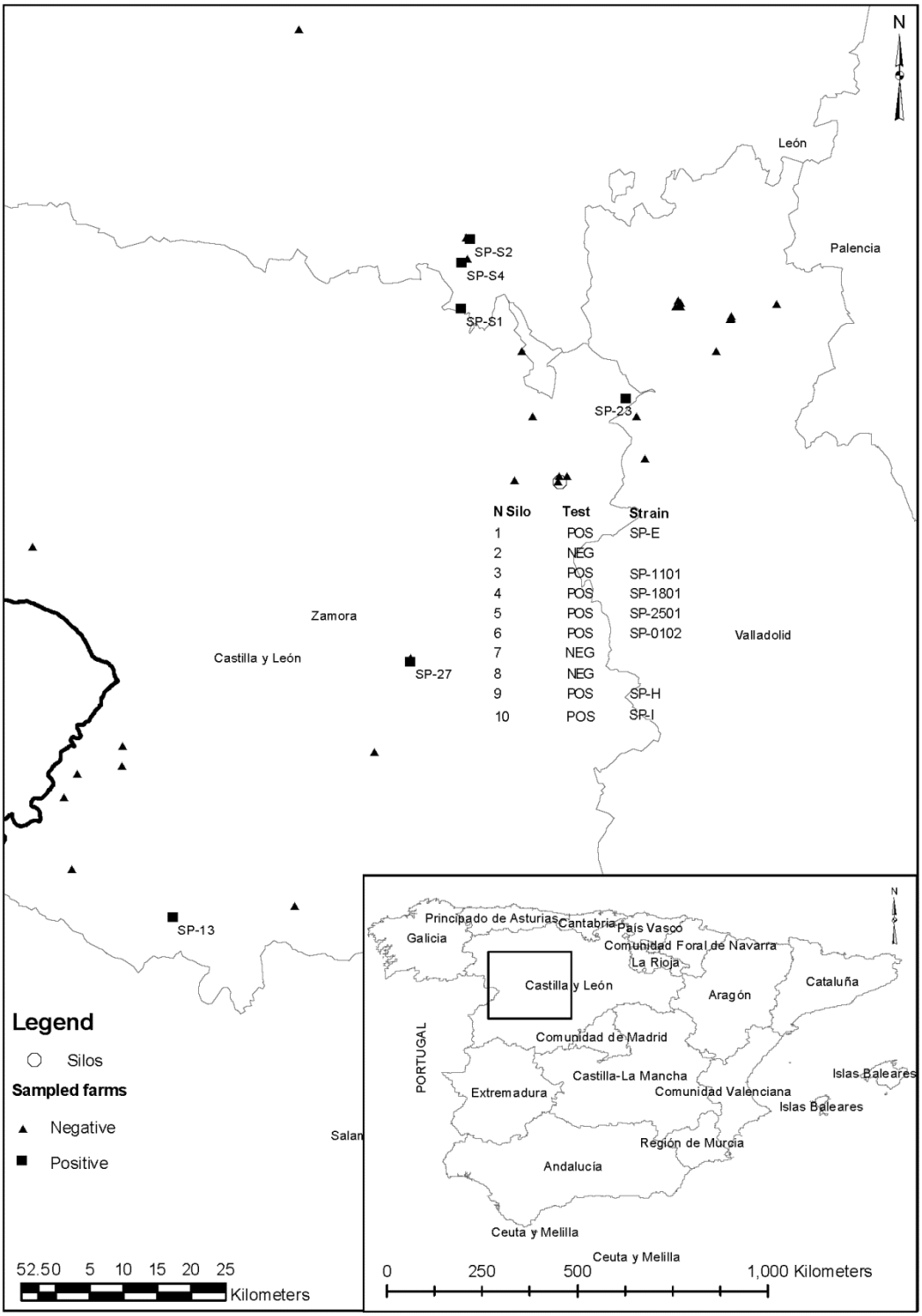
INFORMACIÓN COMPLEMENTARIA C2

Mapa de localización de silos y explotaciones incluyendo resultados positivos y negativos.

INFORMACIÓN COMPLEMENTARIA C2. Mapa de localización de silos y explotaciones incluyendo resultados positivos y negativos. Cortesía de Rafael J. de Garnica García.



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ORIGINAL ARTICLE

Isolation, molecular characterization and antimicrobial susceptibilities of isolates of *Mycoplasma agalactiae* from bulk tank milk in an endemic area of Spain

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antimicrobial susceptibility, contagious agalactia, *Mycoplasma agalactiae*, pulsed field gel electrophoresis.

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Abstract

Aim: To isolate and characterize strains of *Mycoplasma agalactiae* from bulk tank and silo ewes' milk.
Methods and Results: Thirteen mycoplasma isolates were obtained from samples of sheep milk taken from bulk tank and large silos and identified as *Myc. agalactiae* by PCR-DGGE. The isolates were typed by pulsed field gel electrophoresis (PFGE), SDS-PAGE and immunoblot. The *in vitro* activity of 13 antimicrobials of veterinary interest was tested against these isolates. Results showed that the most effective compounds against *Myc. agalactiae in vitro* were clindamycin, an antibiotic not previously described as a suitable contagious agalactia (CA) treatment, with Minimum Inhibitory Concentration (MIC) values of <0·12 μg ml^{-1}, and quinolones, with MIC values <0·12–0·5 μg ml^{-1}, which are used as standard treatments against CA.
Conclusions: Based on the *in vitro* assay, clindamycin, quinolones, tylosin and tilmicosin would be appropriate antimicrobials for CA treatment. The isolates were mostly resistant to erythromycin, indicating that it would not be a suitable choice for therapy. The isolates showed common molecular and protein profiles by PFGE and SDS-PAGE, with minor differences observed by immunoblot analysis, suggesting a clonal relationship among them.
Significance and impact of the Study: This study demonstrated the importance of the appropriate selection of antimicrobials for treatment of CA.

Introduction

Mycoplasma agalactiae is the main causative agent of contagious agalactia (CA), a syndrome that affects sheep and goats and is characterized by mastitis, absence or reduction of milk production, rapid spread and long persistence in affected areas. In most cases, infected animals recover rapidly from acute signs but develop chronic disease with excretion of the agent, mainly in milk and other secretions sometimes persisting for years (Bergonier *et al.* 1997). CA is of great economic importance in Mediterranean countries as there may be losses of up to 30% in milk production as a result of irreversible changes to the mammary gland. In addition, young animals may develop arthritis and in some cases septicaemia resulting in death or culling of the affected animal (Bergonier *et al.* 1997; Corrales *et al.* 2007).

Due to these characteristics, antimicrobials used for CA treatment should have the following properties: very low Minimum Inhibitory Concentration (MIC), long persistence in infected tissue, excretion in milk and easy diffusion from blood to mammary tissue (Bergonier *et al.* 1997). Antimicrobials chosen traditionally for CA treatment are tetracyclines, macrolides (tylosin and tilmicosin), fluoroquinolones, erythromycin and florfenicol, but this treatment is largely based on studies that have been carried out on *Mycoplasma mycoides* subspecies *mycoides* large colony type, now known as *Myc. mycoides* subspe-

cies *capri* (Manso-Silvan *et al.* 2009), which is an important aetiological agent for CA in goats but it is rarely found in sheep. Moreover, the efficacy of antimicrobial therapy under field conditions is limited (Bergonier *et al.* 1997; Giguère *et al.* 2006). Animal health and welfare, as well as the economic importance of CA, make it necessary to determine which antimicrobial treatments are effective against *Myc. agalactiae in vivo*.

The description of the genome sequence of *Myc. agalactiae* reference strain PG2 (Sirand-Pugnet *et al.* 2007) has enabled the development of several diagnostic and molecular typing methods, such as VNTR analysis (McAuliffe *et al.* 2008) and MLST (McAuliffe *et al.* 2011) that are able to provide useful epidemiological information. *Myc. agalactiae* also has variable surface proteins (Glew *et al.* 2000; de la Fe *et al.* 2006) that may contribute to its pathogenicity and hinder the development of effective vaccines (Dybvig and Voelker 1996; Flitman-Tene *et al.* 2000; Glew *et al.* 2000).

Despite the legal and health measures taken in Spain and other European countries to prevent the spread of CA, which includes border movement controls, epidemiological monitoring, vaccination or antimicrobial treatment, a total of 931 outbreaks were reported in Spain between 2010 and 2011, according to data of the World Organization for Animal Health (http://web.oie.int/wahis/public.php?page=disease; accessed December 2012); 924 (919 sheep/5 other species) of the 931 outbreaks were declared in the Castilla y Leon region. This number of infected sheep is considerably higher than the 17 reported outbreaks in the same region in 2007.

The objectives of this work were to study the prevalence of *Myc. agalactiae* in samples of bulk tank and silo sheep milk and to characterize the isolates by antimicrobial and molecular typing.

Materials and methods

Sampling

From November 2009 to March 2010, 45 samples of sheep milk from the Castilla y León region were processed for this work. Thirty-five were obtained from tanks at dairy farms suspected of having or having had CA and the other 10 were collected from cooling silos at a collection centre where milk from a total of 400 flocks from different collection routes was stored. In both cases, samples were taken aseptically in sterile containers according to the standards recommended by the American Public Health Association (White *et al.* 1992). Samples of 50 and 500 ml were collected in farms and silos, respectively, and stored at 4°C until processed in the laboratory within the next 24 h.

Isolation and culture of *Mycoplasma agalactiae*

Upon receipt in the laboratory, samples were homogenized by slowly inverting the container 25 times. An aliquot of 100–200 μl of milk was inoculated in vials containing 3 ml of Eaton′s Broth (Nicholas and Baker 1998) and incubated at 37°C in a 5–10% CO_2 atmosphere for 24 h with periodic agitation. After the first incubation, 1–2 ml of culture was filtered using 0·45-μm Minisart filter (Sartorius AG, Goettingen, Germany) and inoculated into a fresh broth and incubated until a pH change and opalescence was seen (Nicholas *et al.* 2008). Then 25 μl of each broth was subcultured on Eaton's agar (Nicholas and Baker 1998), using the same incubation conditions. Plates were inspected after 3–7 days under 40× magnification and a single typical 'fried-egg' colony was picked using a sterile tip and incubated in Eaton's broth vials, until turbidity, to obtain a pure culture. Aliquots in stationary phase were frozen at −80°C in 20% glycerol until required.

DNA extraction

The DNA of each strain was extracted from 1-ml culture which was centrifuged at 13 000 ***g*** for 5 min in 1·5-ml microtubes. The pellets obtained were washed three times with Phosphate-Buffered Saline (PBS; Oxoid, Basingstoke, UK) pH 7·4. Washed pellets were subjected to a standardized protocol of extraction (GenElute DNA extraction kit; Sigma-Aldrich, Poole, UK) according to the manufacturer's instructions.

PCR and denaturing gradient gel electrophoresis identification

16S rDNA PCR and denaturing gradient gel electrophoresis (PCR-DGGE) were performed to identify isolates and to ensure that they were pure *Myc. agalactiae* cultures according to a published protocol (McAuliffe *et al.* 2003). The profiles obtained were compared with *Myc. agalactiae* PG2-type strain and other *Mycoplasma* species that may affect small ruminants including *Myc. mycoides* subsp. *capri*, *Myc. mycoides* subsp. *mycoides* SC, *Myc. arginini, Myc. capricolum* subsp. *capricolum, Myc. putrefaciens* and *Myc. ovipneumoniae*.

MIC testing

Minimum inhibitory concentration testing was carried out using a microbroth dilution method following the guidelines of Hannan (2000). Thirteen antimicrobials of veterinary use were tested. These antimicrobials were tylosin, tilmicosin, erythromycin, tulathromycin,

danofloxacin, enrofloxacin, marbofloxacin, lincomycin, clindamycin, oxytetracycline, spectinomycin, chloramphenicol and florfenicol at specified concentrations on Sensititre plates (Trek Diagnostics, East Grinstead, West Sussex, UK). Eaton's broth medium without antimicrobials and phenol red was used to culture the *Myc. agalactiae* strains for the MIC inoculum (Nicholas and Baker 1998). The inoculum concentration was standardized by adjusting the optical density (OD) of the broth medium to an OD at 450 nm of 0·1, which was determined to be equivalent to approximately 10^8 colony-forming units (CFU) ml^{-1}. Fresh culture was serially diluted in Eaton's broth to obtain a final concentration of 10^5 CFU ml^{-1}. Wells were inoculated with 10 μl of inoculum added to 190 μl of Eaton's medium. Microplates were incubated statically for 72 h at 37°C with 5% CO_2. To assess killing effect after incubation, the Sensititre plates were centrifuged at 800 ***g*** to concentrate *Myc. agalactiae* cells at the bottom of the wells. Cell growth was examined using an inverted mirror in a light box. Absence of growth was considered negative and the first negative well for each antimicrobial was taken as the MIC value. Results were recorded on standard worksheets, and MIC values are given as MIC range ($\mu g\ ml^{-1}$) MIC_{50} and MIC_{90} ($\mu g\ ml^{-1}$) in Table 2.

Pulsed field gel electrophoresis

Confirmed *Myc. agalactiae* strains were reconstituted in 3 ml of Eaton's broth and incubated until growth was observed and then subcultured into 20 ml of the same medium for a further 24–48 h to obtain an appropriate cell concentration. Cultures were then centrifuged at 6000 ***g*** and 4°C for 30 min. Pellets were carefully suspended in 2 ml of PBS pH 7·4 and centrifuged again at 13 000 ***g***. This wash was repeated three times with the final pellet being resuspended in 250 μl of PBS pH 7·4 and stored in ice to preserve the cells integrity. Equal volumes of 2% low melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA) and cell suspension were mixed and allowed to solidify in the pulsed-field moulds at 4°C. Plugs were lysed in 2-ml microtubes at 45°C for 48 h using 500 μl of lysis buffer (10 mol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 1% *N*-lauryl sarcosine and 1 mg ml^{-1} proteinase K). After lysis, the buffer was carefully removed and plugs were washed four times with 2 ml of TE (10 mmol l^{-1} Tris-HCl plus 1 mmol l^{-1} EDTA) and stored at 4°C until being digested with the restriction enzyme. Plug slices were digested overnight with 30 U of *Sma*I (Takara Bio, Otsu, Shiga, Japan) following the manufacturer's instructions. Using the CHEF DR III System (Bio-Rad Laboratories), digested plugs underwent electrophoresis for 18 h at 14°C in a 1% agarose gel in TBE buffer (0·1 mol l^{-1} Tris, 0·1 mol l^{-1} boric acid and 2 mmol l^{-1} EDTA, pH 8) using pulse times of 4–40 s. A *Salmonella* serotype Braenderup restricted with *Xba*I (Takara Bio) was used as a molecular weight standard (http://www.pulsenetinternational.org; accessed December 2012). Gels were stained with ethidium bromide, destained in distilled water for 30 min and observed under UV transillumination. Gel pictures were analysed using GelCompar II software (Applied Maths, St-Martens-Latem, Belgium).

SDS-PAGE and immunoblotting

Mycoplasma isolates, including reference strain PG2, were cultured in 20 ml of Eaton's broth for 7 days at 37°C with 5% CO_2. Cell pellets were obtained by centrifuging cultures at 12 500 ***g*** at 4°C for 30 min. Pellets were washed three times with PBS pH 7·4 and finally resuspended in PBS at 1/200 of the original volume.

Protein concentration was estimated using the BCA protein assay (Thermo Scientific, East Grinstead, West Sussex, UK) according to the manufacturer's instructions. Ten micrograms of protein from each sample was mixed with an equal volume of 2× Laemmli buffer (Laemmli 1970), boiled for 10 min and then cooled in ice. Samples were loaded and electrophoresed in a Protean II Vertical Electrophoresis Cell (Bio-Rad Laboratories) in 1·5 mm discontinuous gels with 4% stacking gel and 10% resolving gel at a constant voltage of 200 V. Protein bands were visualized by staining with Coomassie PhastGel Blue R-350 (GE Healthcare Life Sciences, Buckinghamshire, England, UK).

Immunoblot was performed by transferring electrophoresed proteins onto 0·45-μm nitrocellulose membranes (Bio-Rad Laboratories) in a semi-dry device (Wolf Laboratories, York, UK) at 15 V for 30 min. Membranes were stained with 2% Ponceau S Red in 1% acetic acid for 1 min to confirm the transfer of proteins. After washing with PBS to eliminate red staining, membranes were blocked using blocking buffer (1 mol l^{-1} Glycine, 1% egg albumin, 5% powdered skimmed milk, 0·1 mol l^{-1} PBS) overnight at 4°C. Afterwards, membranes were washed twice with PBS plus 0·1% Tween 20 and a third time only with PBS. Then, primary antibody reaction with a reference rabbit antiserum against *Myc. agalactiae* PG2 (University of Aarhus, Denmark; Freundt 1983) was performed at 37°C for 1 h with continuous agitation at a concentration 1/200. After three washes with PBS plus 0·1% Tween 20 and a last one with PBS, membranes were incubated as before in a 1/1000 dilution of peroxidase-conjugated Protein G (Thermo Scientific) and washed again as above. Antigenic bands were revealed by soaking the gel in the substrate 4-chloro-1-naphthol at

room temperature. The reaction was stopped with deionized water when bands had developed sufficient intensity.

Results

Mycoplasma agalactiae isolation

After several culture and isolation steps, 13 strains were obtained, thus isolation percentage from bulk-tank sheep milk constituted a 28·8% of total sampling. All the isolates were confirmed as pure cultures by specific PCR-DGGE. Strains, their source and location where samples were taken, are listed in Table 1. Additional information about isolates (complete characteristics and positive and negative sampling locations) is available in Table S1 and Figure S1.

Antimicrobial MIC

Once the plates were incubated and centrifuged, clear deposits of cells were observed in wells where the antimicrobial was not effective. The results are summarized in Table 2. Among the 13 antimicrobials used, clindamycin was the most effective agent inhibiting the growth of 100% of strains with MIC <0·12 μg ml^{-1}. The quinolones, danofloxacin and enrofloxacin, inhibited most isolates at a MIC $\leq$0·25 μg ml^{-1} followed by marbofloxacin which presented a MIC between 0·5 and 2 μg ml^{-1}. Remarkably, all strains were resistant to erythromycin showing MIC >32 μg ml^{-1} in all cases. Intermediate MIC values between 1 and 2 μg ml^{-1} were obtained for the other macrolides, tylosin and tilmicosin, and for lincomycin, for most strains. Nevertheless, two strains gave MIC values of 8 μg ml^{-1} when tested against tilmicosin. Tulathromycin was found to have a less uniform response than the other macrolides (MIC between 1 and 8 μg ml^{-1}). Chloramphenicol, florfenicol and spectinomycin showed quite high values (MIC$_{90}$ $\leq$ 8 μg ml^{-1}).

Table 1 Field strains of *Mycoplasma agalactiae*, source and geographical origin

Strain	Source	Province
SP-S1	Tank	León
SP-S2	Tank	León
SP-S4	Tank	León
SP-13	Tank	Zamora
SP-23	Tank	Zamora
SP-27	Tank	Zamora
SP-0102	Silo	Zamora
SP-1101	Silo	Zamora
SP-1801	Silo	Zamora
SP-2501	Silo	Zamora
SP-E	Silo	Zamora
SP-H	Silo	Zamora
SP-I	Silo	Zamora

Table 2 MIC range, MIC$_{50}$ and MIC$_{90}$ values (μg ml^{-1}) of the antimicrobial agents studied

Antimicrobials	MIC range	MIC$_{50}$	MIC$_{90}$
TYL	0·5–2	1	2
TLM	0·5–8	1	8
ERY	8 to >32	>32	>32
TUL	1–8	4	8
OXY	1–8	8	8
DAN	0·25–0·5	0·25	0·5
ENR	<0·12–0·5	0·25	0·5
MAR	0·5–2	0·5	0·5
LIN	0·5–2	1	1
CLI	<0·12	<0·12	<0·12
SPT	2–8	2	8
CHL	8	8	8
FLO	2–8	8	8

TYL, tylosin; TLM, tilmicosin; ERY, erythromycin; TUL, tulathromycin; OXY, oxytetracycline; DAN, danofloxacin; ENR, enrofloxacin; MAR, marbofloxacin; LIN, lincomycin; CLI, clindamycin; SPT, spectinomycin; CHL, chloramphenicol; and FLO, florfenicol; MIC, minimum inhibitory concentration.

Pulsed field gel electrophoresis

*Sma*I restriction profiles of 13 *Myc. agalactiae* isolates showed no differences in their band pattern. A single profile of six fragments of estimated sizes consistent to those reported by McAuliffe *et al.* (2008) for Spanish, Greek, Italian, Macedonian and Portuguese strains was observed at approximately 466, 174, 132, 91 and 11 kbp. The variable fragment described elsewhere (Tola *et al.* 1996, 1999; McAuliffe *et al.* 2008) was estimated to be 58 kbp for the isolates characterized in this work.

SDS-PAGE and immunoblotting characterization

Protein electrophoretic profiles obtained by SDS-PAGE analysis were homogeneous within tank isolates and compared with type strain PG2 even though some slight differences of intensity were observed in low molecular weight proteins (<20 kDa) and at approximately 50 kDa for strain SP-S4 from a tank in Leon. Some minor differences were also observed in SP-2501 from a Silo in Zamora at approximately 66 and 36 kDa. Immunoblot test (Fig. 1) revealed a similar homogeneity for high molecular weight antigens. Some differences in staining intensity were observed at approximately 36–37 kDa, but in contrast to SDS profiles, an evident heterogeneity of presence or absence of bands and/or intensity was observed in antigenic molecular masses below 30 kDa.

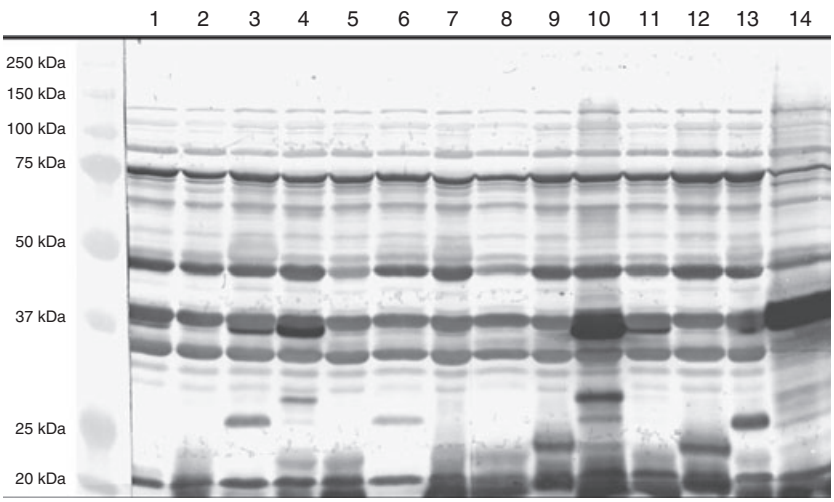

Figure 1 Immunoblot analysis. Strains SP-S1 (lane1), SP-S2 (lane 2), SP-S4 (lane 3), SP-H (lane 4), SP-I (lane 5), SP-27 (lane 6), SP-13 (lane 7), SP-1101 (lane 8), SP-1801 (lane 9), SP-2501 (lane 10), SP-0102 (lane 11), SP-23 (lane 12), SP-E (lane 13) and PG2-type strain (lane 14).

These lower molecular weight bands separated the studied strains into four different patterns: Profile 1 (strains SP-S1, SP-S2, SP-I, SP-1101, SP-0102, SP-13), profile 2 (strains SP-S4, SP-27, SP-E), profile 3 (strains SP-H, SP-2501) and profile 4 (strains SP-1801, SP-23).

Discussion

Mycoplasma agalactiae isolation was obtained in a 28·8% of milk samples of which 70% (7/10) of samples from silos and 17% (6/35) from tanks were positive.

These rates are higher than previous figures obtained for sheep and goat milk in Spain (Gonzalo *et al.* 2002; Contreras *et al.* 2008) and can be related to the high number of outbreaks recorded in Castilla y León revealing this region is an endemic area.

Regarding the antimicrobial activity, these results demonstrate the effectiveness of quinolones *in vitro* against *Myc. agalactiae*, which are included as standard treatments against CA, and show agreement with Hannan (2000); Loria *et al.* (2003) and Antunes *et al.* (2008) on the susceptibility of field isolates of *Myc. agalactiae*. Clindamycin was the most effective antimicrobial in all cases, but there is no previously published data to compare with this study. Erythromycin has been considered as an option for the treatment for CA (Bergonier *et al.* 1997; Antunes *et al.* 2008). Although other mycoplasmas have been reported to be susceptible, all strains of *Myc. agalactiae* in the present study were resistant, which is in agreement with the work of Antunes *et al.* (2008). In contrast to the results of previous studies, isolates in this study presented much higher oxytetracycline MIC values and slightly higher MIC values in the case of tylosin, although Loria *et al.* (2003) reported one isolate with an MIC of 2 μg ml^{-1}. Both of these antimicrobials are treatments of choice against the disease. Results obtained on Sicilian strains by Loria *et al.* (2003) were similar for lincomycin and enrofloxacin. According to suggested published MIC breakpoint values for *Myc. agalactiae* (Hannan 2000), most of the strains are classified as sensitive or intermediate resistant for oxytetracycline and tylosin where the intermediate values are suggested at 8 μg ml^{-1} and $\leq$2 μg ml^{-1}, respectively. Spectinomycin MIC values were similar to those of Antunes *et al.* (2008). A relatively variable response was seen to tulathromycin, which belongs to a group of new-generation macrolides, and depended on the strain (MIC range between 1 and 8 μg ml^{-1}) giving higher MIC_{50} and MIC_{90} values compared with other antimicrobials of the same family.

It is very important to know which antimicrobials of veterinary use are effective against *Myc. agalactiae* isolated from sheep farms, especially in affected areas of Spain and neighbouring Mediterranean countries where CA is endemic. It is well known that *in vitro* sensitivity of antimicrobials does not always correspond to the effectiveness of treatment in the field, but it is highly probable that antimicrobials with high MIC values are likely to be ineffective in treating the affected animal.

Molecular typing of the isolates revealed high levels of similarity among the strains. These results are in agreement with the data published in a global VNTR and MLST typing study (McAuliffe *et al.* 2011) where isolates characterized in this work were also included. Strains isolated from tank milk presented a similar pattern belonging to a unique European majority VNTR profile (VNTR profile 1121). Limited variation was observed when using MLST, dividing isolates into three sequence types (*ST-5*,

10 strains; *ST-16*, 2 strains and *ST-17*, 1 strain) all belonging to the same clonal complex.

These results were consistent with those reported for other isolates from Spain and other European countries by Tola *et al.* (1996) and de la Fe *et al.* (2006) and were in agreement with the opinion of de la Fe *et al.* (2006) about the low relatedness of the reference strain PG2 compared with the isolates from Spain. In addition, a further knowledge on conserved and variable strong immunogenic proteins could be of help in the design of effective vaccines against CA.

In conclusion, the isolates of *Myc. agalactiae* characterized in this work came from an endemic area and shared a common molecular profile, as revealed by PFGE and SDS-PAGE, with limited antigenic variability as visualized by immunoblot analysis. Clindamycin and quinolones showed great effectiveness against the isolates and may be considered for standard treatment for CA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Map showing the position of sampled farms and silos including positive and negative locations for isolates.

Table S1 Characteristics of field strains of *Mycoplasma agalactiae*.

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Multilocus sequence typing of *Mycoplasma agalactiae*

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Mycoplasma agalactiae is the main cause of contagious agalactia, a serious disease of sheep and goats, which has major clinical and economic impacts. We have developed a multilocus sequence typing (MLST) scheme using the sequenced genomes of the *M. agalactiae* strains PG2 and 5632. An MLST scheme based on the genes *gltX*, *metS*, *gyrB*, *tufA* and *dnaA* was designed and in total 3468 bp of sequence were analysed for each strain. MLST offers a highly discriminatory typing method for *M. agalactiae* and was capable of subdividing 53 strains into 17 distinct sequence types, largely according to geographical origin. MLST detected unexpected diversity in recent isolates from Spain, identifying two novel outliers, and enabled typing of novel Mongolian isolates for the first time. Genetic diversity in the sequenced regions was largely due to mutation, with recombination playing a much smaller role. A web-accessible database has been set up for this MLST scheme for *M. agalactiae*: http://pubmlst.org/magalactiae/. MLST offers a robust, objective molecular epidemiological tool for *M. agalactiae* that that enables interlaboratory comparison of data.

INTRODUCTION

Known for nearly 200 years, contagious agalactia is primarily a disease of dairy sheep and goats characterized by mastitis, arthritis and keratoconjunctivitis (OIE, 2003). It is mainly caused by *Mycoplasma agalactiae* but in recent years, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *capri* and *M. putrefaciens* have also been isolated in many countries from goats with mastitis, arthritis and, occasionally, respiratory disease. The clinical signs of the infections are sufficiently similar to those of contagious agalactia for the Office International des Epizooties to include them as causes of this listed disease (OIE, 2003).

M. agalactiae is prevalent worldwide but causes particular problems around the Mediterranean basin, where it has a major clinical and economic impact on the small-ruminant dairy industry (Bergonier *et al.*, 1997). In most cases, infected hosts spontaneously recover from acute clinical signs within a few weeks but develop a chronic infection accompanied by shedding of *M. agalactiae* in milk and/or other body secretions for years without presenting any clinical signs; these asymptomatic carriers can transmit the bacteria to other susceptible animals and cause acute disease (Bergonier *et al.*, 1997).

As *M. agalactiae* shows differing prevalence across the world, and as it is currently absent from some countries, notably the UK, there is a pressing need for molecular epidemiological techniques which enable a high degree of strain differentiation, allowing the tracing of the source of disease outbreaks. Early studies showed that *M. agalactiae* was largely homogeneous, with PFGE analysis revealing no variation in over 80 Italian isolates although some degree of antigenic variation was evident (Solsona *et al.*, 1996; Tola *et al.*, 1996, 1997). Insertion sequence analysis of *M. agalactiae* isolates of wide geographical origin found some degree of variation, with some isolates containing and others lacking the IS element ISMag, although subtyping isolates was not possible (Pilo *et al.*, 2003).

Recently the genome of the *M. agalactiae* type strain PG2 has been sequenced (Sirand-Pugnet *et al.*, 2007), making it highly amenable to analysis using sequence-based typing methods such as variable number of tandem repeats (VNTR) analysis and multilocus sequence typing (MLST). We have previously described the identification and characterization of VNTRs within the *M. agalactiae* PG2 genome, the selection of four VNTRs which showed most

Abbreviations: HR, homologous recombination; MLST, multilocus sequence typing; ST, sequence type; VNTR, variable number of tandem repeats.

intraspecific variation, and their use as a molecular epidemiological tool for the analysis of 88 *M. agalactiae* strains (McAuliffe *et al.*, 2008). VNTR analysis revealed unexpected diversity among *M. agalactiae* strains, with 14 different profiles seen (McAuliffe *et al.*, 2008).

MLST utilizes the sequences of internal fragments of several housekeeping genes for unambiguous characterization of bacterial isolates. For each gene, different sequences are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST) (reviewed by Maiden, 2006).

Although the number of nucleotide differences between alleles is ignored when assigning alleles for epidemiological purposes, the embedded sequence data can provide information about the population structure of the species and the relevant contribution of mutation and recombination in the genetic diversity seen (Feil *et al.*, 2001; Maiden, 2006). In addition to its use for determining evolutionary relationships between bacteria, MLST offers many advantages over other molecular typing schemes as it is portable and unambiguous, and as data are shared via the internet the results are directly comparable between laboratories around the world.

Originally developed for *Neisseria meningitidis* (Maiden *et al.*, 1998), MLST has subsequently been applied to numerous bacterial species, including the porcine pathogen *Mycoplasma hyopneumoniae* (Mayor *et al.*, 2008). The present study describes the development and application of a novel MLST scheme to *M. agalactiae* and a comparison of MLST with a previously developed VNTR scheme.

METHODS

Mycoplasma culture and DNA extraction. *M. agalactiae* isolates (as listed in Table 3: see Results) were stored at −80 °C and grown in 3 ml aliquots of Eaton's broth medium (Nicholas & Baker, 1998) for 24 h. A loopful of the culture was then plated on Eaton's solid medium and incubated at 37 °C, 5 % CO_2 for between 48 and 72 h. Single colonies were picked and grown in 3 ml Eaton's broth until growth was detected and then aliquots were frozen in 15 % (v/v) glycerol at –80 °C until required.

Confirmatory tests. DNA from each strain was extracted from 1.5 ml of culture using a Genelute gDNA extraction kit (Sigma), following the manufacturer's instruction. To confirm the identity of the isolates as *M. agalactiae*, PCR based on the *uvrC* gene (Subramaniam *et al.*, 1998) and 16S rRNA gene PCR DGGE (McAuliffe *et al.*, 2005) were performed.

Primer design and PCRs. Genes were chosen for MLST based on the sequenced genome of *M. agalactiae* type strain PG2 (Sirand-Pugnet *et al.*, 2007). The gene targets and primer sequences are listed in Table 1. Gene targets were chosen so that they were distributed evenly within the genome as calculated by the distance from the origin of replication. Initially primers (primer set 1) were chosen so that they amplified the whole of the gene. Initial screens looked at genes *gyrB*, *tpi*, *gltX*, *recA*, *uvrC*, *tufA*, *dnaA* and *metS*. Primers were chosen so that they had an optimum annealing temperature of 55 °C. All PCRs were carried out using the cycling conditions of denaturation at 94 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 60 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min; samples were kept at 4 °C until analysis. For the PCR, 1 µl extracted DNA was added as a template to 49 µl of a reaction mixture containing 10 mM Tris/HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 0.2 mM each deoxynucleoside triphosphate, 0.5 mM each primer and 0.5 U Taqgold (Applied Biosystems). Samples were checked for correct amplification by electrophoresis on a 2 % agarose gel followed by visualization with ethidium bromide under UV illumination.

Initial MLST studies on a subset of eight strains using primer set 1 gave long reads encompassing most of the whole coding region for *dnaA*, *gltX*, *gyrB*, *metS*, *tufA*, *tpi*, *recA* and *uvrC*. Analysis of sequences showed some variation in *dnaA* between 300 and 900 bp, in *gltX* between 350 and 950 bp, in *gyrB* between 1000 and 1300 bp, in *metS* between 500 and 1000 bp and in *tufA* between 300 and 1100 bp. No variation was seen in *tpi*, *recA* or *uvrC*. Based on these sequences, a new set of internal primers was designed to ensure that high-quality forward and reverse sequence was obtained over the variable regions

Table 1. Primers based *on M. agalactiae* PG2 genome used for initial screen (1), for subsequent MLST analysis (2) and alternative primers designed based on *M. agalactiae* 5632 genome (3)

Gene target	Forward	Reverse	Product size (bp)
gyrB (1)	tagcaaaacaccctcaaact	attttcaggattcattgtgg	1968
gltX (1)	taggtggtgctagaactgct	tgctcaatgtaagttgttgc	1392
tpi (1)	ccgtcagaattgacagaaat	tagaaaagccaatccacaat	777
tufA (1)	attttgaccgtagcaaagaa	gaccacctgtaacgtctgtt	1191
dnaA (1)	aaccccaaactcacaatatg	attgctctgtttctttttcg	1401
uvrC (1)	tgttttgtatgttggcaaag	tagcaacattaaatggcaca	1716
metS (1)	gcatcagaaagattcattg	ctgctgctgctaataaggat	1550
recA (1)	tgctcaatgtaagttgttgc	agtgatgttcttgccttgat	993
gyrB (2)	caatacacatcaaccttcca	agcagagttaccttcgacaa	519
gltX (2)	gccttgcctacaaatcttat	atagttgcttaagcgcaaac	792
tufA (2)	gaacatgattactggtgctg	acggccttttttcaaattcta	781
dnaA (2)	taacgtaaccccaaactcac	cataattcaggcgtcatctt	762
metS (2)	gcctgtaaattagcccttct	ttttaaccaaaaatcaagctg	756
gltX (3)	ggcgaaagaagtcagttaga	gagcagtttctgaaggctta	1080

Table 2. Details of the five loci used in the *M. agalactiae* typing scheme, including genetic variation, where *H* is genetic diversity, and d_N/d_S is the ratio of non-synonymous to synonymous mutations

Locus	Gene function	Start position (in PG2 genome)	Seq. size (bp)	Nucleotide position within gene	No. of alleles	*H*	d_N/d_S
dnaA	Chromosomal replication initiation protein	1	672	238–909	8	0.5035	0.0193
metS	Methionyl-tRNA synthetase	237325	687	451–1137	6	0.2227	0.0508
tufA	Elongation factor Tu	382492	681	331–1011	5	0.3404	0.0231
gltX	Glutamyl-tRNA synthetase	669536	699	373–1071	5	0.2227	0.0729
gyrB	DNA *gyr*ase subunit B	854497	420	901–1320	4	0.6016	0.0197

in *gyrB*, *gltX*, *metS*, *tufA* and *dnaA*. These primers (primer set 2) are shown in Table 1. It was found the set 2 *gltX* primers that were designed based on the *M. agalactiae* PG2 genome did not yield amplicons for two strains (L9 and 281F03) and no sequence data could be produced. Therefore additional primers were designed based on the *M. agalactiae* 5632 genome (Nouvel *et al.*, 2010). These primers are also given in Table 1 (set 3); they provided high-quality forward and reverse sequence in the region of interest for both L9 and 281F03.

PCR purification and DNA sequencing. PCR products were purified using a Qiaquick PCR purification kit (Qiagen), quantified by running on 2 % agarose E gels with an E gel quantitative ladder (Invitrogen). Sequencing reactions were then carried out using the BigDye v3.1 kit (Applied Biosystems) and samples were run using the ABI Prism 3730 Genetic Analyzer. Sequences were analysed and contigs generated and aligned against a reference sequence using SeqScape v2.6 (Applied Biosystems). Any apparent polymorphisms in sequence were confirmed by manually analysing the sequencing traces.

Allele and ST assignment. Allele numbers were assigned to sequences. For each locus, distinct allele sequences were assigned arbitrary allele numbers in the order of identification. Each genotype was therefore designated by five numbers (e.g. 1-3-2-4-1) that constituted an allelic profile or sequence type (ST; e.g. ST-3). The STs were assigned arbitrary numbers in the order of description.

Data analysis. MLST data analysis was performed using the non-redundant database (NRDB) at pubMLST (http://pubmlst.org/analysis/) to assign sequence types. Dendrograms to show relatedness between strains based on allelic profiles were constructed using the PHYLIP neighbour-joining methods at http://pubmlst.org/analysis/.

Simpson's index of diversity, which is based on the probability that two unrelated strains will be placed into different typing groups, was calculated according to Hunter & Gaston (1988). Simpson's index of diversity ranges from 0.0 to 1.0, where 0.0 means that all strains in a population are of an identical type and 1.0 indicates that all of the strains in a population are different and can be distinguished from one other.

BURST analysis to reveal the relationship of MLST sequence types and to analyse clonal complexes was carried out with eBURST V3 (accessible at http://eburst.mlst.net).

The 'standardized' index of association (I_A) was used to test the null hypothesis of linkage equilibrium for multilocus data and therefore determine the relative contribution of mutation and recombination to the diversity seen by MLST. I_A is zero for linkage equilibrium and deviation from this indicates a degree of linkage disequilibrium. Analysis was done using the LIAN program available at http://pubmlst.org/analysis/.

VNTR analysis. VNTR analysis was carried out on new strains not included in our previous study as was described previously (McAuliffe *et al.*, 2008). Four targets were assessed (VNTR 5 and VNTR 19 in predicted lipoproteins, and VNTR 14 and VNTR 17 in intergenic regions). VNTR profiles were recorded as character data using allelic profiles and then dendrograms were constructed using neighbour-joining with PHYLIP hosted at http://pubmlst.org/analysis/.

RESULTS

Analysis of sequence variation in housekeeping genes

Genetic variation was seen in the genes *gyrB*, *gltX*, *metS*, *dnaA* and *tufA*. An online database for this MLST scheme has been set up using specially designed software, BIGSdb (Jolley & Maiden, 2010) and is available at http://pubmlst.org/magalactiae/. The greatest variation was seen in *metS*, with 36 polymorphisms found giving 6 different alleles. Twenty-three polymorphisms were found in *dnaA* giving 8 different alleles, 11 polymorphisms in *tufA* giving 5 different alleles, 3 polymorphisms in *gltX* giving 5 different alleles and 21 polymorphisms in *gyrB* giving 4 different alleles. The genetic diversity (*H*) was calculated for each locus using LIAN: *dnaA* had a diversity of 0.5035, *gltX* of 0.2227, *gyrB* of 0.3404, *metS* of 0.2227 and *tufA* of 0.6016 (Table 2).

Determination of the index of association

LIAN analysis gave a standardized index of association of 0.2318. Essentially this value of I_A shows that the genetic diversity seen is due to mutation and not recombination and the genetic variation seen is in linkage disequilibrium.

Determination of allelic profiles and sequence types for strains

Sequences were analysed using the NRDB at http://pubmlst.org/analysis/ and it was found that there were 17 STs among the 51 strains (as summarized in Table 3). ST-5

Table 3. Description of strains used in this study, their ST, allelic profile, BURST type and VNTR profile

Strain	Origin	Year of isolation	BURST group*	ST	Allelic profile					VNTR profile
					dnaA	*gltX*	*gyrB*	*metS*	*tufA*	
NCTC 10123 (PG2)	USA	1983	CC1	1	1	1	1	1	1	1111
222F03	Greece	2003	CC1	2	1	1	1	2	1	1111
233F03	Greece	2003	CC1	2	1	1	1	2	1	1111
240F03	Greece	2003	CC1	2	1	1	1	2	1	1111
244F03	Sardinia, Italy	2003	CC2	3	1	1	1	2	2	1021
247F03	Sardinia, Italy	2003	CC2	3	1	1	1	2	2	1021
235F03	Greece	2003	AG	4	1	1	2	2	1	1121
259F03	Portugal	2003	CC2	5	1	1	2	2	2	1121
269F03	Portugal	2003	CC2	5	1	1	2	2	2	1121
271F03	Portugal	2003	CC2	5	1	1	2	2	2	1121
289F03	Spain	2003	CC2	5	1	1	2	2	2	0121
1640/98	Sicily, Italy	1998	CC2	5	1	1	2	2	2	1011
14SR97	Macedonia†	1997	CC2	5	1	1	2	2	2	1121
S2	Spain	2010	CC2	5	1	1	2	2	2	1121
I	Spain	2010	CC2	5	1	1	2	2	2	1121
27	Spain	2010	CC2	5	1	1	2	2	2	1121
13	Spain	2010	CC2	5	1	1	2	2	2	1121
1101	Spain	2010	CC2	5	1	1	2	2	2	1121
1801	Spain	2010	CC2	5	1	1	2	2	2	1121
2501	Spain	2010	CC2	5	1	1	2	2	2	1121
0102	Spain	2010	CC2	5	1	1	2	2	2	1121
E	Spain	2010	CC2	5	1	1	2	2	2	1121
23	Spain	2010	CC2	5	1	1	2	2	2	1121
165/99	Sicily, Italy	1999	CC2	5	1	1	2	2	2	1012
250F03	Italy	2003	CC2	5	1	1	2	2	2	1012
256F03	Italy	2003	CC2	5	1	1	2	2	2	1012
243F03	Sardinia, Italy	2003	CC2	5	1	1	2	2	2	1121
287F03	Spain	2003	CC2	5	1	1	2	2	2	1121
501F03	Sicily, Italy	2006	CC2	5	1	1	2	2	2	1121
Lo Faso	Sicily, Italy	2000	CC2	5	1	1	2	2	2	1121
263F03	Portugal	2003	CC2	5	1	1	2	2	2	1121
234F03	Greece	2003	AG	6	1	1	2	4	1	0121
249F03	Sardinia, Italy	2003	CC2	7	3	1	2	2	2	
224F03	Greece	2003	AG	8	1	2	2	3	1	1131
225F03	Greece	2003	AG	8	1	2	2	3	1	1131
227F03	Greece	2003	CC1	9	1	3	1	2	1	1101
401	Mongolia	2006	AG	10	2	1	2	2	1	1122
752	Mongolia	2006	AG	10	2	1	2	2	1	1122
14	Mongolia	2006	AG	10	2	1	2	2	1	1122
334	Mongolia	2006	AG	10	2	1	2	2	1	1122
44	Mongolia	2006	AG	10	2	1	2	2	1	1122
356	Mongolia	2006	AG	11	2	1	2	2	3	1112
564	Mongolia	2006	AG	11	2	1	2	2	3	1112
659	Mongolia	2006	AG	11	2	1	2	2	3	1112
666	Mongolia	2006	AG	11	2	1	2	2	3	1112
281F03	Spain	2003	Singleton	12	4	4	3	5	4	1001
568	Mongolia	2006	AG	13	5	1	2	2	1	1122
572	Mongolia	2006	AG	14	5	1	2	2	3	1112
L9	Gran Canaria, Spain	Unknown	Singleton	15	6	5	4	6	5	2001
S1	Spain	2010	CC2	16	7	1	2	2	2	1121
H	Spain	2010	CC2	16	7	1	2	2	2	1121
S4	Spain	2010	CC2	17	8	1	2	2	2	1121

*AG, ancestral group; CC, clonal complex. †Republic of Macedonia.

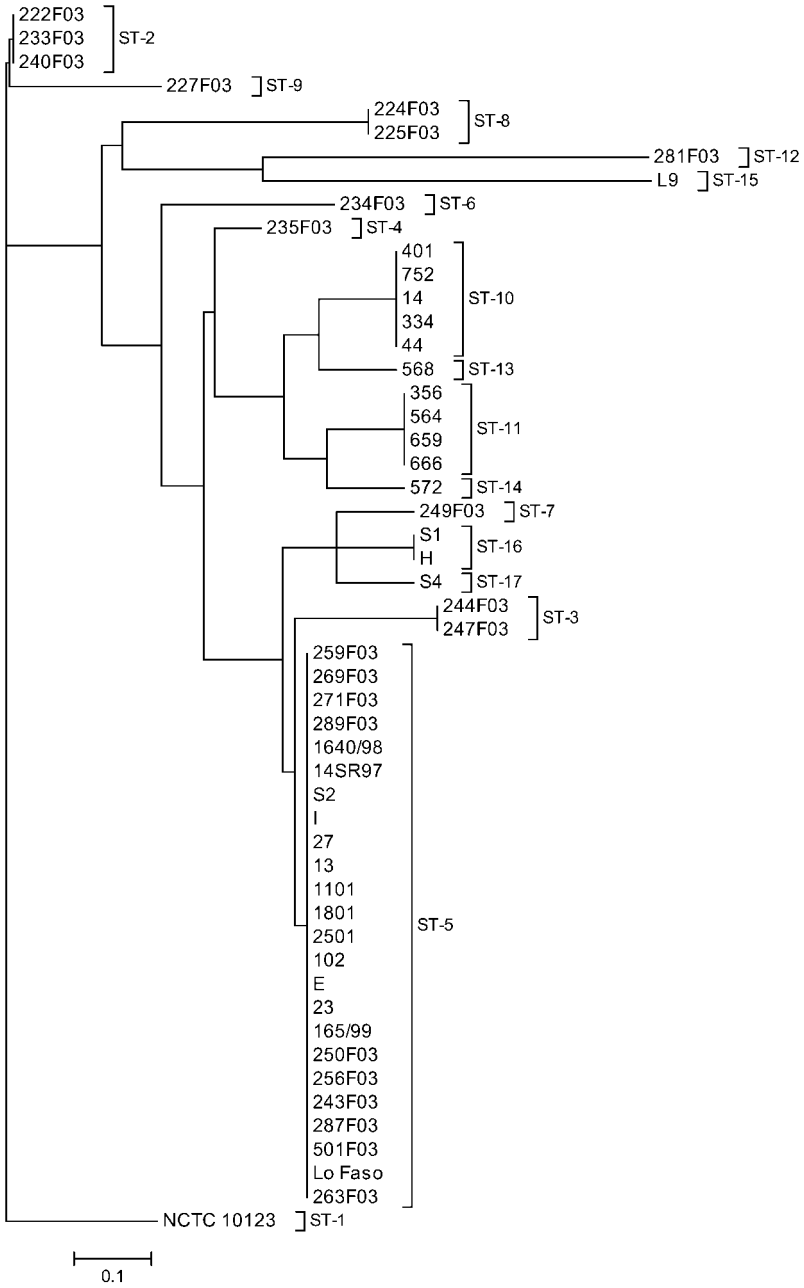

Fig. 1. Dendrogram of MLST allelic profiles of isolates as determined using neighbour-joining analysis (scale bar represents number of differences). Pairwise distances were calculated using the unweighted pair group method with arithmetic averages (UPGMA). ST designations are indicated for each branch.

with the allelic profile of 11222 was the most numerically dominant ST and corresponded to 24 strains of European origin. Spanish strains were diverse, representing 5 STs. Greek strains represented 5 STs, Mongolian strains represented 4 STs, and Sardinian strains represented 3 STs. All other Italian strains, the single Macedonian strain tested and Portuguese strains were characterized as ST-5. A dendrogram of the MLST allelic profiles is shown in Fig. 1.

Determination of BURST types

Three clonal complexes were found using BURST analysis. For this study, clonal complexes are defined as a group of multilocus genotypes in which every genotype shares at least three of the five loci in common with at least one other member of the group. The most likely ancestral genotype was identified by comparing in turn each genotype within a clonal complex with all other genotypes within the clonal complex. The ancestral genotype was defined as the genotype within the clonal complex that differs from the highest number of other genotypes in the clonal complex at only one locus out of five. To put it another way, the ancestral clone is that genotype defining the highest number of single-locus variants, or SLVs. SLVs are identical to the ancestral genotype at four loci, but differ at the fifth. The ancestral group comprised ST-4, which contained a single Greek strain 235F03. SLVs of the ancestral group were ST-6 from Greece and ST-10 and

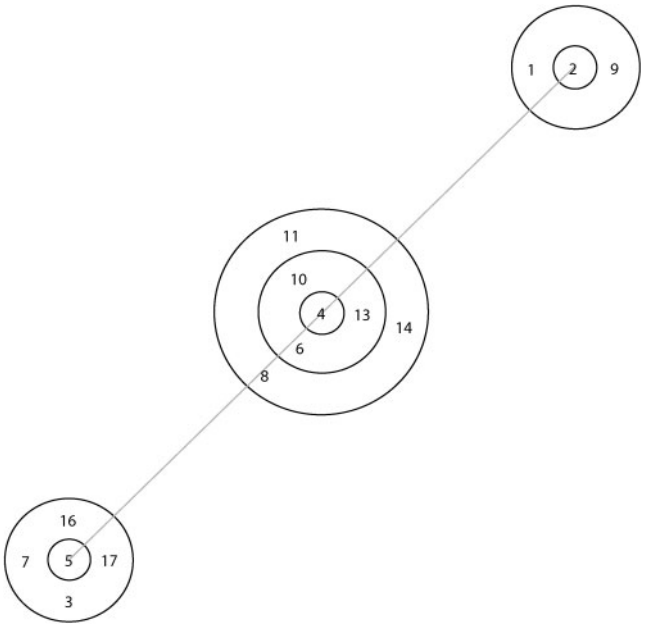

Fig. 2. BURST analysis of *M. agalactiae* STs. The assigned ancestral genotype is within the central ring (ST-4). The central group is thus considered to be the ancestral group (AG), with single-locus variants (SLVs) and double-locus variants (DLVs) shown in concentric circles. Two additional clonal complexes were identified, CC1 (centred on ST-2) and CC2 (centred on ST-5). ST-2 and ST-5 are both SLVs of the ancestral genotype ST-4.

ST-13 from Mongolia. Double-locus variants (DLVs) of the ancestral group were ST-8 from Greece, ST-11 from Mongolia and ST-14 from Mongolia. Clonal complex 1 was centred on ST-2 from Greece and SLVs were ST1, the type strain from the USA and ST-9 from Greece. Clonal complex 2 was centred on ST-5, which contained strains of multiple European origins, and SLVs were ST-3 from Sardinia, ST-7 from Sardinia, and ST-16 and ST-17 from Spain. ST-12 and ST-15 (Spain) were not linked to the clonal complex and were singletons (Fig. 2).

VNTR analysis

As was seen previously (McAuliffe *et al.*, 2008) *M. agalactiae* isolates were relatively heterogeneous, with 12 different profiles obtained using VNTR analysis; these are summarized in Fig. 3. Analysis of the 12 strains of *M. agalactiae* from Mongolia produced a unique set of profiles not observed before in any other *M. agalactiae* strains with either a 1112 or 1122 profile given.

Group 1 contained three Greek strains and the Spanish type strain. Groups 2 and 3 comprised five and six Mongolian strains respectively. Group 4 comprised two Greek isolates. Group 5 was a single Sicilian strain. Group 6 contained three Italian strains. Group 7 was a single Spanish isolate. Group 8 also consisted of a single Spanish isolate, L9. Group 9 contained two Greek strains, and the closely related group, group 10, also contained a (single) Greek strain. Group 11 contained a Spanish strain and a Greek strain, and group 12 was a large indistinguishable group of 23 European strains of multiple origins.

Congruence between MLST and VNTR analysis

MLST was capable of differentiating some strains which were indistinguishable using VNTR analysis. For example, VNTR group 1 could be separated into two groups using MLST analysis, with the type strain found to be distinctly different from all other strains. Strains in VNTR group 6 could also be differentiated using MLST analysis. Similarly, strains found to be identical by VNTR analysis (VNTR group 11, and strains S1, H and S4 in VNTR group 12) could be distinguished using MLST analysis. Conversely, a single strain 1640, which was different from all other strains using VNTR analysis, was found to fall into the large homogeneous European group using MLST analysis. Overall, the methods placed 75 % of strains into equivalent groups.

Determination of Simpson's index of diversity

Simpson's index of diversity was calculated to be 0.7843 for MLST analysis of these strains and 0.7746 for VNTR analysis.

DISCUSSION

To our knowledge this study is the first to undertake MLST analysis of the important small-ruminant pathogen *M. agalactiae*. Of the eight genes initially examined, five (*gyrB*, *gltX*, *metS*, *dnaA* and *tufA*) showed some degree of variation and were selected for inclusion in this MLST scheme. However, the other three genes that were investigated (*recA*, *tpi* and *uvrC*) were completely invariant over the entire region of sequence coverage (3486 bp). This suggests that these loci are under stabilizing selection, that is, the sequence is optimal for the function that it performs. Any other variation is deleterious and therefore it will not persist in the population. Nonetheless, this MLST scheme of five loci (3160 nt) offers a highly discriminatory typing method for *M. agalactiae* and was capable of subdividing 53 strains into 17 distinct sequence types, largely according to geographical origin. MLST detected unexpected diversity in recent isolates from Spain, identifying two novel outliers, and also enabled typing of novel Mongolian isolates for the first time. The analysis of the Mongolian isolates is interesting as as they form a distinct branch (Fig. 1) and it can therefore be speculated that there are different lineages prevalent in Asia compared to Europe.

Previous comparative genome sequence analysis has indicated significant genome variation between *M. agalactiae* strains: an additional 95 genes (representing an extra 130 kbp) found in a field strain when compared to the PG2

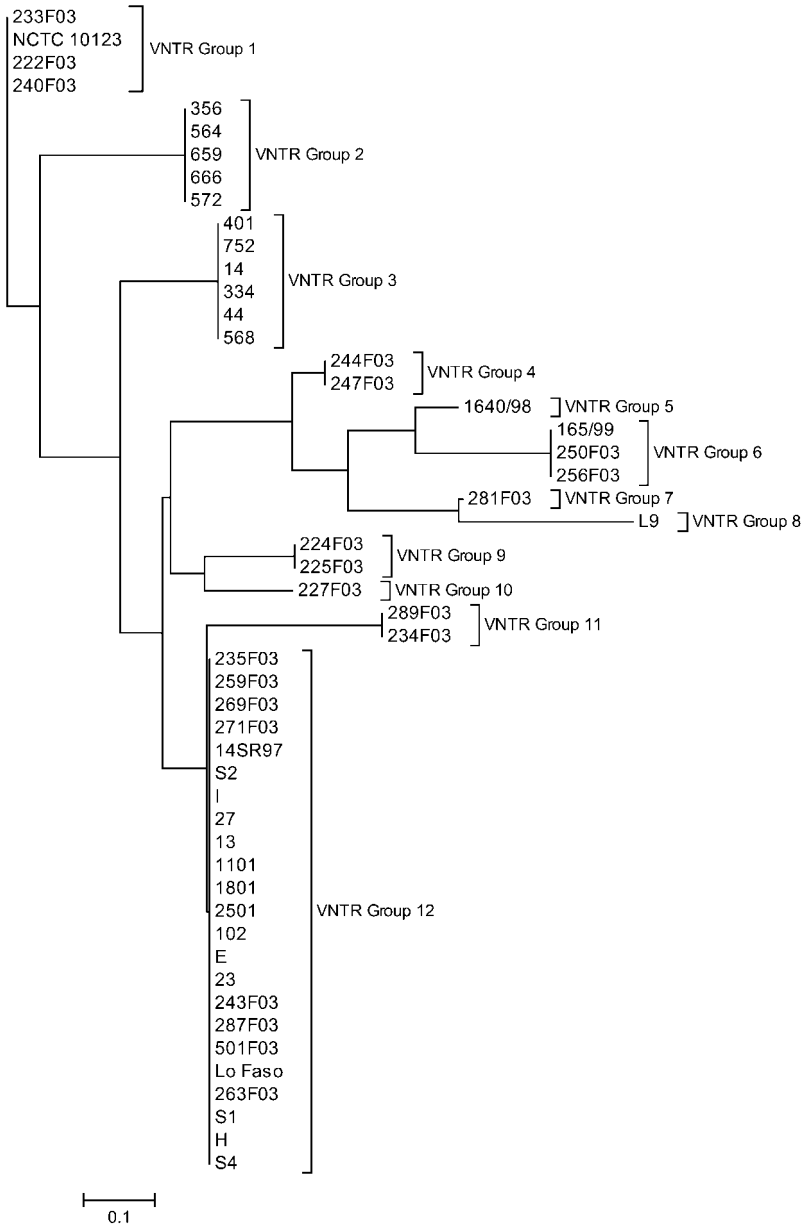

Fig. 3. Dendrogram of VNTR analysis of *M. agalactiae* strains. VNTR profiles were recorded as character data using allelic profiles and then dendrograms were constructed using neighbour-joining (scale bar represents number of differences). Pairwise distances were calculated using the unweighted pair group method with arithmetic averages (UPGMA). VNTR groups are designated for each branch.

type strain (Nouvel *et al.*, 2010). However, these genes are clearly non-essential and as such could be considered to be 'accessory genes'. The MLST work here demonstrates that sequence variation also occurs within housekeeping genes, indicating that the core genome is also variable. However, the large majority of variation for all five loci was synonymous (Table 2), meaning that there was a smaller amount of variation in the expressed proteins. Each locus was shown to be under purifying selection by means of a one-tailed *Z*-test ($P<0.005$ for all loci), indicating that the coded proteins are essential and conserved for their respective functions. This is not unexpected for an organism with a minimal genome.

MLST analysis detected a large homogeneous group of European strains that were indistinguishable; a similar finding was also made using VNTR analysis (McAuliffe *et al.*, 2008), despite there being some isolates that differed using the two methods. Large groups such as this may be more successful (see below) or this result may indicate that these strains have been oversampled. It has previously been described how highly successful clones will become widespread in a population (Vos & Didelot, 2009). Therefore these clonal strains may offer a selective advantage and studies should be undertaken to determine if they are significantly more persistent, more virulent or more recalcitrant to antibiotics than other strains.

Two strains (L9 and 281F03 from Spain) were found to be substantially different from all other strains studied and as such were singletons which did not fit into any clonal complex. Some regions from these strains could also not be

amplified using primers based on the PG2 genome and required alternative primers based on the 5632 genome. These strains represent unusual isolates and are likely to be related to the unusual field strains described previously (Nouvel *et al.*, 2010), which possess additional genes, mobile genetic elements, gene families encoding surface proteins and integrative conjugative elements. The impact of these genomic differences on virulence and persistence of strains is worthy of future study.

Analysis was also carried out to determine what the relative contributions of mutation and homologous recombination (HR) were in the genetic diversity seen among *M. agalactiae* strains. It was found that diversity was largely due to mutation, with recombination playing a much smaller role. However, as described by Feil and co-workers (Feil *et al.*, 2001; Feil & Enright, 2004), with the possible exception of *Salmonella enterica* (Selander & Smith, 1990), few bacterial species appear to be truly clonal, such that recombinational exchanges are absent, or so rare that they are observed only in genes under strong diversifying selection. In these species, clones should be stable because diversification at housekeeping loci depends almost entirely on the accumulation of point mutations, but, with increasing ratios of recombination to point mutation, bacterial clones should become increasingly transient. Care must be taken in the interpretation of values of HR, as Maynard Smith *et al.* (1993) first pointed out that the over-representation of closely related, high-frequency (epidemic) clones in a sample will lead to an inflated estimate of clonality of the population as a whole. Oversampling of a single clone in an epidemic population structure will therefore result in an underestimation of HR. Nonetheless, ST-5 was represented by isolates from temporally and spatially separated disease outbreaks, suggesting that this type may be stable and more pathogenic than other potential variants.

Interestingly, MLST has only been carried out on one other mollicute species, *Mycoplasma hyopneumoniae*, so it is difficult to test statistically whether HR is reduced in certain phylogenetic or ecological groups such as the mollicutes. HR was actually found to be relatively high for *M. hyopneumoniae* (Mayor *et al.*, 2008) but as has been shown for other phyla, wide variation in HR rates can occur among species belonging to the same phylum or division (Vos & Didelot, 2009).

This study has also confirmed the utility of VNTR analysis as a rapid screening tool for molecular epidemiology purposes and it gave results that were largely congruent with the MLST analysis. Differences may largely be due to MLST being based on sequences of genes under purifying selection whist VNTR regions are by their nature likely to be under different levels of selection. As such MLST is likely to be more representative of evolutionary lineages, whilst VNTR could reflect local adaptations. However, MLST was capable of greater discrimination and has the added advantages of also providing meaningful information about population structure and being totally objective and subject to easy inter-laboratory comparison.

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CONCLUSIONES

- La conservación de la leche a 4 ºC evidenció un incremento significativo de los recuentos de los grupos bacterianos de mesófilos, psicrotrofos y coliformes a lo largo del tiempo, mientras que el recuento del resto de variables microbiológicas estudiadas (termodúricos, *E. coli*, *S. aureus* y *Str. agalactiae*) se mantuvo constante. Este aumento de concentración de los tres primeros grupos bacterianos, hace aconsejable un rápido procesado de la leche con el fin de evitar alteraciones de la misma y sus productos. El azidiol, combinado con refrigeración a 4 ºC, ha demostrado ser un conservante adecuado para mantener los recuentos microbiológicos iniciales en todos los grupos y especies estudiados en leche de oveja, y particularmente de los mesófilos, psicrotrofos y coliformes cuyos recuentos iniciales se mantuvieron durante 96h. La congelación afectó significativamente a la viabilidad de coliformes, *E. coli*; *S. aureus* y *Str. agalactiae.*
- El recuento de bacterias totales y de células somáticas, tal como es determinado de forma rutinaria en los laboratorios de análisis de leche, constituye una herramienta útil en la monitorización y la mejora continua de la calidad higiénica de la leche en las explotaciones de ganado ovino lechero. Las bacterias psicrotrofas, termodúricas y coliformes se relacionaron significativamente con el RBTLT, mientras que los CGPCN estuvieron asociados tanto con RBTLT como con el RCSLT. Además, las variables RBTLT y RCSLT estuvieron correlacionadas entre sí. Los factores estación anual, terapia de secado y tipo de ordeño son fuentes significativas de variación de los recuentos de distintos grupos microbianos. Así, la flora psicrotrofa y los coliformes presentaron recuentos más elevados en invierno, mientras que los CGPCN fueron mayores en verano y en los rebaños ordeñados manualmente. La terapia antibiótica redujo significativamente el RBTLT y el RCSLT, así como los recuentos de psicrotrofos, evidenciando que algunos patógenos mamarios podrían crecer en condiciones de refrigeración. Futuros estudios serán necesarios con el fin de determinar la influencia de factores de riesgo dentro de la propia explotación que puedan afectar a los recuentos bacteriológicos específicos de la leche de tanque de los rebaños de ovino lechero, con el fin de establecer valores umbral específicos de los distintos grupos microbianos y su influencia en la calidad higiénica de la leche.

- En leche ovina de tanque y de silo se reveló la presencia de una amplia diversidad de CGPCN cultivables, 23 especies, pertenecientes a cinco géneros diferentes. El uso de técnicas moleculares para la identificación de este extenso grupo taxonómico puede ser de interés tanto para los productores como para la industria láctea, de cara al desarrollo de programas de control de calidad, control de mamitis y seguridad alimentaria tanto en leche como en sus productos. Por otra parte, la leche de oveja producida en los países de la Cuenca Mediterránea, puede constituir una fuente de microbiota comensal potencialmente patógena y/o resistente a antibióticos. La ampliación del estudio sobre factores de virulencia y resistencia a antimicrobianos en CGPCN deberá tenerse en cuenta en el futuro.
- La aparición del genotipo *vanC1* en aislados de *E. faecalis* de origen animal enfatiza la necesidad de examinar los genes de resistencia intrínseca y adquirida a glicopéptidos y sugiere el intercambio de genes por conjugación entre *E. faecalis* y *E. gallinarum* de leche ovina de tanque, cuestionando el uso del gen *van*C1 como marcador de especie de *E. gallinarum*. El hallazgo de *E. faecalis* vancomicina resistente en leche de oveja destinada a la fabricación de productos lácteos es significativa ya que *E. faecalis* es el enterococo predominante en los quesos mediterráneos y podría ser transferido al ser humano a través del consumo. Además, la capacidad de esta especie para adquirir y transmitir genes de resistencia sugiere que su presencia debería ser cuidadosamente monitorizada a lo largo de la cadena alimentaria.
- Los aislados de *Myc. agalactiae* caracterizados en Castilla y León proceden de una zona endémica y comparten un perfil molecular común, como revelaron los estudios de PFGE y SDS-PAGE, con una limitada variabilidad antigénica que se visualizó por medio de análisis Immunoblot. La clindamicina y las quinolonas mostraron efectividad frente a los aislados y podrían ser considerados como tratamientos estándar contra la agalaxia contagiosa. El uso de la eritromicina debe ser descartado dada la fuerte resistencia mostrada por los aislados españoles. El análisis MLST ha demostrado ser una herramienta más precisa para evaluar la diversidad de las mismas cepas, identificando dos secuenciotipos de nueva descripción, mientras que el resto de cepas fueron incluidas en el grupo compartido por la mayoría de las cepas Europeas estudiadas hasta el momento.

CONCLUSIONS

- Unpreserved silo sheep milk stored at 4°C significantly increased the concentration of mesophiles, psychrotrophic, and coliform bacteria over time, which makes it advisable to rapidly process the milk stored in silos to avoid its rapid deterioration. However, initial concentration of thermoduric, *E. coli*, *Str. agalactiae* and *S. aureus* remained stable. Storage at 4°C of azidiol-preserved sheep milk was a suitable method to maintain the initial concentration for all studied bacterial groups and species, particularly for mesophiles, psychrotrophs, and coliforms throughout 96 h. Freezing significantly decreased the viability of coliforms, *E. coli*, *Str. agalactiae* and *S. aureus*.
- The BTTBC and BTSCC variables determined in dairy laboratories are very useful tools for continuous monitoring and improvement of milk hygiene quality in dairy sheep. Psychrotrophic, coliform, and thermoduric bacterial groups were significantly related to BTTBC, whereas GPCNC were associated with both BTTBC and BTSCC variables in dairy sheep flocks. In addition, BTTBC correlated with BTSCC. Season, dry therapy, and milking type were important sources of variation for bulk tank milk differential counts; psychrotrophs and coliforms had elevated counts in winter, whereas GPCNC counts were higher in summer and in hand-milked flocks. Further studies are necessary to investigate on-farm risk factors associated with BTTBC in dairy sheep and to define effective discrimination thresholds for specific bacterial counts in bulk tank milk and its influence on milk hygienic quality.
- A wide diversity of culturable GPCNC, including 23 species from five different genera, was revealed in sheep bulk tank milk, normally under or misidentified by common phenotypic methods. It is likely that these species have been found in ovine milk before but have been associated with other taxa within the same genus or others. The molecular approach to the identification of this large taxonomic group can be of help to either farmers or dairy industry in getting more accurate view of Gram positive cocci population in sheep milk regarding mastitis and quality control programms, food safety and new products development. On the other hand, sheep milk

in Mediterranean countries, is used mainly for the fabrication of raw and pasteurized milk cheeses and dairy products that would be a source of GPCNC commensal and potentially pathogenic or drug resistant microbiota. Further studies on virulence factors and antimicrobial resistances of GPCNC in ewe milk are advisable.

- Occurrence of *vanC1* genotype in *E. faecalis* animal isolates emphasizes the need for screening intrinsic and acquired glycopeptide resistance genes and suggests conjugative exchange between *E. faecalis* and *E. gallinarum* from sheep milk calling into question the use of *vanC1* as *E. gallinarum* species marker. The finding of vancomycin resistant *E. faecalis* in sheep's milk intended for manufacturing dairy products is significant, as *E. faecalis* is the predominant enterococcal species in Mediterranean cheeses which could potentially be transferred to humans in comsumption. In addition, the ability of this species to acquire and transfer resistance genes suggests that its presence should be carefully monitored throughout the food chain.
- The isolates of *Myc. agalactiae* characterized in Castilla y León came from an endemic area and shared a common molecular profile, as revealed by PFGE and SDS-PAGE, with limited antigenic variability as visualized by immunoblot analysis. Clindamycin and quinolones showed great effectiveness against the isolates and may be considered for standard treatment for contagious agalactia. Use of erythromycin must be discarded due to the strong resistance showed by Spanish isolates. MLST analysis demonstrated to be a more powerful tool to assess the diversity of the same strains, identifying three isolates with two novel sequence types whereas the rest were included in the group shared by the majority of the European isolates studied until now.