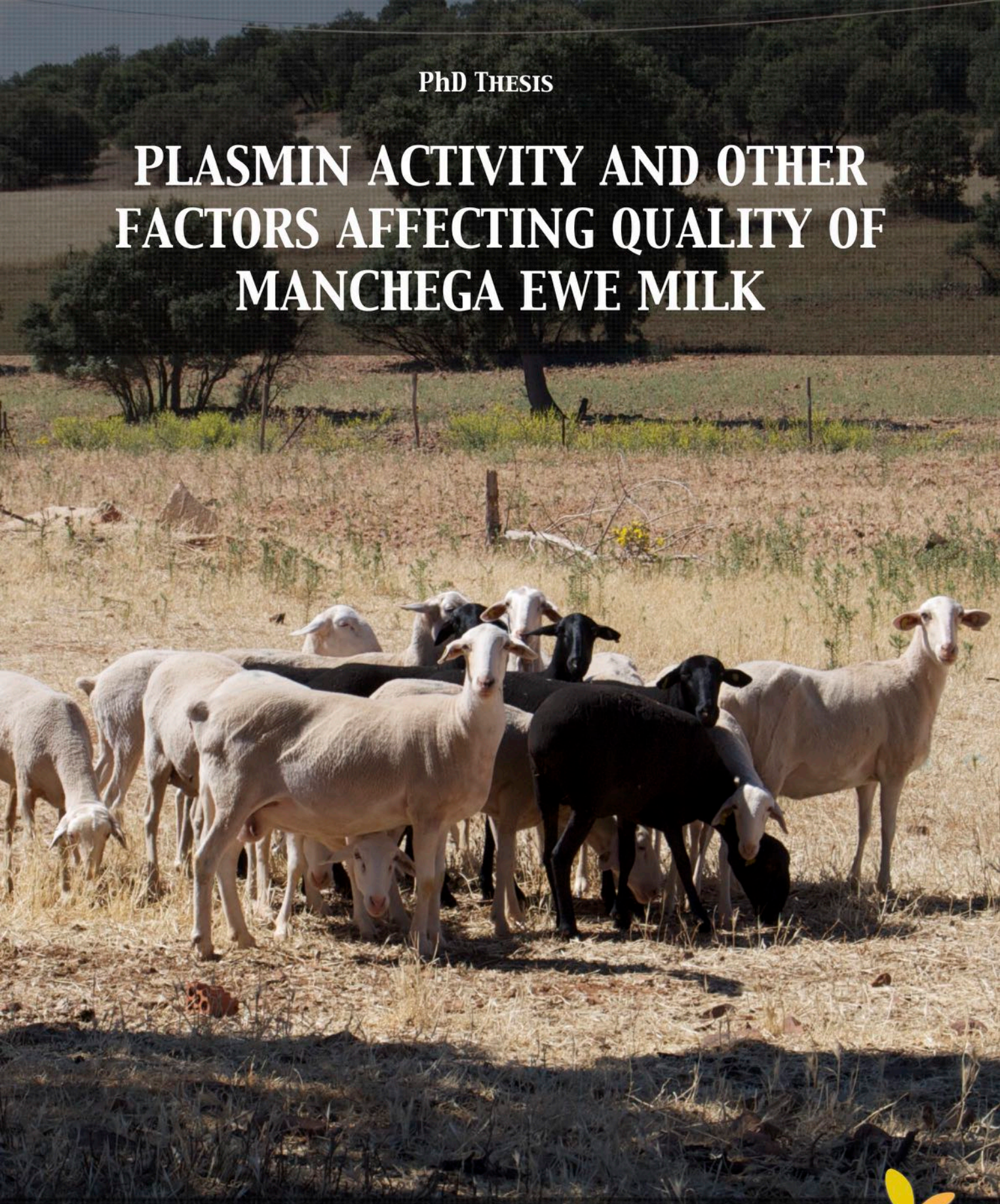
PhD Thesis

PLASMIN ACTIVITY AND OTHER FACTORS AFFECTING QUALITY OF MANCHEGA EWE MILK



JAVIER CABALLERO VILLALOBOS

CEIA3
campus de excelencia internacional agroalimentario

TITULO: *Plasmin activity and other factors affecting quality of Manchega ewe milk*

AUTOR: *Javier Caballero Villalobos*

© Edita: UCOPress. 2016
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

Document layout designed with TEXIS.

PLASMIN ACTIVITY AND OTHER FACTORS AFFECTING QUALITY OF MANCHEGA EWE MILK

Memoria que presenta para optar al título de
Doctor en Biociencias y Ciencias Agroalimentarias

JAVIER CABALLERO VILLALOBOS

Departamento de Producción Animal
Facultad de Veterinaria
Universidad de Córdoba

Octubre 2016

La presente Tesis Doctoral, realizada en el Departamento de Producción Animal de la Universidad de Córdoba bajo nuestra dirección, reúne los requisitos legales necesarios para la obtención del Grado de Doctor.

Es una obra rigurosa, metódica y argumentada en la que se ha realizado un planteamiento hipotético, una profunda revisión bibliográfica, un análisis crítico y unas conclusiones coherentes al trabajo desarrollado. Asimismo, los directores quieren hacer constar que, tras el planteamiento inicial, D. Javier Caballero Villalobos ha ido adquiriendo unos conocimientos y aplicando una metodología científica adecuada cumpliendo con plena dedicación, y muy satisfactoriamente, todas las tareas de investigación que se han desarrollado hasta concluir la presente Tesis Doctoral (revisión bibliográfica, toma y análisis de muestras, estudio estadístico de resultados y obtención de conclusiones).

Durante el desarrollo de la Tesis, el Doctorando ha realizado diversos trabajos y publicaciones derivados de la misma, de gran calidad científica, que se citan a continuación:

- J. Caballero-Villalobos et al.: Factores que afectan a la coagulación de la leche y la calidad del queso en ovino (Póster). En *Creando Redes: III Congreso Científico de Investigadores en Formación en Agroalimentación*. ceiA3, Córdoba (España). Noviembre 2014.
- J. Caballero-Villalobos et al.: Relationship of somatic cell count and composition properties of ewe's milk. *Mljekarstvo* 65 (2), 138-143 (2015).
- J. Caballero-Villalobos: Influencia del pH y del Recuento de Células Somáticas sobre la Coagulación y las Características Tecnológicas en la Leche de Oveja Manchega. En *I Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos*. UCO, Córdoba. Febrero. 2016.
- J. Caballero Villalobos et al.: Plasmin activity in Manchega ewe milk (Póster). *IDF Parallel Dairy Science and Technology Symposia: Dairy Products Concentration and Drying - Cheese Science and Technology*. Dublin (Irlanda). Abril 2016.

Tanto los objetivos, como los resultados obtenidos y las conclusiones, son de aplicación práctica, beneficiosos para el sector y poseen la calidad científica necesaria para que dicha Tesis se tramite a la Comisión de Doctorado.

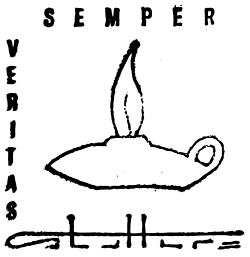
Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, a 10 de Octubre de 2016.

Firma de los directores

Ana Isabel Garzón Sígler **Manuel Sánchez Rodríguez**

To my father and grandad,
who enkindled within me
the flame of knowledge.



Everything great in the world comes from neurotics. They alone have founded our religions and composed our masterpieces. Never will the world be aware of how much it owes to them, nor all they have suffered to bestow their gifts on it.

Marcel Proust

Acknowledgements

And in the end
the love you take
is equal to the love
you make.

The End - Lennon/McCartney

It is not until the very end when you realise the great number of people that, in one way or another (some of them without even being conscious), have been part of this thesis. Ringo Starr once sang about the tasks he would not have been able to accomplish without *a little help from his friends*. So, following his lead, I would like to express my gratitude to all the people who have accompanied me throughout this exciting adventure:

My PhD supervisors **Ana Isabel Garzón** and **Manuel Sánchez**, for introducing me to Animal Husbandry and giving me the chance to start researching. Their guidance and help has been essential during these four years.

Víctor Frías and **Monica Perdices** from *Agrométodos S.A.*, who trusted me and gave me the opportunity to develop this research. Without their continuous support, it would not have been easy to undertake this thesis. To both of them, all my gratitude. May some of the results obtained during this research be useful for future applications inside the company.

Antonio Figueroa, an outstanding co-worker and human being that has been an essential part of every step of this thesis.

Ramón Arias, who scheduled the animal sampling and joined us every time we traveled to Valdepeñas to collect milk.

Professor **Paul McSweeney**, for his invaluable help and advice during my research placement. Thanks to him I had the opportunity to travel and fall in love with beautiful Ireland, a place I will always consider home.

Professors **Patrick Fox** and **Alan Kelly**, for their helpful tips when I was stuck with the plasmin assay.

The outstanding people from UCC, specially the technicians and the folks at Laboratory 227 (**Feli**, **Shoma**, **Lisa** and **Rocco**). Also, I cannot forget to acknowledge **Rodrigo** and **Simone**, whose friendship I will carry forever in my heart.

Dr. **Andrés Luis Martínez**, with whom I spent hours in front of a computer torturing numbers and data. Without him I would not have acquired some statistical skills that will sure be useful in my near future as a researcher.

Professor **Achille Schiavone**, Dr. **Vincenzo Tufarelli** and Dr. **José Humberto Rivas**, for their selfless evaluation of this thesis.

The staff from **AGRAMA** and **CERSYRA**, and **Jesús Romero** from LILCAM, for their collaboration. Also **Ana Mª Rodíguez** and **Cirilo**, from La Nava, who lend a hand during every moment we were working with animals in the farm.

Rocío Jiménez, for being a great colleague and friend. I will always remember those countless cups of tea that helped us relax in between experiments.

The breakfast club (**Inma**, **José Javier**, **Nieves** and **Domingo**), for helping to make work a little bit less stressful.

Paco Delgado, who strongly recommended me to learn LaTeX. **Marco Antonio** and **Pedro Pablo Gómez Martín**, who developed the manual and template (TeXiS) I used to write this thesis. Also, I must acknowledge all the users in the forums, who always had an answer for every doubt and question I came up with while writing code.

Blanca, **Miguel** and **Lala**, who saved my life (or at least my bank account) taking me in their beautiful places and turning *a house* into *a home*.

Ángel Ramiro, for his tips and recommendations on avoiding bureaucratic problems and not dying in my attempt to submit this thesis.

My parents, **José Luis** and **Lourdes**, for instilling in me the values of knowledge, effort and respect. My brother **Pablo**, for being there every time I need him.

Finally, all that is left is to thank **Amalia**, for noticing me among all the other humans in this world.

Abstract

The relationship between plasmin activity and ewe milk composition, rennet coagulation and health of the udder was studied, as well as several inherent and external factors affecting these parameters. Milk from 40 Manchega ewes was collected monthly and analyzed during a complete lactation (5 months). Milk samples were classified by their origin in 3 categories, named PR (primiparous ewes), M1 (multiparous ewes with no previous udder infection) and M2 (multiparous ewes with previous udder infection). Also, according to their SCC (log), milk samples were divided into three groups named LSCCs (<1.6), MSCCs ($1.6 <$SCC <2) and HSCCs (>2). Plasmin activity decreased throughout lactation but was not affected by parity or somatic cell count ($P > 0.05$). A strong negative correlation was found between plasmin activity and protein (specially casein), presumably due to the proteolysis of β-casein. Plasmin also worsened rennet coagulation, increasing rennet clotting time (RCT) and negatively affecting curd firmness (A_{60}), specially in multiparous ewes. However, the good health condition in the herd may have camouflaged some effects of plasmin over renneting. In conclusion, regardless the health condition of the ewes at the beginning of lactation, there is a persisting enzymatic activity probably as a response to a previous udder infection. However, a group of animals with a wider state of health would be needed to investigate further the action of plasmin over milk.

Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Etiam lobortis facilisis sem. Nullam nec mi et neque pharetra sollicitudin. Praesent imperdiet mi nec ante. Donec ullamcorper, felis non sodales commodo, lectus velit ultrices augue, a dignissim nibh lectus placerat pede. Vivamus nunc nunc, molestie ut, ultricies vel, semper in, velit. Ut porttitor. Praesent in sapien. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Duis fringilla tristique neque. Sed interdum libero ut metus. Pellentesque placerat. Nam rutrum augue a leo. Morbi sed elit sit amet ante lobortis sollicitudin. Praesent blandit blandit mauris. Praesent lectus tellus, aliquet aliquam, luctus a, egestas a, turpis. Mauris lacinia lorem sit amet ipsum. Nunc quis urna dictum turpis accumsan semper.

Resumen

Se ha estudiado la relación entre la actividad de la plasmina y la composición de la leche, la coagulación y el estado sanitario de la ubre, así como diversos factores intrínsecos y extrínsecos que pueden afectar a dichos parámetros. Para ello se han analizado muestra individuales de leche de 40 ovejas de raza Manchega durante una lactación completa (5 meses). Las muestras de leche fueron clasificadas según su procedencia en tres categorías: PR (hembras primíparas), M1 (hembras multíparas sin infecciones previas en la ubre) y M2 (hembras multíparas con infección previa de la ubre). Asímismo, en función de su recuento de células somáticas (log) las muestras fueron clasificadas en tres grupos: LSCCs (<1.6), MSCCs (1.6 <SCC <2) HSCCs (>2). La actividad de la plasmina disminuyó con el avance de la lactación, pero no se vio afectada por el número de parto o el recuento de células somáticas (P>0.05). También se observó una fuerte correlación negativa entre la actividad de la plasmina y la proteína (especialmente la caseína), probablemente debido a la proteólisis de β-caseína. Elevados niveles de plasmina también afectaron negativamente a la coagulación, aumentando los tiempos de coagulación y disminuyendo la dureza de la cuajada (A_{60}), especialmente en hembras multíparas. No obstante, las buenas condiciones sanitarias en el rebaño estudiado podrían camuflar ciertos efectos de la plasmina sobre la coagulación. En conclusión, independientemente del estado sanitario de las ovejas al inicio de la lactación, se observa una actividad enzimática persistente, probablemente como respuesta a una infección previa de la ubre. No obstante sería necesaria una población animal con una mayor diversidad sanitaria para investigar más a fondo la acción de la plasmina sobre la leche.

List of acronyms and abbreviations

β-CN Beta-Casein.

α-CN Alpha-Casein.

κ-CN Kappa-Casein.

γ-CN Gamma-Casein.

α-La Alpha-Lactalbumin.

β-Lg Beta-Lactoglobulin.

α_{S1}-CN Alpha$_{S1}$-Casein.

α_{S2}-CN Alpha$_{S2}$-Casein.

A_{30} Curd Firmness At 30 Minutes.

A_{60} Curd Firmness At 60 Minutes.

AGRAMA Select Manchega Sheep National Breeders Association.

ceiA3 Campus De Excelencia Internacional Agroalimentario.

CERSYRA Regional Institute Of Animal Selection and Reproduction Of Castilla-La Mancha.

CMP Caseino-Macropeptides.

CMT California Mastitis Test.

ENAC National Accreditation Entity.

ESROM Manchega Ovine Breed Selection Scheme.

FEAGAS Spanish Federation Of Select Livestock Associations.

HSCCs High Somatic Cell Count Scores.

k_{20} Curd Firming Time.

LF Lactoferrin.

LILCAM Interprofessional Dairy Laboratory Of Castilla-La Mancha.

LP Lactoperoxidase.

LSCCs Low Somatic Cell Count Scores.

M1 Multiparous ewes with no previous udder infection.

M2 Multiparous ewes with a previous udder infection.

MSCCs Mid Somatic Cell Count Scores.

NPN Non-Protein Nitrogen.

PA Plasminogen Activators.

PAI Plasminogen Activator Inhibitors.

PAR Parity.

PDO Protected Designation of Origin.

PG Plasminogen.

PGI Protected Geographical Indication.

PI Plasmin Inhibitors.

PL Plasmin.

PMN Polymorphonuclear Neutrophils.

PP Proteose-Peptones.

PR Primiparous ewes.

RCT Rennet Clotting Time.

SCC Somatic Cell Count.

SCCs Somatic Cell Count Score.

SL Stage of Lactation.

UCC University College Cork, Ireland.

UCO University of Córdoba, Spain.

UHS Udder Health Status.

WMT Wisconsin Mastitis Test.

List of contents

List of figures

List of tables

Part I

INTRODUCTION

Introduction

In Spain, as in many Mediterranean countries, practically the whole production of sheep milk is intended for cheesemaking, wether in its pure form or mixed with goat or bovine milk (Balcones et al., 1996). Cheese production represents a major component of the Spanish ovine industry, and in the last few years has been strengthened by the emergence of the Protected Designations of Origin (PDO) and the Protected Geographical Indications (PGI), which assure control of production and processing factors, and guarantee the consumers a high quality of the products.

Manchega is the most common dairy sheep breed in Spain, with more than 650,000 ewes in production (De La Fuente et al., 2006). The importance of Manchega milk lies in its good cheesemaking aptitude, giving as a result high quality cheeses, with sensorial properties that are greatly appreciated by consumers. This good technological aptitude is evidenced in a short rennet clotting time, firm curds and high yields (Jaramillo et al., 2008). Manchego cheese is a cured hard cheese elaborated exclusively in Castilla-La Mancha. It is the best selling cheese variety in Spain (Ballesteros et al., 2006) and, since 1996, is protected by a PDO that guarantees its quality (Règlement, 1996).

Due to this important cheesemaking activity, selection criteria in this breed have always been focused on the obtention of high yields during milk coagulation. These renneting parameters can be influenced by several factors, which can be genetic, physiological, sanitary or physico-chemical (Bencini, 2002). Many studies (most of them performed on bovine milk) have explored the influence on milk of factors such as temperature (Park, 2007), calcium levels (Nájera et al., 2003), hygienic and sanitary conditions (Albenzio et al., 2004; Le Maréchal et al., 2011) or proteolytic activity (Baldi et al., 1996).

Somatic cell count is used as an indicator to evaluate milk quality and define its price (Kalantzopoulos et al., 2004; Raynal-Ljutovac et al., 2005). Mastitis is the main cause of high somatic cell levels in milk, although other factors

such as oestrum or advanced stages of lactation can act as triggers for high somatic cell counts (Albenzio et al., 2005). Several studies reported that an increment of somatic cells in milk increases the activity of endogenous enzymes, which can induce casein proteolysis, affecting milk renneting properties and reducing cheese yield. Proteolytic enzymes like plasmin are known to modify milk composition, reducing its quality and producing bitter flavours (Fox and Kelly, 2006). Thus, controlling the activity of endogenous enzymes at both farm and industrial levels could lead to improvement of the quality in dairy products, perhaps also reducing production costs (Caballero-Villalobos et al., 2016).

As quality criteria have not been completely defined, milk properties of many Spanish dairy sheep breeds have not yet been studied. Because of this, and the strong economic and cultural impact of Manchega milk on the Spanish dairy industry, the cheesemaking aptitude of this breed needs to be further studied. Thus, the main objective of this PhD thesis is to evaluate some of the main factors that impact on the cheesemaking aptitude of Manchega ewe milk.

Part II

LITERATURE REVIEW

Chapter 1

Manchega breed

CONTENTS

1.1. General aspects and farming system

Manchega breed is named after the region of La Mancha, located in the southern sub-plateau of the Iberian Peninsula. In the Official Catalogue of Spanish Livestock Breeds it is classified as a local enhancement breed. It represents one of the most important national breeds, due both to its census and its production. Also, great importance lies in its adaptation to a dry and difficult environment, and in its influence on the improvement of other Spanish local ovine breeds, such as Castellana, Aragonesa, Alcarreña or Segureña.

The farming system is semi-extensive, based on continuous pasturing during the whole year to maximize the use of natural resources. However, while in the sheepfold, feed is supplemented with concentrate, hay and agricultural by-products. As herds are more specialized towards milk production, the stabling periods tend to increase. Ewes have a continuous ovarian cycle, so lambing goes on throughout all year. First gestation takes place when ewes are 10-13 months old, and fertility rates reach 100 %.

1.2. Origin and historical background

Manchega ewe derives from *Ovis aries ligeriensis*. This ancestor, originally from France, crossed the Pyrenees and travelled through the Iberian Peninsula to finally settle in the natural region of La Mancha. Since this moment, Manchega became a sedentary breed, faithful to the land that would forever be its home.

It is a proven fact that the early settlers of La Mancha domesticated Manchega sheep and worked to improve the breed, avoiding crossbreeding. This has helped to maintain its purity and original attributes, as well as its unique characteristics, which have hardly changed throughout time.

1.3. Morphological characteristics

There are two different varieties of Manchega: the white variety and the black variety (figure 1.1). Both have identical morphologic, functional and genetic characteristics, and only differ in the color of skin and wool (Sánchez-Belda, 1979). The black variety has currently been granted special protection as an endangered indigenous breed. The rules of procedure of the Herd Book of the Spanish Manchega Breed (AGRAMA, 1977) qualify the animals according to their breed standard, describing the following morphological prototype:

Figure 1.1: White and black varieties of Manchega sheep.

1. **General appareance**: convex shape, more pronounced in males than in females. Proportions tending to the predominance of longitudinal diameters. Strong sexual dimorphism.
2. **Head**: with a convex fronto-nasal line, middle sized and in harmony with the body volume, and completely uncovered by wool. Big and slightly lop ears. Lips, nose and mucous membranes must be visible and depigmented in the white variety, though slight pigmentation is admitted in the black variety.
3. **Neck**: cylindrical and rightly connected to the head and the trunk of the body. Without any vertical skin folds or jowl. It may or may not present wattles.
4. **Trunk of the body**: long, deep and provided with wide ribs. Horizontal dorso-lumbar area. Rump wide and squared, horizontal or slightly angled. Deep thorax. Chest wide and rounded. Well-proportioned belly.
5. **Udders**: similar in volume and development, with skin uncovered by wool. Teats well-proportioned and positioned.
6. **Testicles**: symmetrical in size and position, without any wool.
7. **Limbs**: well planted and with length consistent with body development. Back correctly connected to the trunk of the body. Buttocks and thighs wide and muscular. Thin carpus and tarsus. Hooves strong and symmetrical, with a proportionate size.
8. **Skin, mucous membranes and integuments**: the skin is thin and with no folds in any region of the body. All areas with no wool are covered by thin and bright hair. In the white variety, skin, mucous membranes and integuments must be depigmented; however mild pigmentations may be acceptable if their tone and extension is discreet. Pigmentation affecting skin and hair is acceptable just in ewes, only if it is diffuse or distributed as small moles. In the black variety, the presence of black patches in the forehead and the the terminal area of the tail is acceptable.

9. **Fleece**: it must be be evenly white in the white variety and black in the black variety. Semi-closed or closed. It must cover the trunk of the body, and may reach the nape of the neck, but must leave uncovered the anterior third of the tracheal border. In the forelimbs it may reach the upper third, and in the hind limbs two thirds of the leg. The belly may or may not be covered by wool.
10. **Wool strands**: rectangular or slightly trapezoidal shape, with hair or medullar fibers inside the fleece.

1.4. Geographic distribution and census

Manchega breed is present all over the country, and can be found in 42 of the 50 Spanish provinces (particularly in the autonomous regions of Madrid, Comunidad Valenciana and Castilla y León). However, the highest concentration involves Castilla-La Mancha (figure 1.2). This autonomous region hosts 762 farms, with a total census of 529,505 dairy sheep in 2015 (table 1.1), and a milk production that has gradually increased over the last years to reach 73 million litres of milk.

Figure 1.2: Dispersion of the Manchega breed in Spain.[1]

[1]Source: Spanish Federation Of Select Livestock Associations (FEAGAS).

Table 1.1: Manchega sheep in the region of Castilla-La Mancha between 2011 and 2015.

	2011	2012	2013	2014	2015
No. Sheep	569,084	554,205	520,225	526,952	529,505
No. Farms	917	877	798	776	762
Production (millions of litres)	53	58	60	65	73
Estimated average (litres/ewe)	93	105	115	124	138
No. Sheep ESROM	140,660 (25%)	137,638 (25%)	130,007 (25%)	127,293 (24%)	133,518 (25%)

ESROM = Selection Scheme For Manchega Ovine Breed.

The region of La Mancha is located in the southern sub-plateau of the Iberian Peninsula and is characterized by a flat relief that descends towards the Atlantic. It constitutes a high plain settled on chalky and clay soils, and the grounds designated for pastures consist of substrates rich in limestone and marl. The region presents an extreme continental climate with great oscillations, alternating between very cold winters and warm summers where temperatures can reach values close to 40ºC. The daily temperature range in this region is 30ºC, and the annual range can be of 50ºC. Rainfall is low, and the environment is extremely dry, with a relative humidity of 65 %. In fact, La Mancha was named by the Arabs, who called it *Al Mansha* or "waterless land".

A vegetation capable to withstand the strong heat of the summer months and the devastating winter frosts developed over time. Numerous species of grass and leguminous plants grow in this apparently hostile environment, and represent the nutrition basis of Manchega sheep, adapted to this ecosystem since time immemorial.

1.5. Manchego cheese

Manchego cheese is probably the most important and best known product of the agri-food sector of Castilla-La Mancha. Since 1996 it is covered by the Protected Designation of Origin (PDO) *Queso Manchego*, through the Regulation 1107/96 of the European Commission. It is also the first PDO certified by the the National Accreditation Entity (ENAC).

Figure 1.3: Mark of the PDO *Queso Manchego*.

The industrial structure of this PDO has a vital importance, with more than 80 dairies that produced over 12 million kg of quality mark cheese in Spain in 2014. This was economically valued in 132 million euros, which represents over half of the production of quality mark cheese in Spain. Additionally, it is important to highlight the large export volume of Manchego cheese (more than 50 % of the whole production), being the USA the main importer.

Milk (raw or pasteurized) used to elaborate this cheese must come exclusively from Manchega ewes bred in the region of La Mancha: a surface of 4,419,763 hectares that includes municipalities of the provinces of Albacete (21.66 %), Ciudad Real (33.16 %), Cuenca (22.13 %) and Toledo (23.05 %). Thus, the cheese manufacturing and ripening area is the same as the area of production.

Figure 1.4: Production area of the PDO *Queso Manchego*.

The history of Manchego cheese is as old as the breed after which it was named, and its production and quality have been kept unaltered over the years. The first known evidences regarding its consume and manufacture date back to several centuries before Christ. The processing methods are not yet clear, but those archaic methodologies would not differ much from the ones followed nowadays.

Some archeological remains also suggest the existence of a cheese made from milk from a local sheep breed during the Bronze Age, which could be considered as a predecessor of the current Manchega. The soil and climate of the region of La Mancha (section 1.4), have influenced natural selection, so Manchega sheep is greatly adapted and produces milk that confers cheese some peculiar characteristics of color, smell, flavour and texture. These features provide an unique product that has been unsuccessfully attempted to elaborate in other parts of the world, as it has been impossible to imitate all the factors involved outside the region of La Mancha.

The PDO *Queso Manchego* describes this product as:

> *"A cheese made of pressed paste, elaborated from Manchega sheep milk. With a minimum ripening of 30 days for cheeses with a weight below 1.5 kg, and of 60 days for the rest, and a maximum ripening time of 2 years.*

Its appearance is cylindrical, with perceptibly flat sides, a maximum height of 12 cm, a maximum diameter of 22 cm and a weight from 0.4 to 4 kg. The rind is hard and free from parasites, an presents a yellowish color, or dark greenish when the molds developed during ripening are not cleared from the surface. Paste is firm and compact, with a color that varies from white to yellowish ivory, and can present small eyes unequally distributed.

It has a strong lactic smell, and an intense and persistant acidity that evolves towards spicy notes in cured cheeses. Flavour is acid, strong and tasty, and also turns into spicy in cured cheeses. It has a mild an peculiar residual flavour, distinctive of Manchega sheep milk, and presents low elasticity, with a buttery and slightly floury perception, that might be granular in matured cheeses."

The Product Specification of the PDO (BOE, 13/11/2007) describes the physico-chemical characteristics of the milk used in the elaboration of Manchego cheese (table 1.2), and also gathers the basic requirements that endorse the origin of the product.

1. Milk must be obtained exclusively from Manchega ewes of registered farms, located in the production area.

2. Extraction, cooling, storage, collection and transportation of milk must be thoroughly monitored.

3. Cheese will be manufactured in registered dairies, located in the processing area. Dairies must provide systems to guarantee independent processing of the cheese (from the reception of raw materials to the shipping of the product).

4. Ripening and storage will be accomplished in registered premises, to guarantee identification and separation of qualified cheeses from other products.

5. The final product will be submitted to physico-chemical and organoleptic analyses in order to certify its quality.

6. Once these tests are concluded, the product will be released to the market, and its origin will be warranted by a numbered label.

7. In order to obtained the license for the PDO conformity mark and to be certified by the Regulating Board of the Protected Designation of Origin *Queso Manchego*, farmers and manufacturers must apply control systems to guarantee the conformity with these technical specifications.

Table 1.2: Analytical characteristics of the milk used to manufacture PDO Manchego Cheese.

PARAMETER	PERCENTAGE	DETERMINATION METHOD
Fat	6.5 % minimum	Gerber method (B.O.E. 20-07-77)
Protein	4.5 % minimim	Kjeldahl method (B.O.E. 20-07-77)
Usable dry matter	11 % minimum	Desiccation in stove (B.O.E. 20-07-77)
pH	6.5-7	Direct measurement - pH meter
Freezing departure point	≤-0.550ºC)	Cryoscope
Medicinal products	Absence	Inhibition of growth of *Bacillus sterotermophilus*

Source: Product Specification of the PDO *Queso Manchego* (BOE, 13/11/2007).

In 1974, several farmers joined forces and created the *Select Manchega Sheep National Breeders Association (AGRAMA)*, to improve productivity and quality of Manchega breed industry. This institution, with social headquarters in Albacete (Spain), has been officially recognized by the Ministry of Agriculture, Fisheries and Nutrition. It currently strives to optimize the Herd Book, and broadcast the improvement program based on the *Manchega Ovine Breed Selection Scheme (ESROM)*, developed in conjunction with the Office of Agriculture and Environment of Castilla-La Mancha.

Figure 1.5: Logo of AGRAMA.

At present, almost half a century since its foundation, AGRAMA has not only managed to define and maintain the profile of Manchega breed, but has also actively participated in the establishment and consolidation of the PDO *Queso Manchego* and the Protected Geographical Indication (PGI) *Cordero Manchego*.

Chapter 2

Sheep milk and its composition

CONTENTS

2.1. Definition and general characteristics

The concept of **milk** is defined by FAO (1999) in *Codex Alimentarius* as

> *"the normal mammary secretion of milking animals obtained from one or more milkings without either addition to it or extraction from it, intended for consumption as liquid milk or for further processing".*

Likewise, *Codex* defines **milk product** as

> *"a product obtained by any processing of milk, which may contain food additives, and other ingredients functionally necessary for the processing".*

Veisseyre (1988) describes milk as a white and opaque fluid, two times more dense than water, with a slightly sweetened flavour and a rather scarce smell. It represents a complex physico-chemical system, and can be summarily considered as an emulsion of fat in an aqueous solution containing several components, some of them dissolved and others in colloidal dispersion.

Although milk is conventionally defined in a general manner, there are specific variations between milk from different species, which endow it with distinctive features. Assenat (1991) overviewed the main differences in ewe milk compared to the rest of domestic ruminants:

- Its color is pearl white, similar to porcelain.
- Opacity is higher than in milk from other species.
- Viscosity is also higher, due to its large amount of fat.
- It has a particular smell, rather faint in milk collected in good conditions.
- Organoleptic properties of sheep milk make it different from other drinking milks. Thus, compared to the sweetened flavour that it has in common with other milks, it presents a characteristic smell and a higher creaminess due to high fat concentration.
- It is specially resistant to bacterial growth in the first hours after its collection, as it shows immune activity. In addition, sheep milk has twice mineral content than cow milk, clearly presenting greater buffer activity, whitch represents an advantage concerning storage and preservation.
- It is specially rich in cheesemaking components (fat and protein). Normally, making use of the same amount of milk, twice the quantity of cheese is obtained with sheep than with cow milk.

- Milk coagulation results in strong curds, much firmer than what the cheese yield ratio between ewe and cow milk (1/2) would imply.
- Cheese made from sheep milk has a particular appearance and flavour: the paste is generally whiter and the development of bitter flavours is unusual. These features are attributed to the lower proportion of α-Casein respecting total casein, and to the differences in fatty acids composition of triglycerides in sheep milk.

2.2. Factors affecting milk production and composition

2.2.1. Inherent factors

They are defined as those factors that are intrinsic to the animal and cannot be easily modified.

2.2.1.1. Genotype and reproductive potential. Breed effect

It is a known fact that there are existing differences concerning milk yield and composition among different dairy breeds of sheep. Although most of these differences seem to be environmental, there is an important genetic component. According to Molina (1987), a breed's "genetic potential" for dairy production is known as the amount of milk it is capable to produce when its genotype is expressed in optimal environmental conditions.

2.2.1.2. Stage of lactation

Milk production in sheep reaches the maximum yield in the first weeks after lambing, and gradually decreases from this point to the dry period (figure 2.2). As lactation advances, the main components in milk (except lactose) follow a trend similar to the one described for production, but in reverse direction, and reach their minimum values when milk yield is maximum (Gallego et al., 1991).

2.2.1.3. Age and parity

Young animals generally produce less milk than old ones, and reach their highest productions between the third and fourth lactation (Busetti, 2007). According to Purroy (1982), ewes considerably increase their milk production from the first to the second lactation, and slightly less between the second and the third. From this point, milk production is constant until the sixth or

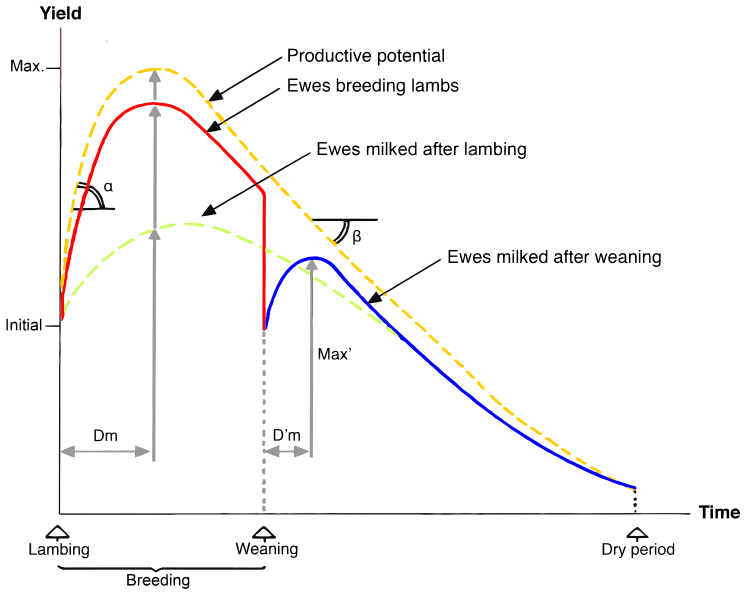


Figure 2.1: Lactation curve in sheep.[1,2]

eighth lactation, where it begins to fall. Gallego et al. (1991) reported that in Manchega ewes, milk production increases about 38 % between the first and the fifth lactation, and then diminishes.

2.2.1.4. Number of lambs

Twin births generally induce a higher milk production. Some authors reported a yield enhancement of 48 % in multiple lambing ewes compared to those that delivered a single lamb (Real Ortellado, 1999). Nevertheless, this increase in milk production seems to be more related to the number of nursed lambs rather than to the born ones. Therefore, ewes nursing two or more lambs produce a higher amount of milk (Molina, 1987).

2.2.1.5. Anatomy and morphology of the udder

The size of the udder and the cistern depth, as well as the teat length and angle, are the main anatomic factors that have an impact on milking. It is generally accepted that the higher the volume of the udder, the greater milk production (Gallego, 1991).

[1] Source: Gallego et al. (1994).
[2] Dm = Days before maximum milk yield.

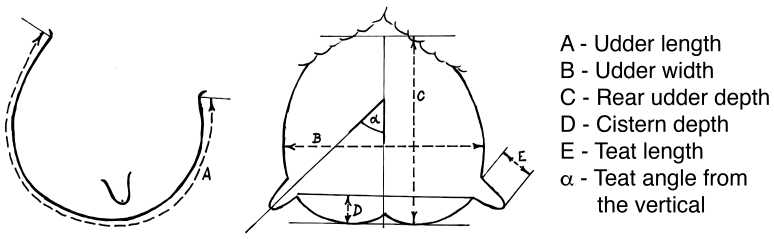


Figure 2.2: Udder measurements.[3]

2.2.2. External factors

They are defined as those factors that can be modified through management practices or by human action.

2.2.2.1. Ewe-lamb relationship and weaning

Sheep are regarded as "primitive" dairy animals. This means that milk is hard to express during milking and its secretion is strongly conditioned by the presence of the lamb (Molina, 1987). Weaning induces a decrease in milk production due to stress caused by the separation of the ewe from the lamb, and an existing adaptation to milking. This yield reduction in Manchega has been stipulated in 30-40 %, percentage that subsequently increases once the ewe adjusts to milking (Caja, 1991).

2.2.2.2. Milking method and milking interval

Milking has a great influence on milk yield and composition, as its expression is necessary for the maintenance of lactation (Garzón, 1996). In ewes, the synthesis of milk in the udder diminishes when the milking interval is longer than 16 hours, and it is inhibited after 24 hours (Molina, 1987).

It has often been reported that mechanical milking produces an incomplete emptying of the udder in comparison with manual milking, diminishing the quantity and quality of milk (Garzón, 1996). However, Gallego et al. (1991) reported that only fat concentration is affected. The most evident difference between mechanical and manual milking is the microbiological content of milk, as operators increase the amount of germs in almost 50 % (Gallego et al., 1991).

[3] Source: Milerski et al. (2006).

2.2.2.3. Nutrition and feeding

According to Molina (1987), nutrition is the most important of all the external factors affecting lactation, as it covers conservation and production requirements and restores body reserves. The impact of nutrition on milk production is revealed in the last third of pregnancy and, obviously, during lactation. In this sense, nutrition and milk production are closely related, as its necessary to provide animals with a complete and balanced diet in order to develop their productive potential. Nutrition can also affect to a lesser extent milk composition, almost exclusively fat concentration.

2.3. pH

The pH of sheep milk has a similar value to that found in milk from other species, and can provide precise information about milk freshness. In standard conditions, the pH of raw milk is neutral or slightly acid, but if lactic bacteria have taken action, lactose deteriorates and converts into lactic acid, causing a decrease in pH (pH<6.5). Contrastingly, values of pH above 7.0 suggest that milk exhibits alkaline compounds, a typical sign of mastitis (Gorsaud, 1991).

2.4. Carbohydrates

Lactose is the major carbohydrate in milk, and one of the main components of total solids (Park, 2007). It is a non-permeable disaccharide synthesized from glucose and galactose in the mammary gland, with the active participation of α-lactalbumin (Larson and Smith, 1974). Lactose is found in several concentrations in milk from all mammals except for seals (Park, 2007). Its content in sheep milk stands around 4.44 %, and may vary between 3.70 and 5.01 % (Molina, 1987).

According to Ramos and Juarez (2003), the lactose rate in ewe milk is slightly lower (22-27 % of total solids) than in bovine milk (33-40 %). However, this fact does not represent a disadvantage in cheesemaking, as lactose has a relative importance from a nutritional and technological point of view, and its concentration in ewe milk is more than enough to assure lactic fermentation Assenat (1991).

The importance of lactose relies in its contribution to preserve the osmotic balance between blood plasma and alveolar cells (Larson and Smith, 1974).

In this sense, the synthesis of milk is strongly influenced by lactose, which maintains osmolality and regulates the amount of water and ions, as well as the amount of milk released (Pulina and Nudda, 2004).

Other minor carbohydrates, such as glucose and galactose, are also present in milk, as well as several oligosaccharides and sugar derivatives. In addition, other carbohydrates bind to proteins in order to form milk glycoproteins (Schlimme and Buchheim, 2002).

2.5. Milk fat

The amount of fat in milk is frequently known as butyric rate, and comprises the ensemble of all lipids that, through hydrolysis of esters, generate fatty acids (Garzón, 1996). It should be noted that the color of fat in sheep milk is white, due to the absence of carotene (Assenat, 1991).

The proportion of fat in milk from domestic ruminants can present significant variations, finding the highest average values in sheep milk (around 7.19 %), versus the lower values found in cow (3.87 %) and goat (3.38 %) milk (Gorsaud, 1991). However, as in other mammals, average composition of sheep milk is a purely theoretical concept, since it changes throughout lactation and is also affected by several factors such as breed, nutrition, management of the animals, etc. (Garzón, 1996).

Fat content in milk is structured as an emulsion of fat globules (1-8μ diameter) in the aqueous plasma, surrounded by a lipid and protein membrane negatively charged. This membrane confers stability and prevents outflow, assuring electrostatic repulsion between fat globules (Serrano, 1999). Some authors have reported that the fat globule in sheep milk seems to be smaller than in other species (Le Mens, 1991). This appears to improve digestibility and the efficiency of lipid metabolism in comparission with fat from bovine milk (Park, 1994).

The global composition of milk fat in domestic ruminats (sheep, goat and cow) is quite similar, featuring three kind of compounds:

- **Triglycerides** (98.0 %): normally sheep milk fat presents a lower proportion of long chain triglycerides and a higher proportion of short chain triglycerides than bovine milk fat. It also presents a high content of saturated fatty acids of 6-12 carbon atoms (capric and caprylic acid),

and a low rate of myristic (C14:0) and palmitic (C16:0) acid (Routaboul, 1981).

- **Phospholipids** (0.5 %): 30.8 % lecithins, 45.0 % cephalin and 24.2 % sphingomyelins (Baliga and Basu, 1955).
- **Other fat-soluble compounds** (1.0 %): Wohlt et al. (1981) reported an average cholesterol value of 60mg/100g in sheep milk.

This fat composition confers milk specific characteristics regarding smell and flavour. In addition, the distinctive smell in dairy products is due to spontaneous lipolysis as a consequence of the high lipase concentration in milk (Molina, 1987; Serrano, 1999).

2.6. Nitrogenous substances (proteins and urea)

Two types of nitrogenous substances are found in milk: proteins and compounds consisting of non-protein nitrogen (NPN), which represent 95 % and 5 % of total nitrogen, respectively.

2.6.1. Whey proteins

This soluble fraction of milk proteins involves about 17-22 % of the total protein in ewe milk. The main proteins in whey are alpha-lactalbumin (α-La) and beta-lactoglobulin (β-Lg), although other proteins are present in whey in lower concentrations (immunoglobulins, serum albumin, proteose-peptones, etc.) (Park et al., 2007). Sheep whey is, therefore, rich in protein (almost as twice as bovine whey). Consequently, when submitted to thermocoagulation processes, whey is frequently directed to the production of derived products, such as cottage cheese, *bruccio* and *riccota* (Molina, 1987).

2.6.1.1. α-Lactalbumin

α-Lactalbumin (α-La) is a calcium metalloprotein with a single strong calcium binding site, and constitutes the second most abundant protein in whey, representing approximately 20-25 % of all whey protein (Jenness, 1982; Permyakov and Berliner, 2000). α-La has a high content of tryptophan (*Trp*), an essential amino acid with potential benefits for the production of serotonin, regulation of sleep, and mood improvement under stress. It also induces the synthesis of lactose in the udder, so its role is essential for the secretion of milk (Moatsou, 2010).

Two variants of α-La have been reported in sheep milk (Chiofalo and Micari, 1987; Erhardt, 1989). The A variant is usually the most common, and the B variant is rare, and seems to appear only in specific breeds (Selvaggi et al., 2014).

Table 2.1: Milk protein genetic polymorphisms in sheep.[4]

PROTEIN	VARIANTS	REFERENCES
α_{S1}-Casein	A, B, C, D, E, F, H, I	Chianese et al. (1996) Pirisi et al. (1999) Wessels et al. (2004) Giambra et al. (2010)
α_{S2}-Casein	A, B, C, D, E, F, G	Chianese et al. (1993) Chessa et al. (2003) Picariello et al. (2009) Giambra and Erhardt (2012)
β-Casein	A, B, C, X, Y	Chianese (1997) Chessa et al. (2010)
κ-Casein	Monomorphic	Jolles et al. (1974) Bastos et al. (2001) Ceriotti et al. (2004) Feligini et al. (2005) Pariset et al. (2006)
α-Lactalbumin	A, B	Chiofalo and Micari (1987) Erhardt (1989)
β-Lactoglobulin	A, B, C	Bell and McKenzie (1967) King (1969) Kolde and Braunitzer (1983) Erhardt (1989) Ali et al. (1990) Recio et al. (1997) Ramos et al. (2009)

2.6.1.2. β-Lactoglobulin

β-Lactoglobulin (β-Lg) is the main whey protein in ruminant milk, representing almost 50 % of the total whey protein. It is present in the majority of species, except for mouses and humans. So far, three genetic variants of this protein have been described in literature (table 2.1).

[4]Source: Selvaggi et al. (2014).

Bell and McKenzie (1967) and King (1969) proved an existing polymorphism of this protein in several breeds of sheep. Kolde and Braunitzer (1983), Gaye et al. (1986) and Ali et al. (1990) described two variants (A and B) that only differ in an amino acid in position 20 (variant A presents *Tyr* and variant B presents *His*). Both variants are present in almost all sheep breeds (Amigo et al., 2000). Further on, Erhardt (1989) described a new variant of β-Lg in German Merino breeds. This variant, named C, is an alteration of variant A (*Arg* is replaced by *Glu* in amino acid position 148). It has more recently been isolated in Spanish Merino by Recio et al. (1995). Thus, variant C has only been identified in varieties of Merino (Barillet et al., 2004).

β-Lg is the most hydrophobic of all whey proteins, and has the ability to interact with several hydrophobic molecules, particularly retinol and fatty acids. This feature, probably related to its biological function, confers β-Lg good emulsifying properties.

2.6.1.3. Other whey proteins

Serum Albumin and **Immunoglobulins** are not specific to milk, and are considered equivalent to those found in blood (Park et al., 2007).

Proteose-Peptones (PPs) represent 10 % of whey protein and consist of 38 compounds (Buccioni et al., 2013). Most of them result from the cleavage of beta-casein (β-CN) by plasmin. PPs are heat-stable and soluble in acids, but their specific biological functions have not been yet described (Ohno et al., 2010). The main components are known as *component 3 (PP3)*, *component 5 (PP5)*, *component 8 fast (PP8f)*, and *component 8 slow (PP8s)*, based on their electrophoretic mobility (Andrews and Alichanidis, 1983.). *PP3* is of great interest due to its functionality, particularly because of its emulsifying properties and its biochemical role as a regulator of spontaneous lipolysis (Girardet and Linden, 1996). This fraction also seems to be responsible of foaming in milk (Zhang and Goff, 2004).

Lactoferrin (LF), an 80 kDa glycoprotein belonging to the group of the transferrins (iron-binding proteins), is another soluble protein present in whey. LF comprises around 1-2 % of whey protein and is mainly found in colostrum. It represents an important component of the inborn immunity system, presenting anti-inflammatory and antioxidant activities, and also inhibiting bacterial, viral and fungal growth (Rodríguez-Franco et al., 2005). Although most of the published studies have been performed on human and bovine milk, some authors have reported a higher antimicrobial activity of LF in small ruminant

milk (Recio and Visser, 2000).

Lactoperoxidase (LP) is a glycoprotein naturally present in milk, representing 0.5 % of whey protein. It is identical to that found in saliva and gastric juice (FAO-OMS, 2005). By itself it does not have an antibacterial effect, but in the presence of hydrogen peroxide and oxidated thiocyanate (commonly present in milk), it develops a strong bacteriostatic effect (FAO, 2000).

Lysozyme is an enzyme naturally present in milk, and represents less than 0.1 % of whey protein, conferring properties that increase immunity. This protein inactivates numerous microorganisms, attaching to the bacterial wall and breaking the β-14 bond between N-acetylmuramic acid and N-acetylglucosamine. Gram-negative bacteria are generally more vulnerable to its action, so lysozyme is used to prevent butyric swelling of cheese, as it is active against clostridia (Martínez et al., 2010).

Caseino-Macropeptides (CMP) are soluble fragments derived from the action of chymosin over κ-casein during cheesemaking, and can also be present in whey (Park et al., 2007).

2.6.2. Caseins

Caseins are phosphoproteins present in milk and synthesized in the mammary gland as a response to the lactogenic hormones and other stimuli (Ginger and Grigor, 1999). They are associated to calcium phosphate in a complex known as caseinogen, which precipitates at pH 4.6. Caseins interact with each other and, together with calcium and magnesium fractions in milk, form a coloidal dispersion, consisting in spheral particles called micelles. The average diameter of the casein micelle is 130 nm, but it may vary between 60-450 nm.

Although at first they were considered homogeneous, Linderstrøm-Lang and Kodama (1925) proved that there were two well differentiated groups of caseins: a first group constituted by those that precipitated in the presence of calcium (calcium sensible caseins) and a second group that incorporated those that were not sensible to this element. However, with the further development of molecular biology in the decades of 1970 and 1980, different kinds of caseins were discovered. These proteins, classified originally according to their electrophoretic mobility, differ due to effects of post-translational processing, alternative splicing of the gene product or genetic polymorphisms (Ng-Kwai Hang and Grosclaude, 1992). Therefore, from a structural point of view, there are 3 types of caseins, each one consisting of about 200 amino acids

Table 2.2: Characteristics of whey proteins.

PROTEIN	PROPORTION IN WHEY	PROPORTION IN SKIM MILK	MOLECULAR WEIGHT ($\times 10^3$)	COFACTORS
α-Lactalbumin	20-25 %	0.7 g/l	14	
β-Lactoglobulin	50 %	3.3 g/l	18	
Serum albumin	5 %	0.3 g/l	66	
Proteose-peptones	10 %	1.0 g/l	4-40	
Immunoglobulin		700 mg/l		
IgG_1 IgG_2 IgA IgM			153-163 146-154 385-417 960-1000	
Xanthine oxidase			275-300	FAD
Lactoferrin	1-2 %	100 mg/l	76.5	HCO_3^-
Lactoperoxidase	<0.5 %	30 mg/l	78	SCN^- / H_2O_2
Lysozyme	<0.1 %	0.13 mg/l	14.3-14.6	SCN^- / HCO_3^-

Source: own compilation, based in Martínez et al. (2010).

(table 2.3). These caseins are known as alpha-casein (α-CN), β-casein (β-CN) and kappa-casein (κ-CN). The commonly known as gamma-caseins (γ-CNs) are, in fact, fragments derived from the proteolytic action of plasmin over β-CN.

AMINO ACID	%	AMINO ACID	%
Alanine	5.6%	Leucine*	10.3%
Arginine*	2.8%	Lysine*	7.0%
Asparagine	3.7%	Methionine*	2.8%
Aspartic	3.3%	Phenylalanine	3.7%
Cysteine	0.5%	Proline	7.9%
Glutamine	6.5%	Serine	7.5%
Glutamic	11.7%	Threonine*	2.8%
Glycine	4.2%	Tryptophan*	0.9%
Histidine*	2.3%	Tyrosine	4.7%
Isoleucine*	5.6%	Valine*	6.1%

(*Essential amino acids)

Table 2.3: Average amino acid proportion in caseins.

All caseins have different genetic variants, induced by amino acid substitution or, in some cases, by deletion (table 2.1). Since 1984, by decision of the *American Dairy Science Association Committee on Nomenclature and Classification*, it is accepted that the nomenclature developed for the bovine caseins may be adopted for investigation of milk proteins in other species (Selvaggi et al., 2014).

2.6.2.1. α-Casein

Alpha$_{S1}$-casein (α_{S1}-CN) is a structural component of the casein micelle and plays a functional role in cheese curd formation (Selvaggi et al., 2014). It is a deeply phosphorylated protein, and is highly soluble in the presence of calcium (Farrell et al., 2004). α_{S1}-CN consists of 214 amino acids, and exists as a number of distinct genetic variants, known as A, B, C, D, E, F, H and I (table 2.1), which have been discovered by performing protein electrophoresis (Chianese et al., 1996; Pirisi et al., 1999; Wessels et al., 2004; Giambra et al., 2010).

Ovine alpha$_{S2}$-casein (α_{S2}-CN) is the most heterogeneous fraction of caseins (Selvaggi et al., 2014). Seven variants have been described for α_{S2}-CN, named from A to G (Chessa et al., 2003; Picariello et al., 2009; Giambra and Erhardt, 2012), including a low molecular weight variant reported by Chianese et al. (1993) in Manchega breed. So far, the role of α_{S2}-CN in casein micelles has not been studied in detail (Selvaggi et al., 2014).

2.6.2.2. β-Casein

β-CN is considered calcium-sensitive, as it precipitates in the presence of low concentrations of this cation. Its importance relies in the determination of the surface properties of casein micelles. Also, β-CN is essential for rennet curd formation (Pearse et al., 1986).

Ovine β-CN is formed by 209 amino acids, and a non-genetic polymorphism occurs due to varying degrees of phosphorylation. Five genetic variants (table 2.1) have been described (Chianese, 1997; Chessa et al., 2010). β-CN is the main substrate of plasmin in milk. Its cleavage produces γ_1-CN, γ_2-CN, γ_3-CN and proteose-peptones (Fox and Kelly, 2006).

2.6.2.3. κ-Casein

κ-CN represents around 15 % of total casein in sheep milk. It consists of 169 amino acid residues, and is only soluble in the presence of calcium, because its phosphate content is lower than in other caseins (Selvaggi et al., 2014).

κ-CN is considered a monomorphic casein, as no genetic variants have been found (Jolles et al., 1974; Bastos et al., 2001; Ceriotti et al., 2004; Feligini et al., 2005; Pariset et al., 2006). However, as reported by Park (2007) it can present non-genetic polymorphisms due to three glycosilation sites (Thr_{135}, Thr_{137} and Thr_{138}) and two phosphorylation sites (Ser_{151} and Ser_{168}).

2.6.3. Urea

Urea is an organic compound consisting of carbon, nitrogen, oxygen and hydrogen, with a molecular weight of 60. It represents the main fraction of NPN, with a variable concentration between 20 and 75 % (Journet et al., 1975). This proportion is directly related to protein and energy intake (Geerts et al., 2004).

In ruminants, urea is synthesized in the liver from nitrogen derived from amino acid oxidative deamination, and is excreted in urine (Pazzola et al., 2011). However, a fraction of this urea goes through the rumen, from where it passes to the blood system (Cunningham, 2002). Many authors have correlated the concentration of urea in plasma with the concentration in milk, in cows (Broderick and Clayton, 1997), sheep (Cannas et al., 1998) and goats (Sahoo and Walli, 2008).

Standard urea concentration in milk is in a range between 10 and 30 mg/100ml (Marenjak et al., 2004), though studies concerning urea in sheep milk are scarce and less precise than those published for bovine milk (Bendelja et al., 2009). Urea concentration is an indicator of protein metabolism and the nutritional intake of the animal (Giovanetti et al., 2009). Thus, on the basis of the amount of urea in milk it is possible to diagnose issues caused by imbalances in the diet, what can impact on the health and production of dairy animals (Bendelja et al., 2009).

Albeit its presence in milk mainly depends on nutritional factors, it can be influenced by other factors such as breed, season, parity, stage of lactation, milk production, milking time or udder health status (Matutinović et al., 2014).

2.7. Other minor substances found in milk

Salts: they are present in milk both dissolved and in coloidal state (Garzón, 1996). Most of them are mineral salts (calcium phosphate), although other salts (where the anionic fraction is mainly citrate) can be present in milk (Gorsaud, 1991).

Minerals: the main minerals present in sheep milk are sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P) and chlorine (Cl). Also zinc (Zn), iron (Fe), copper (Cu) and manganese (Mn) can be found in smaller amounts. Ewe milk is specially poor in iron, selenium and copper, what may cause particular deficiency diseases in suckling lambs. Phosphorus and calcium play an essential role in the maintenance of the stability of milk proteins, as they are associated to the casein micelle (Garzón, 1996).

Vitamins: references in literature concerning vitamins in sheep milk are scarce (Raynal-Ljutovac et al., 2007). A review published by Paccard and Lagriffoul (2006) for sheep milk proved its high content in B vitamins, specially niacin. Garzón (1996) also reported an important amount of vitamin A (1.46 IU/l), riboflavin (3.82 mg/l) and pantothenic acid (3.64 mf/l). In addition sheep milk lacks carotene (Assenat, 1991), as well as vitamins D and B_6 (Garzón, 1996).

Chapter 3

Quality and cheesemaking aptitude of sheep milk

CONTENTS

3.1. The concept of quality in ewe milk

Quality in sheep milk is a concept difficult to define. It is in fact the consumer, with his final decision, who validates or not the product (Serrano, 1999). This judgement is mainly based on organoleptic and sensorial properties, which constitute hardly classifiable factors. Based on this, Esteban (1991) defined the quality of milk as *"its capacity to satisfy the consumer expectations"*.

According to Garzón (1996), in compliance with diverse objective methods intended to determine this concept, a disctinction can be drawn between:

- **Chemical or compositional quality**: it includes aspects such as fat, protein, lactose or calcium concentrations, pH, and detection of foreign substances (antibiotics, pesticides, blood and gas).
- **Physical quality**: influenced by several properties of milk, such as density, conductivity, boiling and freezing point, heat and alcohol stability, and renneting characteristics.
- **Hygienic and sanitary quality**: established according to total bacterial count, pathogens, lactic acid producers, psychrotrophs, spore forming bacteria and somatic cell count.
- **Organoleptic quality**: defined by smell, flavour, texture and color of milk.

However, the predominant quality standards in the Spanish dairy sheep industry are generally those that tend to increase processing efficiency and diminish sanitary risks, having no control over other parameters (Caja and Such, 1991). Thus, the quality of sheep milk will be determined by:

- Milk chemical composition: fat, protein and total solids.
- Microbial load: total somatic cell count, colony forming units (CFUs) and acidity of milk (Dornic degrees).
- Sheep breed, milk production and collection zones, particularly in the case of PDO cheeses.

3.2. Renneting aptitude of milk

The quality of milk intended for cheesemaking purposes is based in its aptitude to produce a good cheese and satisfactory yields in normal conditions (Toledo, 2013). This depends mostly on chemical composition (especially casein content), microbial load, the nature of bacterial microflora, the lactic bacteria

development capability, and the behavior of milk against rennet (Brule and Lenoir, 1990; Bencini and Pulina, 1997).

Milk may react differently during rennet coagulation. Some milks coagulate slowly, forming soft gels that tend to break easily. This will lead to curds with a high moisture content after gel draining, which will be hard to control during ripening. Other milks, however, coagulate rapidly forming firm gels that are easy to drain. The resulting curds will have an adequate texture and moisture content, and will develop into good quality cheeses (Mocquot et al., 1954).

3.2.1. Characteristics of the curd

The variables to evaluate renneting aptitude of milk can be determined through several techniques and devices: Berridge clotting time method, Plint torsiometer, thromboelastograph torsion viscometers, or oscillatory rheometers. McMahon and Brown (1982) defined the characteristics of the clot using a Formagraph™ torsion viscometer: rennet clotting time (RCT), curd firming time (k_{20}), curd firmness after 30 minutes (A_{30}) and curd firmness after 60 minutes (A_{60}). These parameters are strongly influenced by other factors such as milk composition, pH and health of the udder, and will be described in detail in further chapters (Jaramillo et al., 2008).

3.2.2. Cheese yield

Cheese yield or milk-to-cheese processing efficiency is the mathematical expression of the quantity of cheese obtained from a certain amount of milk, usually 100 litres or 100 kg (Assenat, 1991). According to Pirisi et al. (1994), cheese yield is generally expressed in kg of cheese per 100 litres of milk. Normal cheesemaking practice proves that, due to composition, the double amount of cheese is obtained with the same amount of ewe milk than of cow milk (Assenat, 1991).

As well as the parameters that evaluate the clot (section 3.2.1), cheese yield is mostly affected by milk composition, particularly protein and, to a lesser extent, fat concentration (Pulina et al., 2006; Jaramillo et al., 2008). Other influencing factors are somatic cell count (SCC) and plasmin activity, which reduce cheese yield due to the increase of curd humidity (Pulina et al., 2006) and proteolysis of casein (Srinivasan and Lucey, 2002).

It has so far become clear that there is an existing correlation between cheese yield and renneting parameters. Thus, using a viscometer to measure

the rheological characteristics of milk may somehow predict cheese yield (López Galvez, 1993).

Chapter 4

Hygienic and sanitary quality of milk

CONTENTS

4.1. Mastitis

Mastitis is defined as an inflammatory reaction of the mammary gland (IDF, 1987) and represents an indicator of the udder health status (Pinto et al., 2013). This disease has a wide impact over animal welfare and production, and produces great economic losses in the dairy industry. This is specially evident in subclinical mastitis, which affects around 50 % of dairy ewes, while clinical mastitis only affects 5 %.

4.1.1. Mastitis classification

1. **According to course:**

   a) Acute: they appear frequently after lambing, as a result of a bacterial infection due to wounds in the udder or systemic infections. They are easily recognizable because of the sudden appearance and the physical changes in milk (lumps), so as in the descent of its secretion. They can be followed by anorexia, depression and fever. Severe cases can evolve into acute gangrenous mastitis, where milk secretion stops and a continuous discharge of a bloody exudate is observed in necrotized tissues.

   b) Chronic: swelling of the mammary gland and the cistern is observed, as well as possible tissular edema, aqueous milk, and lumps or clots in the first streams during milking.

2. **According to clinic**

   a) Clinical: characterized by visible abnormalities in milk (lumps, clots or aqueous appearance), as well as by sudden symptoms (pain and swelling) and a descent in milk production. Animals may be systemically affected, presenting fever and dehydration, and refusing to eat.

   b) Subclinical: consisting in an inflammation of the mammary gland with no visible changes in milk or in the udder. Despite affected ewes look apparently normal, their production and quality of milk diminish, and they become a source of infection for other animals in the flock. This type of mastitis is difficult to recognize, and requires special diagnosis tests for its detection.

Table 4.1: Reservoirs and routes of transmission of several microorganisms causing mastitis.[1]

MICROORGANISM	RESERVOIR	TRANSMISSION
Staphylococcus aureus	Infected udder	Milking machine
Streptococcus agalactiae	Infected udder	Milking machine
Arcanobacterium pyogenes	Infected udder, litter	Flies
Streptococcus uberis	Litter	Udder preparation and repose
Pseudomonas	Water	Repose
Enterobacteria	Litter	Udder preparation and repose
Prototheca	Water and syringes	Treatments
Serratia	Litter	Repose and pre-dipping
Yeasts	Environment	Treatments
Bacillus cereus	Mud	Repose
Mycoplasma	Infected udder	Milking machine
Corynebacterium bovis	Infected udder	Bad disinfection

4.1.2. Economic impact of mastitis in sheep

Mastitis is probably the disease that produces the highest economic losses in small ruminant livestock worldwide. Based on the degree of affection, sanitary and economic effects may vary. Clinical mastitis only represents a small part of financial losses, while subclinical cases represent their main source, due to a higher incidence and the difficulty to be identified (Watson and Buswell, 1984; Batavani et al., 2003).

It is subclinical mastitis what produces economic losses directly related with production. Infected ewes reduce significantly their milk production compared to healthy animals. Starvation may occur in some cases when milk yield is severely diminished, causing death or deficient growth/development of the lambs (Torres Mañas, 2003).

On the other hand, hygienic and sanitary quality of mastitic milk strongly affects processing in the cheese industry, giving as result a worsening of the quality and conservation of the products (Pellegrini et al., 1996). Several factors such as an increase of plasmin activity in milk from animals with udder infections, may have an effect on cheese yield due to proteolysis of caseins (Albenzio et al., 2009).

All these factors concern profitability of the flocks and farms, and compel to apply mastitis control and prevention specific programs (Tardáguila, 1999). Thus, other financial losses come up from added costs due to medical

[1] Source: www.solomamitis.com

treatments, disposal of milk with antibiotic residues, and an increase of animal replacement in the flock (Torres Mañas, 2003). Therefore, specific medical treatments are only considered if the cost does not exceed the economic value of the affected animal (Suárez, 2007).

4.1.3. Control of mastitis in dairy sheep

To control mastitis in the flock it is essential to avoid new infections and to reduce prevalence (Torres Mañas, 2003). Treatment and cure of mastitis, as well as prevention of its propagation, are more efficient if applied during the drying of the ewes, mainly in those cases where the disease is revealed subclinical.

It is crucial to treat with antimicrobial drugs in the moment the ewes are dried. The implementation of this treatment may be total (all ewes and udders are treated) or selective (only infected animals are treated). However, in flocks with strong seasonal production and long dry periods, total treatment has shown to be more effective (Torres Mañas, 2003).

To avoid mastitis transmission it is also essential to implement a good milking routine: the first batch of animals to be milked shall be the one with the healthy ewes, followed by the batch of ewes positive to mastitis tests. Animals in treatment must be isolated from the rest and milked by hand, considering that these isolation periods must be longer in sheep than in cows. The milking machine must be often thoroughly inspected, replacing clusters and milk tubes, and avoiding bad habits such as over-milking or removing the teat cups without turning off the vacuum (Suárez, 2007).

4.1.4. Diagnostic methods for mastitis

1. **Visualization and palpation:** having access to the clinical, productive and reproductive history of the animals is basic before examining the mammary gland. Palpation of lymphatic nodes and the udder (both full and freshly milked) must be performed to verify existing abnormalities, since a decrease in milk production or visible physical changes may represent the first evidence of mastitis.
2. **Physical tests:**
   a) Milking bowl test: based on the direct observation of lumps when milk is poured over a black fabric covering the milking bowl.
   b) Black fabric test: based on the detection of milk lumps by passing the first streams through a black fabric mesh during milking.

c) Test cup: the first streams of milk are poured into a dark background container, so that clots, lumps, or any alteration in color and density can be evidenced.

3. **Chemical tests:**

   a) Electrical conductivity of milk: milk conductivity increases with mastitis due to the increment of sodium (Na^+), chloride (Cl^-) and potasium (K^+). These changes in conductivity can be converted into a computer readable signal and, therefore, quantified (Pyörälä, 2003).

   b) pH indicator paper: a few drops of milk are poured over the indicator paper. Animals with a pH of milk above 7 are suspected to have mastitis.

   c) Whiteside test: it is based on an excess of leukocytes in milk. A small amount of NaOH is placed on a glass, and 5 drops of fresh milk are added, mixing with a small glass rod for about 20 seconds. Milk negative to mastitis remains liquid, while positive milk forms a precipitate with variable density, depending on the level of infection.

4. **Biological tests:**

   a) California Mastitis Test (CMT): this test provides a useful technique for detecting subclinical mastitis. It operates by disrupting the cell membrane of any cells present in milk, allowing the DNA to react with the test reagent forming a gel (White et al., 2005). Milk samples (2 ml) are drawn into a four-well plastic paddle and a special reagent (CMT-Test) is added while agitating gently. The level of mastitis is categorized visually according to the viscosity of the reaction: negative (0), trace (T), weak positive (1), distinct positive (2) and strong positive (3).

   b) Wisconsin Mastitis Test (WMT): designed to be performed in the laboratory. The procedure is similar to CMT, but results are quantitatively measured depending on viscosity. Milk (2 ml) is pipetted in a graduated tube and a 2 ml mixture of CMT-Test reagent and deionized water (1:1) is added. After being stirred for 10 seconds and left at room temperature, results are obtained by comparing the mixture in the tube with a specific table published for this test.

   c) Somatic Cell Count (SCC): it allows to evaluate with more precision the health and functional status of the udder. Several methods can determine directly or indirectly the concentration of somatic cells in milk, such as direct counting with a microscope, Fluoro-opto-

electronic cell-counting (Fossomatic™), or the Counter Coulter.

5. **Microbiological tests:** isolation and culture of microorganisms represent an important path to identify the etiological agent causing mastitis. However, despite of the low investment in material and equipment, results are not immediate, taking minimally 24 hours before they are delivered (Bikker et al., 2014).

4.2. Somatic cells

Somatic cell count is used as an indicator to evaluate milk quality and define its price (Haenlein, 2001; Kalantzopoulos et al., 2004; Raynal-Ljutovac et al., 2005). An increase of somatic cells in milk is a sign of an alteration of the product and a resulting loss of its quality. Mastitis is the main cause of high somatic cell counts in milk, although other factors like oestrous or late lactation may act as triggers (Albenzio et al., 2005).

The cells reflected in these counts (mainly leukocytes or white cells) come from blood and from the epitelial tissue of the mammary gland (cells from the secretory tissue). Around 98 % of somatic cells are polymorphonuclear neutrophils (PMN) and macrophages (Paape et al., 2003) that make their way from blood to the alveolar lumen as a response to the bacterial infection of the udder (Hernandez and Bedolla, 2008). The most numerous cells during mastitis are polymorphonuclear granulocytes, which recognize and phagocytize bacteria.

PMNs represent the first immunologic barrier against bacterial infection of the mammary gland, protecting it through phagocytosis and intracellular death by using bactericidal enzymes and *oxi* radicals (Prin-Mathieu et al., 2002). In pathologic processes, PMNs can represent over 95 % of total cells in milk.

Macrophages are monocytes that migrate from the capillaries to the interior of the mammary gland, initiating inflammation. Not only do they phagocytize bacteria or aged cells, but they present the bacterial antigens to lymphocytes.

Lymphocytes recognize antigens through specific membrane receptors for each invader pathogen. There are two different types of lymphocytes with different roles: B-cells and T-cells. The first ones, which represent about 20 % of the total lymphocytes, recognize the antigen, creating specific antibodies that are locally released. T-cells, on the other hand, destroy the antigens by direct contact, producing lymphokines that activate the major histocompatibility complex.

4.2.1. Causes of high somatic cell counts in milk

1. **Infection of the udder:** the mammary gland may be affected by two different types of microorganisms:

   a) Infectious pathogenic microorganisms: such as *Staphylococcus aureus*, *Streptococcus agalactiae*, etc. Infectious bacteria are spread among the ewe's udders or in between animals in the herd, as a result of a poor practice during milking.

   b) Environmental pathogenic microorganisms: such as other *Streptococcus*, *Corynebacterium bovis* and coagulase-negative *Staphylococcus*. Environmental bateria are secondary opportunistic pathogens present in the surroundings of the animals (skin, manger, puddles, etc.) and they penetrate the udder under particular health conditions.

2. **Age of the animal and stage of lactation (SL):** several authors reported an effect of SL over SCC in dairy cows, due to an increase of mastitis through the productive life of the animals (De Haas et al., 2004; García, 2004). For this reason, multiparous animals generally present higher incidence of mastitis. Also, as milk production diminishes at the end of lactation, somatic cells are concentrated in a lower volume of milk, so SCC/ml increases (Carrión, 2001).

3. **Stress:** any stressful situation affecting the animals may increase somatic cells in milk. Thus, estrus or infectious diseases can raise SCC.

4. **Daily and annual fluctuations:** SCC in milk from evening milking are generally higher due to a low production. This concentration effect is caused by an increase of the milking interval. Also, some authors have recorded higher SCC in summer than in winter (Blowey and Edmondson, 1995).

5. **Lesions of the udder:** these appear due to over-milking, an improper use of the milking machine, or bad conditions of the milk tubes and teat cups. Also improper or poorly designed facilities may cause injuries in the udder.

6. **Physiological variations:** Saran and Chaffer (2000) found that SCC were slightly higher in milk from cows in heat.

4.2.2. Somatic cell levels in ewe milk

The lack of data in small ruminants can lead to errors in the diagnosis of subclinical mastitis and, thus, in the establishment of standard SCC values of

sheep and goat milk (Pinto et al., 2013). Diagnostic limits for SCC in ewe milk are still discussed, specially in European countries (Boyazoglu and Morand-Fehr, 2001; Bergonier et al., 2003). Unlike in bovine milk, where SCC limits have been legally established, there is no EU Directive that applies to sheep milk. Consequently, no legal limits to categorize SCC have yet been established for small ruminants (Paape et al., 2007; Raynal-Ljutovac et al., 2007).

There is a great deal of controversy regarding acceptable somatic cell concentrations in ewe milk. Konig et al. (1985) suggested that SCC around 2000×10^3 cells/ml can be considered normal. Barbosa et al. (1994) recommended a limit of 1500×10^3 cells/ml for bulk tank somatic cell count in small ruminants. To the contrary, Boyazoglu and Morand-Fehr (2001) established this same value as a sign of subclinical mastitis. Bianchi et al. (2004), considered subclinical mastitis when SCC exceeded 500×10^3 cells/ml. Meanwhile, Gonzalo et al. (2000) established 3 sanitary categories regarding bulk tank somatic cell counts in sheep: good health condition (SCC $< 500\times10^3$), with an average of 30 % of the flock affected; intermediate health condition (SCC between 500×10^3 and 1000×10^3), with around 40 % of the flock affected; and bad health condition (SCC $> 1000\times10^3$), with an udder infection rate above 45 % of the flock.

Bulk milk SCC mainly evaluates the level of subclinical mastitis and is used: 1) to determine the prevalence and evaluate control in dairy herds; 2) as an indicator to evaluate the price of milk in payment schemes; 3) as a monitor of hygienic production and milk safety (Smith, 1996).

Chapter 5

Indigenous enzymes: plasmin

CONTENTS

5.1. Proteolysis in milk

Proteolysis potentially affects all dairy products (Saint-Denis et al., 2001a). This results in a lower quality of the products, the development of bitter flavours in UHT milks, a decrease in cheese yield and a degradation of caseins during storage and ripening (Bastian and Brown, 1996; Mara et al., 1998). Proteolysis in milk may have two different origins:

1. Psychrotrophic microorganisms that produce and release heat resistant proteases during refrigerated storage of milk (Fairbairn and Law, 1986).
2. A deterioration of the udder and appearance of mastitis, which produces an increase of endogenous proteases (Le Roux et al., 1995). Thus, high SCC in milk enhance the presence of numerous protease and lipase enzymes that might alter organoleptic properties of the final product.

It is a proven fact that proteolytic enzymes in milk have the following common characteristics (Callejo Ramos, 2015):

1. Their action begins in the second day and is maximum in the fifth day.
2. The optimal pH is 7.8, and at 40-50ºC they reach their highest activity, though they are still active at low temperature and pH.
3. Thermostability reaches 150ºC, what makes them active in pasteurized and UHT milks, with all the problems that this entails.
4. They diminish heat stability of milk, which tends to coagulate.
5. They cause degradation of caseins, what leads to a drop in the yield during cheesemaking and an increase of nitrogen in whey.
6. They modify sensorial properties of milk, which loses freshness, and develops bitter or even rotten flavours.

Since the indigenous milk enzymes have no essential beneficial effect on the nutritional or organoleptic attributes of milk, their destruction by heat is one of the objectives of many dairy processes (Fox and Kelly, 2006).

5.2. The plasmin system

Plasmin is an alkaline serine proteinase, analogous to the blood serum enzyme (Bastian and Brown, 1996). This enzyme is present in milk as its zymogen plasminogen, which activates to plasmin when SCC exceeds 500×10^3 cells/ml.

Milk contains the complete plasmin system (PL system): plasmin (PL), plasminogen (PG) and a complex structure of plasminogen activators (PA), plasminogen activator inhibitors (PAI) and plasmin inhibitors (PI) (Ismail et al., 2006). The whole PL system enters milk from blood, where it plays an important role in the degradation of fibrin clots (Sidelmann et al., 2000). In milk, PL, PG and PA are associated with the casein micelle (Politis, 1996), whereas PAI and PI are soluble in the whey. There are two types of plasminogen activators: a tissue-type PA (t-PA) and a urokinase-type PA (u-PA) Politis (1996). Regarding the inhibitors of the PL system, Christensen et al. (1995) reported at least 6 types of PAI. Two of them have been already isolated in milk and identified as PAI-1 and PAI-2 (Politis, 1996).

5.3. Influence of the plasmin system on fresh milk and cheese

The PL system plays an important role in the breakdown of casein, reducing cheese yield and casein content due to the leakage of proteose-peptones into whey (Albenzio et al., 2009). Plasmin mainly attacks β-CN, α_{S2}-CN and α_{S1}-CN (susceptible in that particular order). However, κ-CN seems to resist its effect, though some experiments have reported that it can be affected under certain conditions (Groves et al., 1998). Plasmin activity tends to increase due to udder infections or advanced stages of lactation (Fox and Kelly, 2006).

In cheese, plasmin-induced proteolysis can contribute to the appearance of some particular desirable flavours and textures during ripening (Ismail and Nielsen, 2010). Meanwhile, other authors associated an intense plasmin activity as a cause of development of bitter peptides (Habibi-Najafi et al., 1996; Sousa et al., 2001). This seems to be more frequent in high-cooked cheese varieties (Fox and Kelly, 2006).

In milk (wether raw, pasteurized or UHT), to the contrary, proteolysis is the cause of undesirable effects. In severe cases, casein hydrolysis induced by plasmin may greatly affect rennet coagulation (Srinivasan and Lucey, 2002; Albenzio et al., 2005). This represents an important issue concerning the dairy sheep industry, as almost all milk production is intended for cheesemaking. Thus, proteolysis causes a reduction of the processing capacity of milk into cheese, as well as changes in its composition and the development of bitter flavours in processed products (Guerrero et al., 2003).

Existing variations in the activity of endogenous enzymes are relevant, not

only due to their physiological role, but as milk quality indicators in terms of coagulation (Albenzio et al., 2009). It is important to investigate if specific enzymatic activities are associated with different types of somatic cells in bulk tank. Controlling plasmin activity could lead to an improvement of quality in the dairy industry, perhaps also reducing production costs. Therefore, proteolysis induced by this enzyme has acquired strong interest from researchers, due to its complexity and versatile effects over the quality of milk and dairy products (Ismail and Nielsen, 2010).

5.4. The structure and biochemical characteristics of plasmin

Plasmin consists of two polypeptide chains, connected by two interchain disulphide bridges (Ogston, 1980):

- A heavy chain (A) with a molecular weight of around 60000 from the NH2 terminal chain.
- A light chain (B), with a molecular weight of about 25000 from the COOH-terminal end.

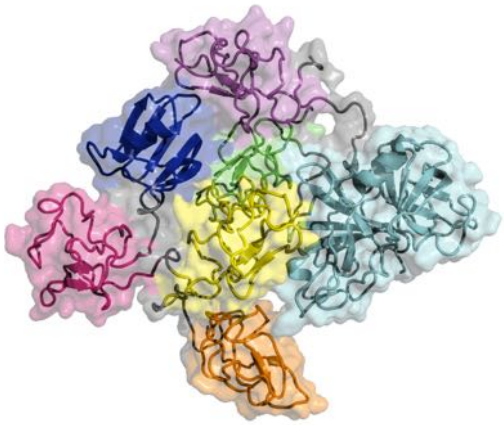


Figure 5.1: Three-dimensional structure of plasmin.

Plasmin exhibits optimal activity at pH 7.5 and 37°C, so the physiological conditions of the udder represent an ideal environment (Gazi et al., 2014). It is considerably stable to heat and partially survives UHT processing. Plasmin is highly specific and preferentially attacks peptide bonds containing Lysine (*Lys*) or Arginine (*Arg*) in the N-terminal end. A substantial number of blood plasma proteins are susceptible to the action of plasmin, including coagulation factors V

and VII, components of the complement system, the hormones ACTH, glucagon and somatotrophin and fibrinogen (Ogston, 1980). However, in milk the main substrate of plasmin is β-CN, from which it produces γ_1-CN, γ_2-CN, γ_3-CN and proteose-peptones (Fox and Kelly, 2006).

5.5. The determination of plasmin in milk

Numerous authors have proposed several protocols to measure plasmin and plasminogen (after its activation into plasmin) activity in dairy products (Saint-Denis et al., 2001a). Most of these assays use synthetic peptide substrates that are hydrolysed to chromogenic or fluorogenic products (see section 5.5.1). However, new methodologies are currently being developed and enable to quickly detect proteolytic activity, such as the *Quantiflour®-P* (Promega Corporation, Madison USA) or the *W2 Optronic's handheld flourometer* (Jiangsu, China). In a recent paper, Bikker et al. (2014) describes the design and synthesis of new taylor made plasmin substrates as a potential diagnostic tool to test for mastitis.

5.5.1. Complications in the measurement of plasmin activity

Investigation of endogenous enzymes is hampered by the fact that each research group uses multiple assay methodologies (Kelly et al., 2006). A good example of these complications involves plasmin, since one fluorimetric and two spectrophotometric assays are currently in use, and employ the following substrates:

a) *N-Suc-Ala-Phe-Lys-7-amido-4-methyl coumarin*[1] (Richardson and Pierce, 1981).

b) *D-Val-Leu-Lys-p-nitroanilide*[2] (Rollema et al., 1983).

c) *HD-Norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide*[3] (Lu and Nielsen, 1993).

Several methods to prepare milk samples for plasmin assays have also been published (Fox and Kelly, 2006). This fact, together with the diversity of peptide substrates and units used to express experimental results (tables 5.1 and 5.2) represent a clear issue when attempting to develop an overview of factors that affect plasmin activity (Kelly et al., 2006).

[1] *S-0763*, fluorimetric.
[2] *S-2251*, spectrophotometric.
[3] *Spectroyme-PL*, spectrophotometric.

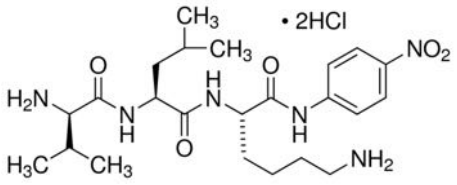


Figure 5.2: *D-Val-Leu-Lys-p-nitroanilide* (S-2251).

Regarding plasmin, the choice of the most suitable technique to measure enzymatic activity is complicated by the fact that, during incubation and storage of milk, plasmin activity might be increased (due to plasminogen activation) or reduced (due to autolysis). In addition, some authors have reported an enhancement of plasminogen activation by two of the most common plasmin substrates: *S-2251* (figure 5.2) and *Spectrozyme-PL* (Kolev et al., 1995).

5.6. Other endogenous proteinases in milk

5.6.1. Cathepsin D

Cathepsin D is a lysosomal aspartic proteinase with a low pH optimum (Hurley et al., 2000). It is the second major proteinase in milk, and its presence was first demonstrated by Kaminogawa and Yamauchi (1972), and subsequently confirmed by Larsen et al. (1993). As well as plasmin, cathepsin D is part of a complex system, including its inactive zymogen, procathepsin D, and other inactive precursors (Fox and Kelly, 2006). The levels of this enzyme in milk are significantly correlated with SCC as it is originated in the lysosomes of somatic cells (Considine et al., 2004). Its concentration in milk is approximately 0.4 μg/ml and it is mainly present in the whey (Larsen et al., 2000).

Cathepsin D contributes to casein breakdown. It can hydrolyse κ-CN and degrades both α_{S1}-CN and β-CN displaying a proteolytic activity similar to chymosin (Albenzio et al., 2009). At considerably high concentrations, it is able to coagulate milk (Hurley et al., 2000). However, coagulation of milk due to cathepsin D is not possible under normal conditions (Moatsou, 2010).

According to Larsen et al. (2000) and Hayes et al. (2001), cathepsin D partially survives pasteurisation, may affect the storage of dairy products, and can induce proteolysis during cheese ripening.

5.6.2. Cathepsin B

Cathepsin B is a cysteine proteinase activated by dithiothreitol (DTT). It has a pH optimum of 6.0 and is inactivated at pH values above 7.0 (Kirschke et al., 1998). This enzyme in milk seems to be heat-stable and can partially survive (at more than 20 %) conventional pasteurisation (Moatsou, 2010).

Cathepsin B cleaves β-CN and α_{S1}-CN at several cleavage sites, which seem to be close or identical to those specific for plasmin, giving as a result a large number of peptides. Therefore, its activity might be significant regarding proteolysis in dairy products (Considine et al., 2004). In addition, the activity of cathepsin B is also comparable to that found for chymosin, as it cleaves similar sites on β-CN and α_{S1}-CN (Moatsou, 2010).

5.6.3. Elastase

Elastase is a neutral serine-type proteinase mainly associated with PMNs (Albenzio et al., 2009). It seems to be present in milk only in cases of high SCC, as it has been isolated from milk during experimentally induced mastitis (Prin-Mathieu et al., 2002).

It specifically cleaves α_{S1}-CN and β-CN, producing an impact on the quality of milk (Considine et al., (2000). Due to this, some authors have associated the action of elastase with the presence of several peptides in high SCC milk (Moussaoui et al., 2003; Nabhan et al., 2004). Elastase can produce hydrolysis of some proteins to fragments that are capable of inhibiting urokinase-type PA (u-PA), and also cleave plasminogen activators (PA) and plasmin (Kelly et al., 2006).

Elastase activity increases constantly during lactation (Moatsou, 2010). These changes in elastase levels in milk are parallel to those found in PMNs, what suggests that elastase concentration could be a reliable indicator of mammary gland involution in healthy udders (Albenzio et al., 2009).

5.6.4. Other minor proteinases

Somatic cells also contain several other proteinases (including cathepsins L and G), which at this time have been scarcely studied (Kelly and McSweeney, 2003).

Table 5.1: Overview of different assays to measure plasmin activity described in literature (I).

ANALYSIS	MEASUREMENT	BUFFER	PREPARATION	REF.
Plasmin, plasminogen	Fluorescence Ex. 380 nm Em. 460 nm	0.4M Sodium citrate	· Dissociation of caseins by sodium citrate. · Centrifugation/skimming to obtain clear sample. · Mixing with buffer and addition of substrate.	Richardson and Pierce (1981)
Plasmin, plasminogen	Spectrometric Abs. 405 nm	40mM Tris-HCl pH 7.4 100mM EDTA 50mM EACA	· Addition of skimmed milk to buffer containing substrate.	Rollema et al. (1983)
Plasmin	Spectrometric Abs. 405 nM	50mM Tris 110mM NaCl 3 mM EACA	· Model study. · Mixing plasmin with buffer and reagents.	Bastian et al. (1991b).
Plasmin, plasminogen	Spectrometric Abs. 405 nM	50mM Tris-HCl pH8 110mM NaCl 50mM EACA	· Centrifugation to obtain casein/plasmin pellet. · Resuspending pellet in extraction buffer. · Centrifugation to remove caseins. · Mixing with substrate buffer.	Politis et al. (1993).

Source: Rauh (2014).

Table 5.2: Overview of different assays to measure plasmin activity described in literature (II).

ANALYSIS	MEASUREMENT	BUFFER	PREPARATION	REF.
Plasmin, plasminogen	Fluorescence Ex. 370nm Em. 440 nm	100mM Tris HCl pH 8 0.4M NaCl 8mM EACA	· Mixing of sample with extraction buffer (EACA and NaCl). · Mixing of sample with substrate buffer. · Removal of turbidity and stopping of enzymatic reaction by Clarifying Reagent. · No information on fat content of milk.	Saint-Denis et al. (2001b).
Plasmin	Spectrometric Abs. 405 nm Abs. 490 nm	50mM Tris 0.1M NaCl Tween 80 pH 7.6	· Model study. · Mixing plasmin with buffer and reagents. · Correction for turbidity.	Prado et al. (2006)
Plasmina	Fluorescence Ex. 380 nm Em. 460 nM	0.4M Sodium citrate Tween	· Dissociation of caseins by sodium citrate. · Centrifugation/skimming to obtain clear sample in presence of Tween.	Iucci et al. (2008)
Plasmin, plasminogen	Spectrometric Abs. 405 nm Abs. 490 nm	0.4M Sodium citrate pH 9 100mM Tris-HCl pH 8 0.4M NaCl 8mM EACA	· Dissociation of caseins by sodium citrate. · Dissociation of caseins and plasmin by EACA and NaCl. · Addition of substrate.	Rauh et al. (2014).

Part III

AIMS

Aims of the doctoral thesis

AIM 1

To study the effect of stage of lactation and parity over composition, renneting properties, hygienic and sanitary parameters, and plasmin activity in Manchega ewe milk.

AIM 2

To determine how the udder health status affects milk composition and coagulation, as well as the enzymatic activity of plasmin.

AIM 3

To evaluate the influence of plasmin activity on milk composition and rennet coagulation.

Part IV

MATERIALS AND METHODOLOGY

Chapter 6

Material

CONTENTS

6.1. Experimental animals

40 Manchega ewes from the province of Ciudad Real (Castilla La-Mancha, Spain) were used in this study. The source of origin of all animals was the farm La Nava Del Conejo, in the region of Valdepeñas.

6.2. Material for milk sample collection

- 100 ml hermetically sealed containers for general tests.
- 50 ml hermetically sealed containers for chemical composition analyses and somatic cell counts.
- Self-adhesive labels for identification.
- Manual milking equipment.
- Azidiol (as a preservative for milk samples).
- Disposable dispensing pipettes.
- Cotton wool and ethanol for disinfection of the udder.
- Container racks.
- Portable coolers and gel ice packs.

6.3. Laboratory equipment and supplies

6.3.1. Rennet coagulation tests

- Formagraph™ viscometer (*Foss Electric*, Hillerød, Denmark), consisting of:
  a) Sample cuvettes, each one with capacity for 10 milk samples.
  b) A service module that heats the sample cuvettes and the milk, and controls the temperature of the device.
  c) A recorder module, consisting of a ten-channel recording system. Each channel comprises a pendulum connected to a computer by a digital circuit.
- Liquid rennet (bovine chymosine 80 % - pepsine 20 %).
- Pipetman Classic™ variable volume micropipettes (*Gilson, S.A.S.*, Villiers-le-Bel, France).
- Multiple spoon apparatus.

6.3.2. Physico-chemical analyses and milk composition

- Crisson Basic 20® pH meter (*Crisson Instruments S.A.*, Barcelona, Spain).
- Milko-Scan™ *FT-6000* (*Foss Electric*, Hillerød, Denmark), consisting of:
  a) An homogenizer and a sampling pump.
  b) A double-beam spectrophotometer, modified so it can detect quick changes in wavelength to obtain separate results for all compositional parameters.
  c) A control unit that converts the signal from the spectrophotometer into a direct reading value (in percentage) to be displayed in the viewing panel or directly transferred to a computer.
- Precisterm® thermostatic bath (*J.P. Selecta S.A.*, Barcelona, Spain), with 40 litres capacity and adjustable temperature (5-110ºC).

6.3.3. Somatic cell count

- Fossomatic™ *Minor cell counter* (*Foss Electric*, Hillerød, Denmark).
- Rainin® *EDP-Plus*™ electronic pipette (*Mettler-Toledo S.A.E.*, Barcelona, Spain).
- Disposable pippete tips.
- 10 ml test tubes.
- Test tube racks.

6.3.4. Determination of ash content

- Analytical laboratory balance (sensitivity 0.1 mg).
- Desiccator with silica gel.
- Laboratory drying oven.
- Muffle furnace.
- Crucibles (65 mm diameter × 34 mm height).

6.3.5. Determination of plasmin activity

- Trisodium citrate 0.4 M (2%).
- 50 mM tris-HCl buffer (pH 7.5).
- *N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amino-4-methyl coumarin*.
- *7-amido-4-methyl coumarin* (AMC, 0.3 nmol)

6.3.6. Urea polyacrylamide gel electrophoresis

- Electrophoresis unit.
- Eppendorf microcentrifuge tubes.
- Pipetman Classic™ variable volume micropipettes (*Gilson, S.A.S.*, Villiers-le-Bel, France).
- Whatman® filter paper No. 1 and No. 113.
- Tris (hydroxymethyl) methyl amine.
- Urea.
- Glycine.
- Potassium hydroxide.
- Bromophenol blue.
- Coomassie brilliant blue G 250.
- 2-Mercaptoethanol.
- Hydrochloric acid (HCl).
- Sulphuric acid (H_2SO_4).
- Trichloroacetic acid ($C_2HCl_3O_2$).
- Acrylamide solution.
- Methylene bisacrylamide (N,N Methylene diacramylide).
- Tetramethylethylenediamine (TEMED).
- Ammonium persulphate.

Chapter 7

Methodology

CONTENTS

7.1. Sample collection and processing

The choice of the farm and the sampling protocol were managed by Regional Institute Of Animal Selection and Reproduction Of Castilla-La Mancha (CERSYRA). All sampled animals came from a single source to avoid the effect of factors such as feeding and nutrition or animal management. Thereby, milk from 40 Manchega ewes was analyzed during a whole lactation (5 months). Samples from left and right udder of each animal were collected monthly and tested in 3 different laboratories:

- **Interprofessional Dairy Laboratory Of Castilla-La Mancha (LILCAM), Spain**: physico-chemical composition and somatic cell count.
- **Dairy Small Ruminants Laboratory, University of Córdoba (UCO), Spain**: rennet coagulation, ash content.
- **Food Science Laboratory 227, University College Cork (UCC), Ireland**: plasmin activity and Urea-PAGE electrophoresis.

7.2. Laboratory methods

Figure 7.1 is a flow chart describing all the tests performed in the laboratory, which will be thoroughly detailed in the following sections.

7.2.1. Rennet coagulation tests

All milk samples were preheated to 32ºC in a thermostatic bath, and gently stirred to homogenize its components. Renneting parameters were monitored using a Formagraph™ viscometer, based on the oscillatory motion of circular pendula immersed in milk during coagulation (figure 7.2).

In the recorder module, samples were brought into contact with the pendulum loops. While the milk remains uncoagulated, insufficient force is transmitted to the pendulum from the linearly oscillating milk to cause the pendulum to move. When coagulation occurs, the resultant increase in viscosity and formation of a curd causes synchronous motion of the pendulum. This information is transferred to a computer, where the result of the pendulum movements is represented in a diagram (figure 7.3).

1. **Rennet clotting time (RCT)**: it represents the time elapsed (in minutes) until the formation of the curd. Its value is determined by measuring

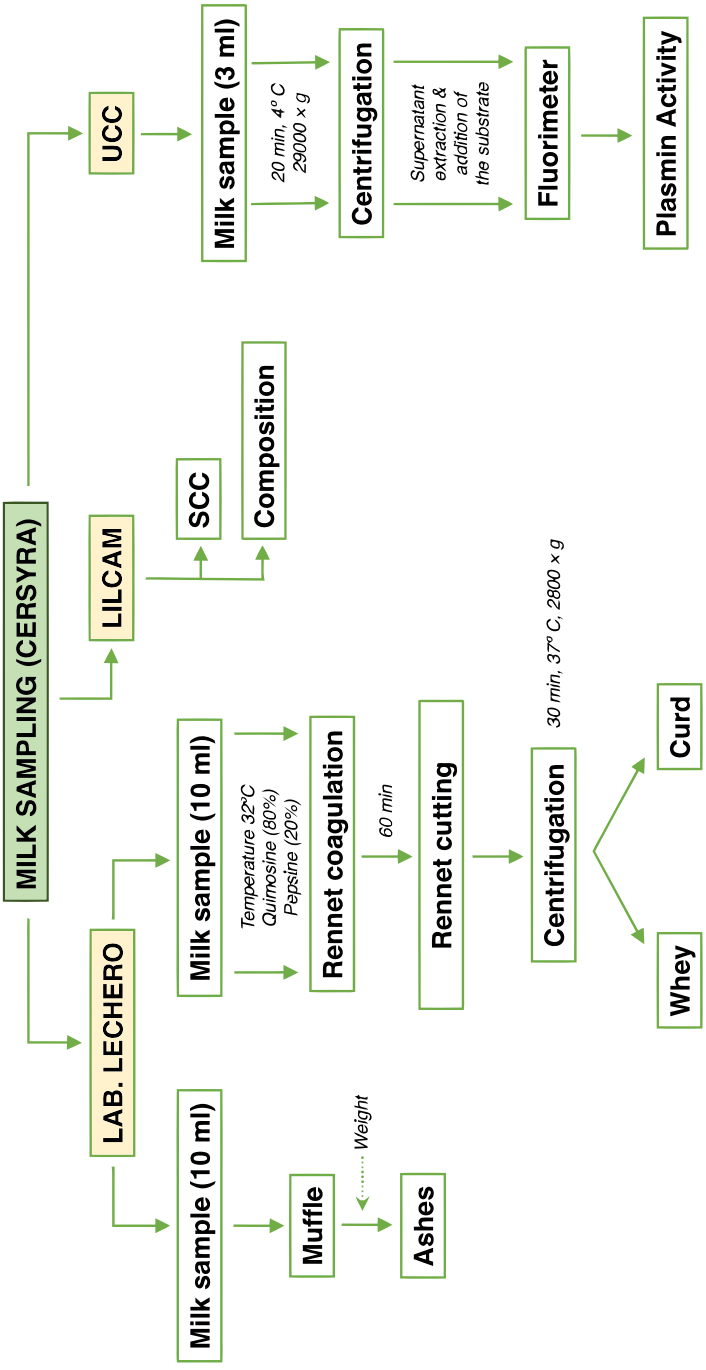


Figure 7.1: Flow chart representing all the performed laboratory tests.

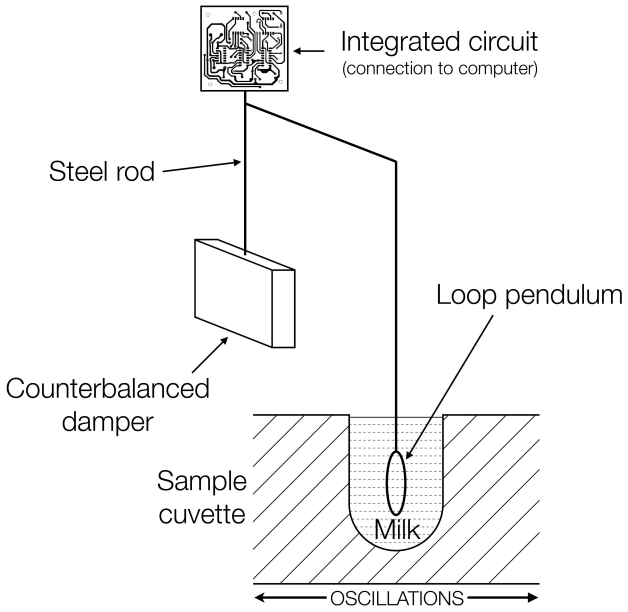


Figure 7.2: Pendulum structure in the Formagraph™.

distance from the origin to the point where the baseline begins to increase in width.

2. **Curd firming time (k_{20})**: it represents the time (in minutes) from the start of gel development until a width of 20 mm is reached on the chart.
3. **Curd firmness at 30 (A_{30}) and 60 (A_{60}) minutes**: they represent the width of the diagram (in mm) at 30 and 60 minutes, respectively.

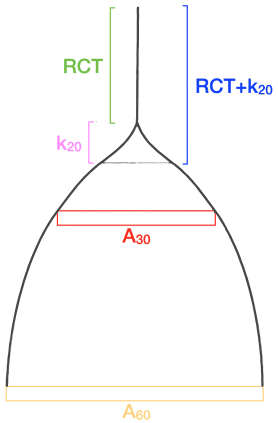


Figure 7.3: Diagram of coagulation and curd firmness as recorded with the Formagraph™.

After performing the analysis (60 minutes), the resulting curds were individually placed in centrifuge tubes and cut with a spatula. All tubes were then centrifuged for 30 minutes at 2800×g and 37ºC, to separate the curd from the whey. Finally, the drained curds were individually weighted to calculate the yield (Serrano, 1999).

7.2.2. Physico-chemical analyses and milk composition

The initial pH of milk was measured with a pH meter at 20ºC while stirring the samples to homogenize all components. After the measurement of pH, all samples were heated to 35-40ºC in a thermostatic bath to increase their temperature in order to perform further analyses.

Milk composition was performed by direct measurement of the percentage of fat, crude protein, lactose, total solids, casein, and urea, using a Milkoscan™ *FT-6000*. This device works with mid-infrared spectroscopy, and is based on the premise that most organic compounds selectively absorb certain wavelenghts within the infrared region of the electromagnetic spectrum.

7.2.3. Somatic cell count

Somatic cell count was measured using a Fossomatic™ *Minor cell counter* (figure 7.4). This device is based in the principle of DNA staining with ethidium bromide. Once the genetic material is stained, a blue light beam strikes over the sample, and the fluorescence emission is registered by a fluorescence detector. A signal is sent to the computer, and the operating result is displayed on the screen. This fluorescence emission is proportional to the amount of DNA in the sample and, therefore, to somatic cell concentration in milk.

To perform this test, 2 ml of deionized water were added to 2 ml of milk. This dilution was made to avoid the obstruction of the device due to the high density of ewe milk, since the Fossomatic™ is optimized to analyze bovine milk.

After the sample intake, ethidium bromide is added to milk. Stained cells are counted in a small chamber directly connected to the computer for data processing (figure 7.5). Once the analysis is performed, Fossomatic™ automatically activates an internal cleansing program that makes use of two different detergents (*Clean I* and *Clean 2*) to remove residual cells from the circuit.

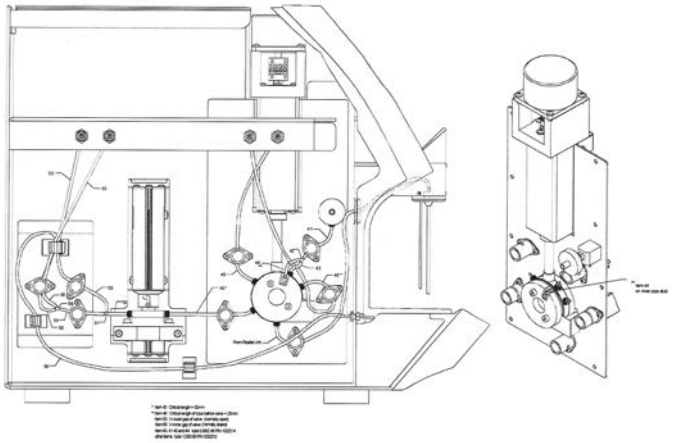


Figure 7.4: Fossomatic™ *Minor Cell Counter.*

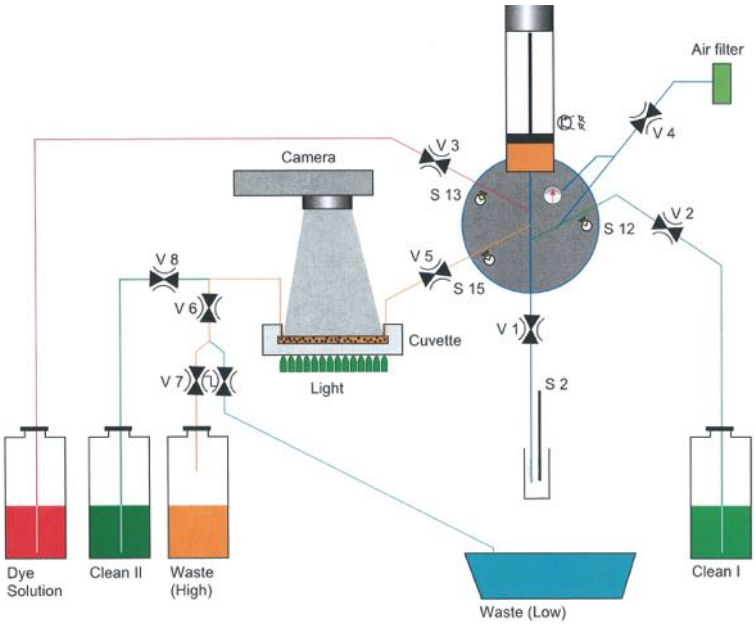


Figure 7.5: Internal components of Fossomatic™.

7.2.4. Determination of ash content

Ash content in milk is defined as the resulting product of the incineration of total solids, expressed in weight percentage.

Frozen milk samples (-20ºC) were defrosted in a cooling chamber and then kept for 30 minutes at room temperature until analysis. Milk (10 ml) was pipetted into crucibles and dried in a laboratory oven for 24 hours to obtain total solids. After this time, crucibles were put into a muffle furnace for 2 hours at 550ºC. Finally, crucibles were stored in a desiccator with silica gel for around 30-45 minutes. The crucibles were then weighted and the ash content was calculated according to the following formula:

$$Ash\,(\%) = \frac{M - m}{p} \times 100$$

M = weight of the crucible + ash (once cooled).
m = weight of the crucible (empty).
p = weight (g) of 10 ml milk.

7.2.5. Determination of plasmin activity

Plasmin activity was measured following the method developed by Richardson and Pierce (1981), using as a substrate the peptide *N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amino-4-methyl coumarin*.

As described in chapter 5, plasmin is a serine proteinase with trypsin-like specificity, and cleaves polypeptides preferentially at *Lys-X* or *Arg-X* bonds. When the substrate is cleaved, the fluorophor *7-amino-4-methyl coumarin* is released (figure 7.6), and can be quantified with a fluorimeter at the wavelenghths of 380 nm (excitation) and 460 nm (emission).

1. Milk (3 ml) was added to 1 ml 0.4M trisodium citrate and mixed gently using a Stomacher™.
2. The mixture was centrifuged at 29000×g for 20 minutes at 4°C, and the supernatant was filtered through Whatman® No. 1 filter paper.
3. 825 μl 50mM Tris-HCl buffer (pH 7.5) were added to 50 μl filtrate, and the mixture was incubated for 5 minutes at room temperature.

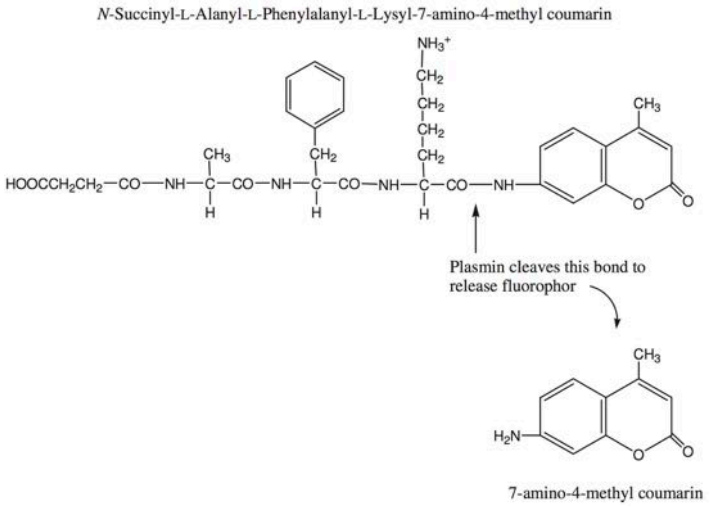


Figure 7.6: Action of plasmin over the substrate.

4. The reaction was initiated by adding 225 μl substrate (5 mg coumarin peptide in 1.33 ml dimethylsulphoxide and 5.33 ml 50mM tris-HCl buffer, pH 7.5).

5. From that moment on, the fluorescence intensity was measured at 5 minute intervals over a period of 35 minutes. For the samples graph, fluorescence units were plotted against time, and the slope of the curve was determined.

6. A standard curve was constructed using *7-amido-4-methyl coumarin (AMC)*. A standard solution of *AMC* was prepared by dissolving 13.4 mg *AMC* in 5 ml dimethyl sulphoxide. Once dissolved the solution was diluted to 250 ml with 0.05M Tris-HCl buffer pH 7.5, to give a concentration of 300×10^{-6}M *AMC*. The solution was protected from light and stored at 40ºC. Further dilution (1 in 100) with Tris buffer gave 300×10^{-8}M (solution A). This solution was diluted 1 in 10 with Tris buffer to give 300×10^{-9}M (solution B). Several dilutions of solution B were used to draw the standard curve by plotting a graph of fluorescence units against *AMC* concentration, checking it was linear.

The slope of the samples curve (determined in step 5) was plotted on the x-axis of the standard curve. It was extrapolated from this point across until it hit the line of the graph and then down to the y- axis. The y-axis value was then multiplied by the dilution factors to give a result in nmols *AMC* min^{-1} ml^{-1}. Plasmin activity was then determined, defining one unit of plasmin activity as the activity necessary to release 1 nmol *AMC* min^{-1} ml^{-1} milk under the conditions of the assay.

Dilution factors:
50 μL filtrate (1100/50).
3 ml milk + 1 ml trisodium citrate (40/30).

7.2.6. Urea polyacrylamide gel electrophoresis

7.2.6.1. Stock solutions

The chemicals described in section 6.3.6 were used to prepare the stock solutions.

1. **Acrylamide solution (40 %):** stored at 4ºC.
2. **Stacking gel buffer:**
   - 4.15 g Tris.
   - 150 g urea.
   - 2.2 ml concentrated HCl.

   Dissolved in, and made up to 500 ml with deionized water, adjusting the pH to 7.6 with 2N HCl.
3. **Separating gel buffer:**
   - 32.15 g Tris.
   - 192.85 g urea.
   - 2.86 ml concentrated HCl.

   Dissolved in and made up to 500 ml with deionized water, adjusting the pH to 8.9 with 2N HCl.
4. **Electrode buffer:**
   - 15 g Tris.
   - 73 g glycine.

   Dissolved in and made up to 5 litres with deionized water, adjusting The pH to 8.4 with HCl 2N. The resulting solution was stored at 4ºC.
5. **Sample buffer:**
   - 0.75 g Tris.
   - 49 g urea.
   - 0.4 ml concentrated HCl.
   - 0.7 ml 2-mercaptoethanol.
   - 0.15 g bromofenol blue.

   Dissolved in and made up to 100 ml with deionized water while heating slightly. As milk samples were liquid, a double strength sample buffer was prepared to compensate for the dilution. This double strength sample

buffer was prepared by doubling the concentration of all reagents, except for urea (due to its solubility).

6. **Ammonium persulfate:** diluted in deionized water (10 %). 1 ml aliquots were placed into *Eppendorfs* and frozen (-20 °C). One *Eppendorf* was removed for use on each occasion and then discarded.

7. **Staining solution:** 1M sulphuric acid was prepared by carefully adding 54 ml of concentrated acid to approximately 900 ml of deionized water. The solution was allowed to cool and diluted to 1 litre. Then, 2 g of coomassie brilliant blue G 250 were dissolved in 500 ml of water and also diluted to 1 litre. The acid solution was slowly added to the stain solution and hold overnight. The day after, the resulting solution was filtered through Whatman® No. 1 paper and 9 volumes of filtrate were added to 1 volume of 10M potassium hydroxide. Finally, solid trichloroacetic acid was added to this solution in a 5 litre plastic beaker, so that the final concentration of trichloroacetic acid was 12 %.

7.2.6.2. Gel solutions

The gel solutions were prepared just before pouring the gel, which was done the day before running.

1. **Stacking gel solution:**
   - 5 ml acrylamide solution.
   - 45 ml stacking gel buffer.
   - 0.1 g methylene bisacrylamide.

   The above are mixed together and filtered through Whatman® No. 113 filter paper. To the filtrate are added 25 μl of TEMED.

2. **Separating gel solution:**
   - 22.5 ml acrylamide solution.
   - 52.5 ml separating gel buffer.
   - 0.375 g methylene bisacrylamide.

   The above are mixed together and filtered through Whatman® No. 113 filter paper. To the filtrate are added 37.5 μl of TEMED.

3. **Sample preparation:** samples were dissolved in 1 ml sample buffer and heated for a few minutes.

7.2.6.3. Electrophoresis

The electrophoresis unit was assembled according to the manufacturer's instructions. Immediately before use, 282 μl ammonium persulphate solution

were added to the separating gel solution to initiate the polymerization. Two (1 mm thick) gels were then poured so that the level of the top of the gel was such to allow about 2.5 cm of stacking gel below the level of the bottom of the wells. A layer of water was then gently overlaid on top of the gel and the gel was allowed to stand until polymerization was complete (usually 40-60 minutes).

The layer of water was removed using filter paper and 300 μl ammonium persulphate were added to the stacking gel solution. This new solution was poured and a slot-former was placed in position, allowing polymerization (60 min). The slot-former was then removed and the gels were placed in the gel unit, which was filled to the correct level with electrode buffer.

The gels were pre-run at 280 V for 30-40 minutes, and the samples were applied, while the system was cooled by circulating cold water. Samples were run at 280 V through the stacking gel, and at 300 V through the separating gel until the tracking dye front was close to the bottom of the gel slab (4-5 hours).

In the end, the gels were stained by immersing in staining solution overnight, and destained in a few changes of distilled water until the background became clear.

7.3. Statistical processing

To evaluate the effects of lactation and parity (chapters 9 and 11), the statistical analysis performed was the GLM procedure of SAS 9.1. (*SAS Institute Inc.*, Cary, NC). Stage of lactation, parity and their interaction were considered as fixed effects. To compare least squares means of the studied variables, a contrast analysis was performed, declaring statistical significance at $P<0.05$.

To determine the effects of somatic cell count and udder health status (chapters 10 and 11), the GLM procedure of SAS 9.1 was also performed. In this case, somatic cell count range, animal group according to UHS and their interaction were considered as fixed effects. Likewise, a contrast analysis was performed to compare least squares means.

To study the influence of plasmin on milk (chapter 11), linear correlations were calculated between PL and milk composition, and also between PL and rennet coagulation variables.

Finally, to establish a predictive model, a discriminant function analysis was performed (chapter 12), to predict a categorical dependent variable (animal group according to UHS) by one or more independent predictor variables.

Part V

RESULTS AND DISCUSSION

Chapter 8

Descriptive statistics

CONTENTS

8.1. General descriptive data

The mean values obtained for composition, renneting properties and health of the udder are represented in table 8.1. Somatic cell count was normalized and converted to score ($\log_{10}$ SCC) so that all data was adjusted to a gaussian distribution. Hereafter, SCC score (SCCs) will be used in all statistical analysis performed in the following chapters.

Table 8.1: General descriptive data for all analyzed variables.

Variable	Max.	Min.	Mean	Std. Dev.
Fat (%)	13.78	1.75	6.87	2.55
Crude protein (%)	7.87	4.39	5.93	0.68
Total solids (%)	26.97	13.09	18.67	2.77
Lactose (%)	5.87	3.48	4.72	0.55
Casein (%)	6.53	3.73	4.86	0.52
Urea (mg/l)	751.00	177.00	403.89	103.83
Ash (%)	0.12	0.04	0.078	0.008
RCT (minutes)	54.45	8.00	21.96	9.14
k_{20} (minutes)	25.00	1.30	4.28	3.33
$r+k_{20}$	60.00	10.15	26.24	10.63
A_{30} (mm)	55.92	0.28	28.98	16.13
A_{60} (mm)	63.20	7.98	42.16	9.82
Curd yield (g)	4.19	1.51	2.76	0.55
Initial pH	7.68	6.41	6.70	0.17
SCCs	3.78	0.30	1.92	0.53
Plasmin act. (u/ml)	12.67	0.06	1.05	1.30

8.2. Milk composition

The average fat concentration in milk was 6.87 %. This value was slightly lower than others described for this breed (table 8.2), with percentages within 6.68 and 7.81 %.

Crude protein mean value was 5.93 %, very similar to the average obtained by Garzón (1996) and within the range established by the rest of authors. Casein percentage is in accordance with the results obtained in similar studies (Castillo et al., 2008; Jaramillo et al., 2008).

Lactose percentage was of 4.72, very close to the values obtained by Molina (1987), Molina and Gallego (1994) and Castillo et al. (2008), and higher than the value obtained by Jaramillo et al. (2008).

Table 8.2: Average compositional values in milk from Manchega.[1]

Authors	Fat	Protein	TS	Lactose	Casein
Molina (1987)	7.1	5.3	17.6	4.8	NT
Molina and Gallego (1994)	7.5	5.5	18	4.8	NT
Garzón (1996)	8.18	5.83	19.43	4.42	4.74
Castillo et al. (2008)	7.88	6.29	20.1	4.76	4.82
Jaramillo et al. (2008)	8.68	6.39	20.26	4.21	4.97
Ramírez et al. (2008)	7.69	6.2	18.14	NT	4.64
Arias (2009)	7.55	5.92	18.88	4.55	NT
ESROM (2014)	7.3	5.5	NT	NT	NT

NT = Not tested

Finally, total solids (TS) fit the values obtained by other authors for Manchega, with a very similar percentage to the proposed by Arias (2009).

8.3. Renneting parameters

Table 8.3 compiles the mean values for the renneting parameters described by several authors in sheep.

The average RCT was 21.96 minutes, similar to the mean values found by Bencini and O Agboola (2003) and Bianchi et al. (2004). Nevertheless, RCT is lower than the values found by Garzón (1996) and Caballero-Villalobos et al. (2015) in Manchega, or by Serrano (1999) and Toledo (2013) in some varieties of Merino.

Curd firming time (k_{20}) had a mean value of 4.28 minutes and is within the range described by the rest of authors, being very similar to the results obtained by Serrano (1999) and Toledo (2013) in Merino, though far from the values obtained by Pirisi et al. (2000), where k_{20} almost reached 11 minutes.

Mean values for curd firmness at 30 minutes (A_{30}) and curd firmness at 60 minutes (A_{60}) were 2.98 and 42.16 mm, respectively. The value for A_{30} is similar

[1] Source: own compilation.

to the found in Manchega by Garzón (1996) and is, in general, higher than in the rest of the revised articles. On the other hand, A_{60} is similar to the obtained by other authors (Serrano, 1999; Bencini and O Agboola, 2003; Bianchi et al., 2004). These values for curd firmness combined with the low curd firming and rennet clotting times seem to foretell that, generally, curds obtained after enzymatic coagulation of the samples will be firm and easily drained.

Table 8.3: Renneting parameters average values in ewe milk.[2]

Authors	**RCT**	$\mathbf{k_{20}}$	$\mathbf{A_{30}}$	$\mathbf{A_{60}}$
Garzón (1996)	39.52	2.4	30.82	60.65
Serrano (1999)	39.55	4.13	22.27	44.65
Pirisi et al. (2000)	27.41	10.59	23.57	NT
Bencini and O Agboola (2003)	25	1.8	39.7	42.6
Bianchi et al. (2004)	18.77	1.64	NT	43.28
Toledo (2013)	26.74	5.02	22.46	40.73
Caballero-Villalobos et al. (2015)	30.89	6.55	12.12	38.11

NT = Not tested

8.4. Hygienic and sanitary parameters

The average values for the hygienic and sanitary parameters found in literature for sheep milk are represented in table 8.4.

The initial pH mean was 6.70, within the range established by all authors appearing in table 8.4. However, the mean value for SCCs obtained in the present study was 1.92, far below the mean values found in other papers. In fact, the maximum individual value obtained for this parameter in the analyzed samples was 3.78, still below the means obtained by Sevi et al. (2004), Caroprese et al. (2007), Jaramillo et al. (2008) and Koutsouli et al. (2015), which were very similar.

The mean value for plasmin activity was 1.05 units/ml. Nevertheless, values obtained by other authors have not been reflected in table 8.4, due to the different methodologies used in most research works performed in dairy sheep (tables 5.1 and 5.2).

[2]Source: own compilation, based on Toledo (2013).

Table 8.4: Average values for initial pH and SCCs in ewe milk.[3]

Authors	Initial pH	SCCs
Albenzio et al. (2004)	6.75	NT
Sevi et al. (2004)	6.72	5.99
Caroprese et al. (2007)	6.67	5.42
Jaramillo et al. (2008)	6.72	5.23
Koutsouli et al. (2015)	6.62	5.26

NT = Not tested

None of the reviewed papers used the method described by Richardson and Pierce (1981) so, as it has been already detailed in section 5.5.1, it is difficult to numerically compare the values for plasmin activity in sheep, because of both the lack of bibliographic references for this specie and the great diversity of the chemical substrates used in the assay.

8.5. Effect of the udder

No significant differences were found between milk from left and right udder for any of the parameters that were analyzed (tables 8.5 and 8.6). Therefore, from now on, for statistical purposes the udder will be considered as a main biological unit.

Table 8.5: Average composition and sanitary values in left and right udder.

Variable	Left Udder	Right Udder
Fat (%)	6.81	6.92
Crude protein (%)	5.94	5.92
Total solids (%)	18.63	18.72
Lactose (%)	4.72	4.73
Casein (%)	4.86	4.85
Urea (mg/l)	405.45	402.35
Ash (%)	0.08	0.08
Initial pH	6.70	6.69
SCCs	1.91	1.92
Plasmin act. (u/ml)	1.04	1.06

[3]Source: own compilation.

Table 8.6: Average renneting values in left and right udder.

Variable	**Left Udder**	**Right Udder**
RCT (min)	22.54	21.39
k_{20} (min)	4.14	4.41
RCT+k_{20} (min)	26.68	25.80
A_{30} (mm)	28.42	29.53
A_{60} (mm)	41.89	42.42
Curd yield (g)	2.74	2.77

Chapter 9

Effect of stage of lactation and parity

CONTENTS

9.1. Data processing

The statistical analysis performed for the discussion of this chapter was the GLM procedure of SAS 9.1.(*SAS Institute Inc.*, Cary, NC), considering stage of lactation (SL), parity (PAR) and their interaction as fixed factors. The variables included in the analysis where composition parameters, renneting parameters and health of the udder parameters. Least square means were compared using a contrast analysis, and significance was declared at $P<0.05$.

9.2. Effect over milk composition

Table 9.1 shows the least squares means of milk composition variables as affected by stage of lactation and parity. Tables 9.2 and 9.3 represent the values of P obtained in the contrast analysis due to stage of lactation in primiparous and multiparous ewes, respectively.

9.2.1. Fat

Fat content increases gradually and significantly, reaching its highest values in late lactation. Other authors have also described this growth during the milking period (Pugliese et al., 2000; Bianchi et al., 2004; Kuchtík et al., 2008; Bousselmi and Othmane, 2015; Koutsouli et al., 2015).

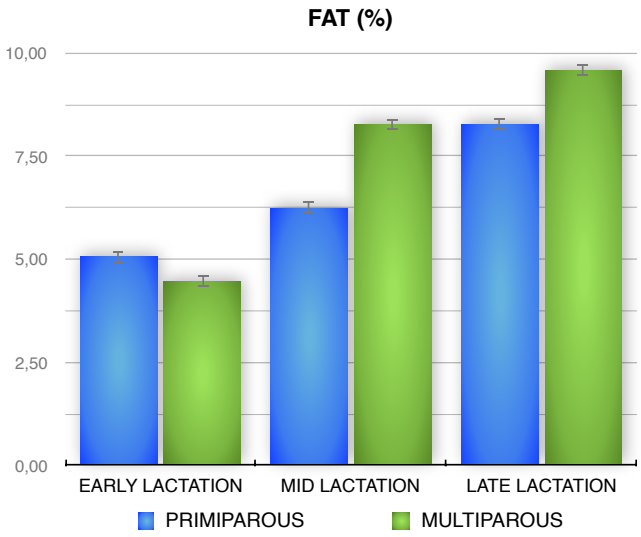


Figure 9.1: Fat content in milk throughout lactation.

Table 9.1: Milk composition (least square means) as affected by stage of lactation and parity.

	EARLY LACT		**MID LACT**		**LATE LACT**		**P**		
	PRIM	MULTI	PRIM	MULTI	PRIM	MULTI	SL	PAR	SL×PAR
Fat (%)	5.04	4.47	6.25	8.27	8.27	9.58	<.0001	0.0159	0.0146
Crude protein (%)	5.28	5.18	5.99	6.37	6.35	6.55	<.0001	0.1503	0.1938
Total solids (%)	16.57	15.85	17.93	19.75	20.27	21.60	<.0001	0.0424	0.0216
Lactose (%)	5.31	5.34	4.49	4.27	4.47	4.20	<.0001	0.006	0.0608
Casein (%)	4.50	4.42	4.72	5.04	5.16	5.30	<.0001	0.1943	0.2098
Urea (mg/l)	464.89	490.25	523.89	449.12	397.08	331.55	<.0001	0.0541	0.0716
Ash (%)	0.08	0.07	0.07	0.08	0.07	0.08	0.4184	0.0016	0.0133

LACT = lactation; PRIM = primiparous; MULTI = multiparous; SL = stage of lactation; PAR = parity.

No significant differences were found for milk fat content between primiparous and multiparous ewes in early lactation, though from mid lactation, fat percentage was significantly higher in multiparous. This increase was also described by Jaramillo et al. (2008) and Bousselmi and Othmane (2015), who found that fat content increases with parity. However, in studies performed by Serrano (1999) and, specifically, in Manchega by Garzón (1996), no significant effect of parity over fat content was observed. Sevi et al. (2000) proposed that the increase of body mass in ewes with more lactations involves a higher availability of body reserves used in the synthesis of milk components. In addition, the greater development of the mammary gland tissue and the synthesis ability of the udder in these animals could as well explain this increase in the fat content.

Table 9.2: Contrast analysis comparing milk composition means among different stages of lactation in primiparous ewes.

	EARLY×MID	**EARLY×LATE**	**MID×LATE**
Fat	0.127	<0.001	0.011
Protein	0.003	<0.001	0.123
Total solids	0.101	<0.001	0.006
Lactose	<0.001	<0.001	0.912
Casein	0.266	<0.001	0.031
Urea (mg/l)	0.156	0.104	0.003
Ash	0.670	0.253	0.403

Values colored in grey are not significant (P>0.05).

Table 9.3: Contrast analysis comparing milk composition means among different stages of lactation in multiparous ewes.

	EARLY×MID	**EARLY×LATE**	**MID×LATE**
Fat	<0.001	<0.001	0.009
Protein	<0.001	<0.001	0.208
Total solids	<0.001	<0.001	<0.001
Lactose	<0.001	<0.001	0.308
Casein	<0.001	<0.001	0.035
Urea (mg/l)	0.078	<0.001	<0.001
Ash	0.009	<0.001	0.105

Values colored in grey are not significant (P>0.05).

9.2.2. Crude protein

A significant increase in crude protein was observed in mid and late lactation, as described by other authors (Bianchi et al., 2004; Kuchtík et al., 2008; Jaramillo et al., 2008; Králícková et al., 2012; Bousselmi and Othmane, 2015). This result, disagrees with the protein values published by Koutsouli et al. (2015), which were significantly higher in early lactation.

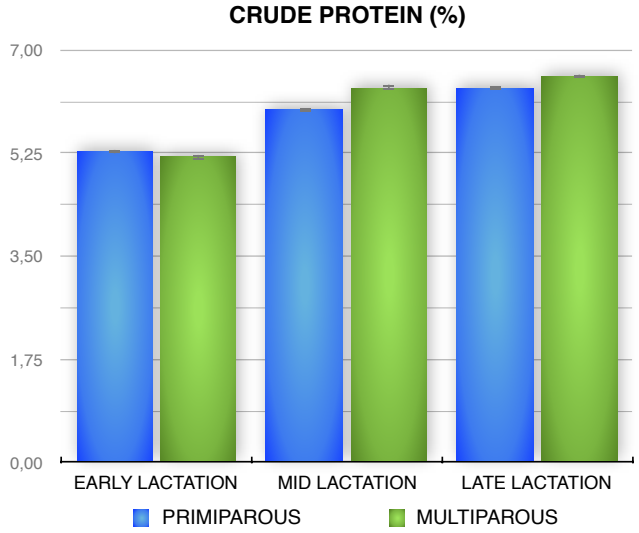


Figure 9.2: Crude protein in milk throughout lactation.

Even so, a higher concentration of protein in multiparous ewes was measured only in mid lactation (figure 9.2). Bousselmi and Othmane (2015), though, reported that, generally, milk from primiparous ewes has lower protein content. Most of the published results also indicate that protein content in milk increases throughout the ewe's productive life (Casoli et al., 1989; Pulina, 1990; Serrano, 1999; Sevi et al., 2000; Pugliese et al., 2000; Jaramillo, 2007).

9.2.3. Lactose

Lactose percentage was significantly higher in early lactation, decreasing in mid lactation from where it remained constant until the end of lactation (table 9.1). This agrees with Pavic et al. (2002) and Bianchi et al. (2004). Koutsouli et al. (2015) found the highest lactose content in mid lactation, from where it diminished significantly along late lactation.

Jenness (1985) proposed that the inverse relation between lactose content and fat and protein percentages might be due to the fact that lactose synthesis in the lactocyte represents the main regulator of water content in milk. Thus, when lactose increases, percentages of fat and protein are diluted. The significant decrease in lactose percentage observed after mid lactation would therefore explain the higher concentration of fat and protein obtained in the last months of lactation, which reaches its highest values in the days before the upcoming dry period.

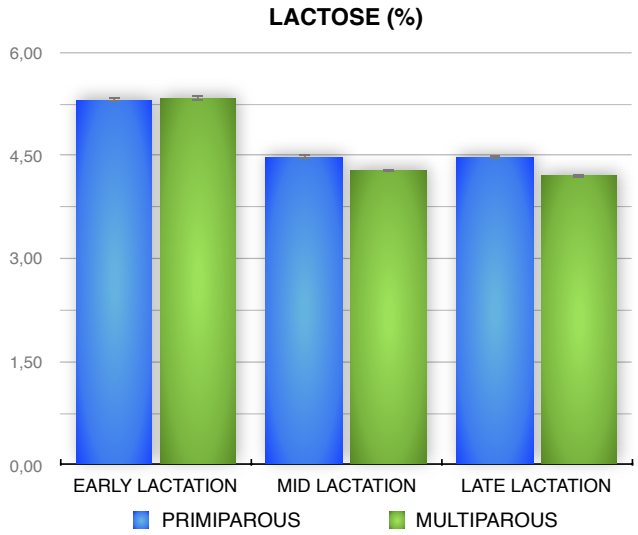


Figure 9.3: Lactose content in milk throughout lactation.

In a recent study, Kuchtík et al. (2008) found that lactose was the milk component that remained more constant during lactation, confirming its role as an osmotic regulator, balancing variations in other milk components.

Lactose content seems to be lower in multiparous ewes from mid to late lactation (figure 9.3), which agrees with the results published by Sevi et al. (2000) and Jaramillo et al. (2008). Other authors did not find changes in lactose due to parity (Králícková et al., 2012).

9.2.4. Total solids

Total solids increased significantly throughout lactation, reaching the highest levels in the last month (tables 9.2 and 9.3). Other authors found similar values (Casoli et al., 1989; Gonzalo et al., 1994; Kuchtík et al., 2008; Ochoa-Alfaro et al., 2009). Contrastingly, the evolution of total solids in the present study differs

from the results reported by Koutsouli et al. (2015), who found significantly lower values in mid and late lactation.

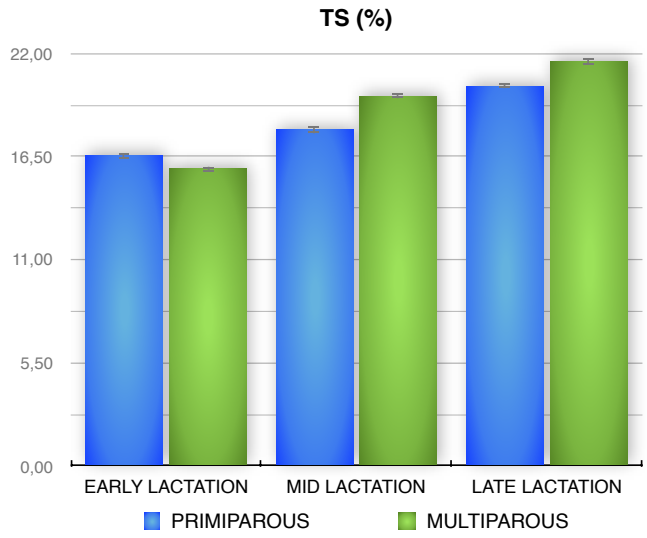


Figure 9.4: Total solids in milk throughout lactation.

Significant differences due to parity were only revealed in mid lactation, where the proportion of TS in multiparous ewes was higher. In addition, in late lactation, a statistical trend towards higher TS was observed in multiparous ewes (figure 9.4). These results agree with those of Bousselmi and Othmane (2015) but disagree with those of Králícková et al. (2012), who did not find an effect of parity over this parameter.

9.2.5. Casein

Regarding stage of lactation, different behaviors were observed in primiparous and multiparous ewes. In the first ones, values for casein content remained constant until mid lactation, and reached significantly higher values in late lactation (table 9.2). In the latter, conversely, casein content significantly increased gradually as lactation progressed (table 9.3). However, both trends are similar to those reported by Sevi et al. (2000), Bianchi et al. (2004), Pugliese et al. (2000) and Kuchtík et al. (2008), who described a progressive increase in casein content along lactation.

Parity did not seem to affect significantly casein content in milk, although in mid lactation, a trend towards higher casein values was revealed in multiparous ewes (figure 9.5).

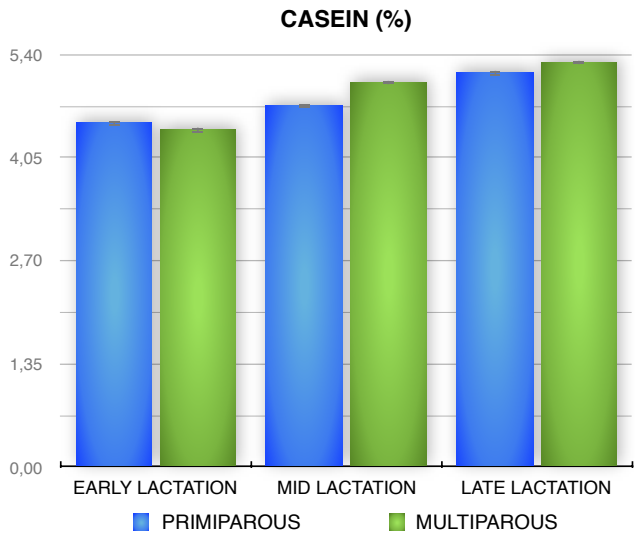


Figure 9.5: Casein content in milk throughout lactation.

9.2.6. Ash

While in primiparous ewes ash content slightly decreased with lactation (with no statistical significance), in multiparous ewes, ash content significantly increased from mid to late lactation. Ochoa-Alfaro et al. (2009) found a gradual decrease in ash, reaching the lowest values in the last week of lactation. These results agree with the trend observed in primiparous ewes.

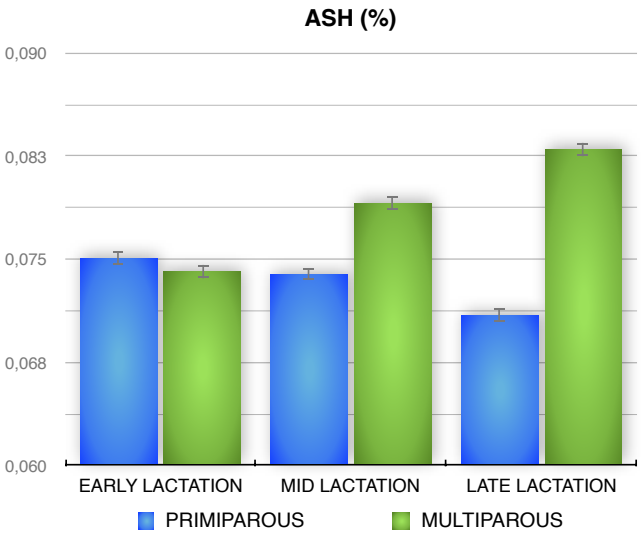


Figure 9.6: Ash content throughout lactation.

Except in early lactation (where all values were quite similar regardless of parity), ash content in milk was significantly higher in multiparous ewes.

9.2.7. Urea

Urea in primiparous ewes initially increased, reaching the highest concentration in mid lactation, where values were significantly higher than in late lactation. This partially agrees with the results obtained by Roy et al. (2003) and Bendelja et al. (2009), who also found the highest concentration of urea in mid lactation milk. However they could not prove that stage of lactation had a significant effect over this parameter.

In multiparous ewes, the proportion of urea in late lactation decreased significantly. These results suggest that milk urea concentration in ewes has an opposite behavior to that observed by other authors in dairy cows, where the concentration of urea seems to increase with stage of lactation (Carlsson et al., 1995; Marenjak et al., 2004).

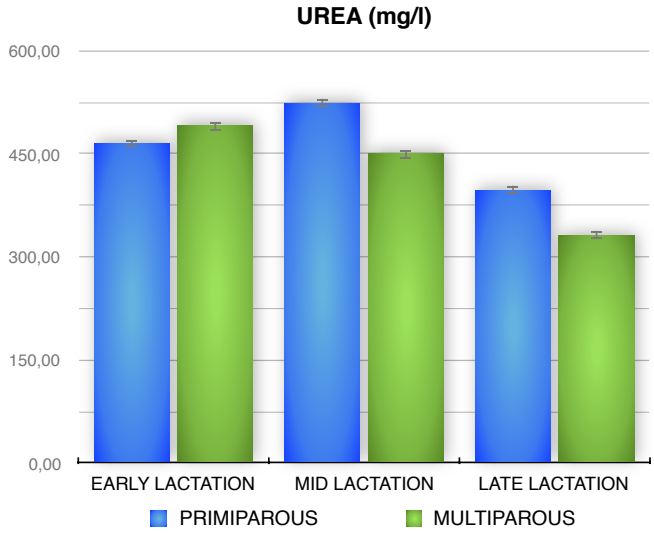


Figure 9.7: Milk urea concentration throughout lactation.

Parity also seemed to have an influence on urea concentration in ewe milk, as values were significantly higher in primiparous ewes, specially in mid lactation. Research performed in other species showed similar results, and a falling trend in milk urea concentration was found as parity increased (Broderick and Clayton, 1997; Roy et al., 2003).

SUMMARY

Stage of lactation (SL) produced an increase in fat, protein, total solids and casein, and a decrease in lactose and urea. Ash content increased in primiparous ewes and decreased in multiparous.

Parity (PAR) increased fat, total solids and ash, and decreased lactose and urea. There was no effect of PAR over protein or casein.

Factor	Fat	Prot	Lact	TS	Cas	Ash	Urea
SL	↑	↑	↓	↑	↑	↑P, ↓M	↓
PAR	↑	=	↓	↑	=	↑	↓

9.3. Effect over rennet coagulation

Table 9.4 represents the least squares means of milk renneting parameters as affected by stage of lactation (SL) and parity (PAR). Tables 9.5 and 9.6 reflect the values of P obtained in the contrast analysis for renetting parameters in primiparous and multiparous ewes.

9.3.1. Rennet clotting time (RCT)

RCT in primiparous ewes decreased in mid lactation, but increased right after, reaching the highest values in the last month of lactation (table 9.5). However, in multiparous ewes, RCT increased gradually with SL, although significant differences were revealed only between early and late lactation (table 9.6). These increasing values of RCT over the course of lactation (figura 9.8) agree with the results published by Garzón (1996) and Jaramillo et al. (2008) for Manchega ewes. Contrastingly, other studies performed with different ovine breeds found that RCT seemed to be shorter as lactation progressed.

Some authors have published mixed results: Caroprese et al. (2007) found that in mid lactation, RCT was shorter, and Pazzola et al. (2014) recorded the highest values for RCT in the same period. Bencini (2002) proposed that these contradictory results may lie in differences in the methodology used to monitorize rennet coagulation (see chapter 3.2.1), suggesting that the initial pH of milk may strongly affect this process.

Table 9.4: Milk renneting properties (least squares means) as affected by stage of lactation and parity.

	EARLY LACT		**MID LACT**		**LATE LACT**		**P**		
	PRIM	MULTI	PRIM	MULTI	PRIM	MULTI	SL	PAR	SL×PAR
RCT (min)	25.23	19.81	19.76	21.99	27.53	26.36	0.0495	0.4674	0.2675
k_{20} (min)	5.03	3.86	3.24	3.92	5.67	5.27	0.0743	0.649	0.4849
RCT+k_{20} (min)	30.25	23.68	23.01	25.99	33.20	31.34	0.0374	0.4528	0.2456
A_{30} (mm)	22.84	32.20	29.49	28.73	24.44	21.46	0.4101	0.62	0.3569
A_{60} (mm)	35.38	43.22	41.00	42.63	36.39	41.81	0.4601	0.0152	0.4191
Curd yield (g)	2.23	2.44	2.25	2.83	3.07	3.35	<,0001	0.0001	0.1754

LACT = lactation; PRIM = primiparous; MULTI = multiparous; SL = stage of lactation; PAR = parity.

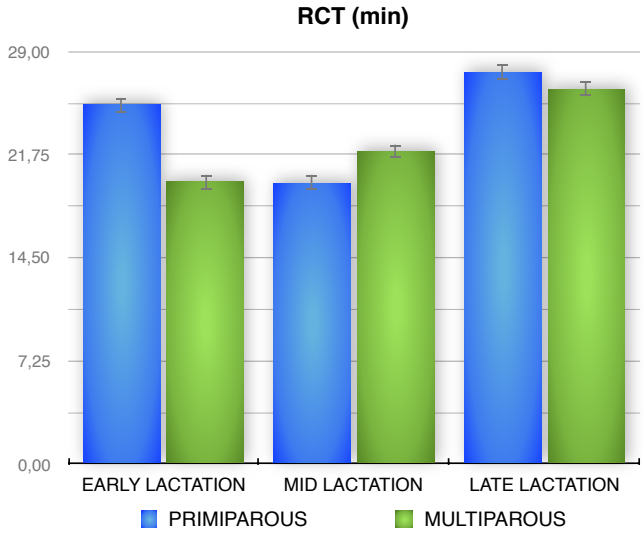


Figure 9.8: Rennet clotting time throughout lactation.

Table 9.5: Contrast analysis comparing means of the renneting parameters among different stages of lactation in primiparous ewes.

	EARLY×MID	**EARLY×LATE**	**MID×LATE**
RCT	0.182	0.584	0.067
k_{20}	0.179	0.636	0.077
A_{30}	0.391	0.841	0.527
A_{60}	0.172	0.811	0.276
Curd yield	0.951	<0.001	<0.001

Values colored in grey are not significant (P>0.05).

Table 9.6: Contrast analysis comparing means of the renneting parameters among different stages of lactation in multiparous ewes.

	EARLY×MID	**EARLY×LATE**	**MID×LATE**
RCT	0.352	0.016	0.107
k_{20}	0.934	0.116	0.141
A_{30}	0.428	0.035	0.154
A_{60}	0.803	0.602	0.965
Curd yield	<0.001	<0.001	<0.001

Values colored in grey are not significant (P>0.05).

Concerning parity, no differences in clotting time were found between milk from primiparous and multiparous ewes. This agrees with data published by Pellegrini et al. (1997) and Jaramillo et al. (2008). Pazzola et al. (2014) found that, in Sarda ewes, RCT seemed to be slightly shorter in primiparous ewes, and confirmed the results observed in dairy cows by Cipolat-Gotet et al. (2012).

9.3.2. Curd firming time (k_{20})

As well as RCT, curd firming time in primiparous ewes became shorter in mid lactation, increasing right after during late lactation (table 9.5). Meanwhile in multiparous ewes, values for k_{20} increased gradually with lactation (table 9.6). However, in both cases these differences were not statistically significant. These results agree with those obtained by Sevi et al. (2004), although other authors (Jaramillo et al., 2008; Albenzio et al., 2009; Abilleira et al., 2010) reported a fall in the values of this parameter along the course of lactation. Pazzola et al. (2014) found the highest curd firming time in mid lactation milk.

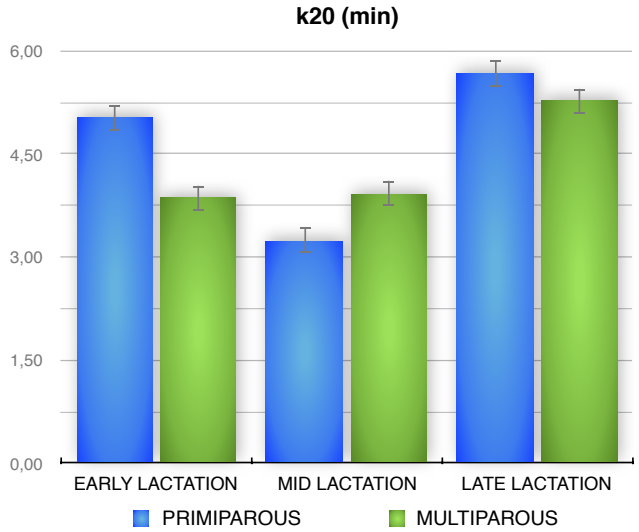


Figure 9.9: Curd firming time throughout lactation.

No significant differences in k_{20} were found due to parity (figure 9.9). These results agree with those of Pellegrini et al. (1997) and Jaramillo et al. (2008). This fact, combined with the trend in RCT, suggests that in dairy sheep, parity has no effect over rennet coagulation.

9.3.3. Curd firmness at 30 and 60 minutes (A_{30} and A_{60})

In primiparous ewes (table 9.5), the lowest values for A_{30} were obtained in early lactation milk. These values increased right after, reaching the highest in mid lactation and gently decreased in the end, with no significant differences. In multiparous ewes (table 9.6), A_{30} decreased with SL, revealing significantly lower values in late lactation than in the beginning. Meanwhile, values for A_{60} remained constant during the whole lactation.

A low firmness of the curd in early lactation milk has been described. The results in the present study agree with those obtained by Garzón (1996) and Caroprese et al. (2007). Other authors reported an progressive increase in curd firmness along lactation (Albenzio et al., 2009; Abilleira et al., 2010) . Only Jaramillo et al. (2008) and Koutsouli et al. (2015) found a decrease in A_{30} throughout time.

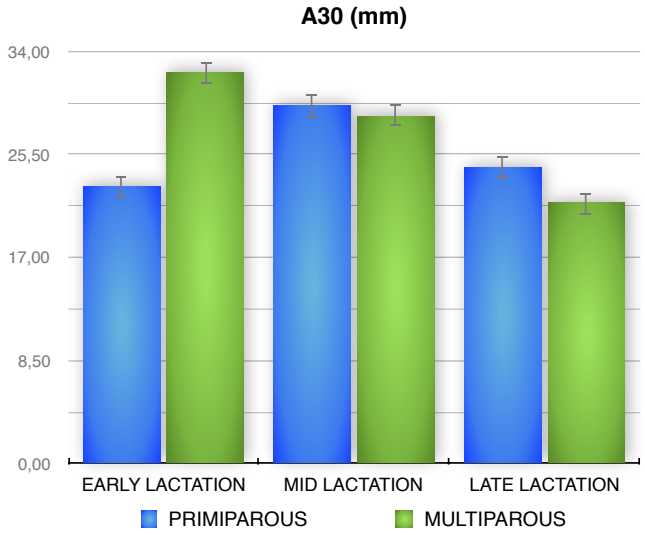


Figure 9.10: Curd firmness (at 30 minutes) throughout lactation.

No significant differences due to parity were revealed for A_{30} throughout lactation (figure 9.10). Conversely, in early lactation, A_{60} was significantly higher in multiparous ewes, although Pazzola et al. (2014) stated that PAR did not affect the firmness of the curd. Some authors obtained higher curd firmness with increasing parity (Sevi et al., 2000).

According to Jaramillo et al. (2008), the wide diversity of results reported for these parameters may be due to the influence that milk composition has over rennet coagulation (specially variables as fat and protein). Remeuf et al. (1991) and Pellegrini et al. (1997) proposed that other factors like the diameter of the

casein micelle or the mineral balance in milk (which may vary with SL), might also affect curd firmness.

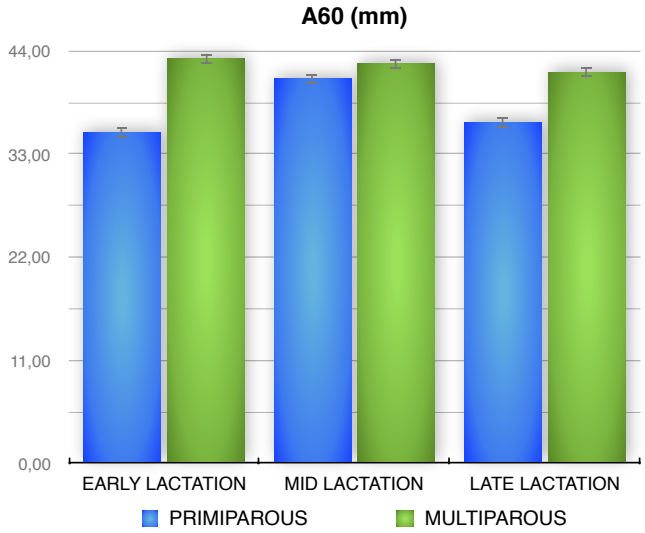


Figure 9.11: Curd firmness (at 60 minutes) throughout lactation.

9.3.4. Curd yield

In primiparous ewes, curd yield remained constant during lactation, increasing significantly in the end. In multiparous ewes, curd yield increased gradually and significantly along the whole lactation, reaching its maximum values in the last days. This is in accordance with the results obtained by Jaramillo et al. (2008) and Bousselmi and Othmane (2015), who found a significant increase of curd yield with SL. Several studies performed in other dairy species consider that this increase in curd yield is due to the increment of total solids reported with the advancement of lactation (Coulon et al., 1998; Fekadu et al., 2005) (see section 9.2.4).

Differences due to parity were only significant in mid lactation. These results agree with those of Bousselmi and Othmane (2015), who found an influence of PAR on curd yield, but could not establish a clear differential pattern in multiparous ewes. However, some authors like Jaramillo et al. (2008) did found that, in dairy sheep, curd yield seemed to increase with PAR.

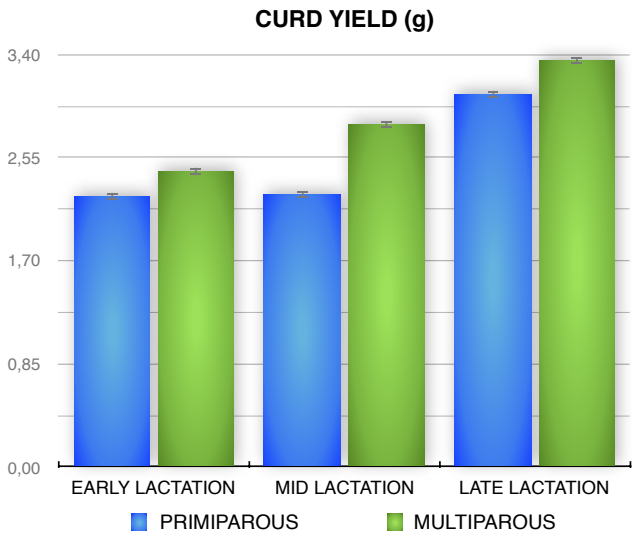


Figure 9.12: Curd yield throughout lactation.

SUMMARY

Stage of lactation (SL) increased rennet clotting time and curd yield, and decreased curd firmness after 30 minutes. Curd firming time and firmness after 60 minutes were not affected.

Parity (PAR) increased curd yield, but did not seem to have any other effect over rennet coagulation.

Factor	RCT	k_{20}	A_{30}	A_{60}	Curd yield
SL	↑	=	↓	=	↑
PAR	=	=	=	=	↑

9.4. Effect over udder health

Table 9.7 shows the mean values of the parameters associated to the health of the udder, according to stage of lactation and parity. Tables 9.8 and 9.9 compile the values of P obtained in the contrast analysis for these parameters in primiparous and multiparous ewes.

Table 9.7: Health of the udder (least squares means) as affected by stage of lactation and parity.

	EARLY LACT		**MID LACT**		**LATE LACT**		**P**		
	PRIM	MULTI	PRIM	MULTI	PRIM	MULTI	SL	PAR	SL×PAR
Initial pH	6.80	6.79	6.83	6.84	6.62	6.58	<.0001	0.6211	0.7207
SCCs	1.83	1.94	1.74	1.66	2.10	2.29	<.0001	0.4137	0.3895
Plasmin activity (u/ml)	0.76	1.69	0.71	1.08	0.46	0.83	0.3128	0.0764	0.6917

LACT = lactation; PRIM = primiparous; MULTI = multiparous; SL = stage of lactation; PAR = parity.

9.4.1. Initial pH

Inital pH of milk samples increased (but not significantly) in mid lactation and decreased significantly in the end, reaching the lowest value in late lactation, both in primiparous and multiparous ewes. However, the evolution of pH described by other authors is the opposite. Pugliese et al. (2000) and Kuchtík et al. (2008) found low pH in mid lactation, from were it increased significantly towards the end of lactation. Likewise, Pavic et al. (2002) and Sevi et al. (2004) observed an upward trend of pH with the advancement of SL. Finally, other authors did not find a significant effect of SL over pH (Ochoa-Alfaro et al., 2009).

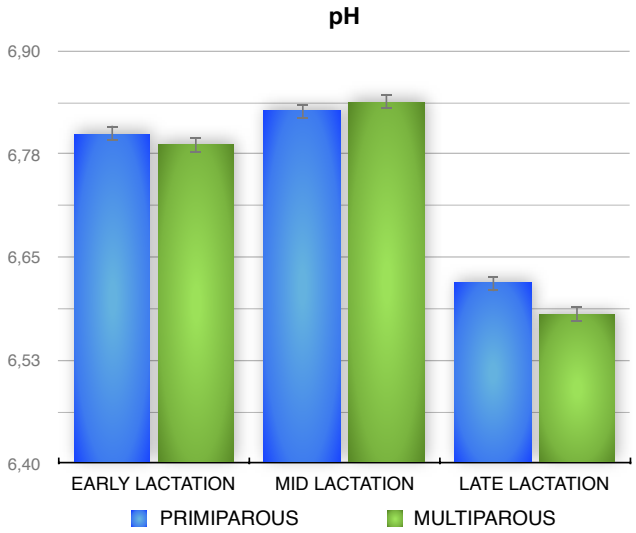


Figure 9.13: Initial pH throughout lactation.

Martini and Caroli (2003) concluded that initial pH of milk is strongly influenced by breed. This could explain the diversity in the pH evolution found in literature.

Concerning parity, Jaramillo et al. (2008) and Bousselmi and Othmane (2015) did not find any differences between primiparous and multiparous ewes. Nonetheless, other authors measured the lowest pH values in the second lactation, and observed that pH increased gradually with PAR (Garzón, 1996; Serrano, 1999; Toledo, 2013).

Table 9.8: Contrast analysis comparing means of milk hygienic and sanitary parameters among different stages of lactation in primiparous ewes.

	EARLY×MID	EARLY×LATE	MID×LATE
Initial pH	0.604	<0.001	<0.001
SCCs	0.587	0.124	0.039

Values colored in grey are not significant (P>0.05).

Table 9.9: Contrast analysis comparing means of milk hygienic and sanitary parameters among different stages of lactation in multiparous ewes.

	EARLY×MID	EARLY×LATE	MID×LATE
Initial pH	0.086	<0.001	<0.001
SCCs	0.006	0.001	<0.001

Values colored in grey are not significant (P>0.05).

9.4.2. Somatic cell count

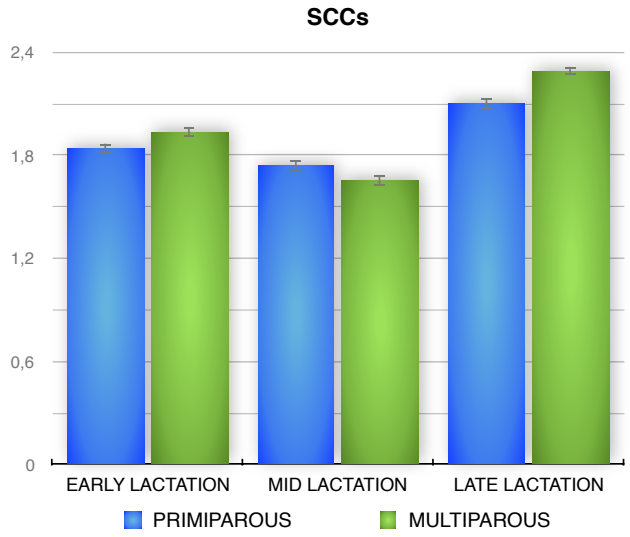


Figure 9.14: Somatic cell count score throughout lactation.

No significant changes in SSCs were found in primiparous ewes until late lactation, where they considerably increased. In multiparous ewes, SCCs diminished significantly with SL, but increased again, also significantly, in late lactation, where it reached the highest levels. This is in accordance with Koutsouli et al. (2015), who also observed a significant increasing trend only

in late lactation. However, other authors did not find an effect of SL over SCCs (Pugliese et al., 2000; Sevi et al., 2004).

No differences in SCCs between primiparous and multiparous ewes were observed, as described also by Sevi et al. (2000). Nevertheless, some authors found that PAR seems to have an effect on SCCs, being significantly higher in multiparous ewes (Králícková et al., 2012).

SUMMARY

Stage of lactation (SL) diminished the initial pH of milk and increased somatic cell count score.

Parity (PAR) did not affect health of the udder parameters in milk.

Factor	Initial pH	SCCs
SL	↓	↑
PAR	=	=

Chapter 10

Effect of somatic cells and udder health status

CONTENTS

10.1. Data processing

Based on the udder health status (UHS), animals were classified in three different categories: primiparous ewes (PR), multiparous ewes with no previous udder infection (M1) and multiparous ewes with a previous udder infection (M2). In addition, three ranges were established for SCCs: low SCCs (SCCs < 1.6), mid SCCs (1.6 < SRCS < 2.0) and high SCCs (SRCS > 2.0).

The statistical analysis used in this chapter was the GLM procedure of SAS 9.1 (*SAS Institute Inc.*, Cary, NC). The range of SCCs, the category according to UHS, and their interaction have been considered as fixed effects. The variables included in the analysis where milk composition parameters, renneting properties and pH. Least square means were compared using a contrast analysis, and significance was declared at P<0.05.

10.2. Effect over milk composition

Table 10.1 shows the least squares means of milk composition variables as affected by SCCs and UHS. Tables 10.2, 10.3 and 10.4 compare milk composition means between different SCCs ranges in PR, M1 and M2, respectively.

10.2.1. Fat

In PR (table 10.2) and M2 (table 10.4), fat content was significantly higher in high somatic cell count scores (HSCCs). Regarding M1 (table 10.3), fat was significantly lower in mid somatic cell count scores (MSCCs). Thus, the effect of SCCs over fat did not follow an apparent pattern. This agrees with Pirisi et al. (2000), (Albenzio et al., 2004) and Pérez-Ramos (2006), who did not found an impact of SCC over fat. On the other hand, Jaeggi et al. (2003), Bianchi et al. (2004) and Revilla et al. (2007) reported a lower fat concentration in high SCC milk, which could be caused by the reduced synthetic and secretory capacity of the mammary gland during mastitis (Raynal-Ljutovac et al., 2007).

UHS only seems to affect fat in low somatic cell count scores (LSCCs), where M1 presented the highest fat content, followed by M2 and then PR.

Table 10.1: Milk composition (least square means) as affected by SCCs and UHS.

	LOW SCCs			MID SSCs			HIGH SCCs			P		
	PR	**M1**	**M2**	**PR**	**M1**	**M2**	**PR**	**M1**	**M2**	**SCCs**	**UHS**	**SCCs×UHS**
Fat (%)	5.51	8.00	6.87	5.70	5.99	6.23	7.82	7.75	7.66	<.001	0.050	0.046
Protein (%)	5.72	6.17	6.10	5.69	5.78	5.73	6.09	6.03	6.02	0.002	0.250	0.225
TS (%)	17.36	19.93	18.77	17.42	17.83	17.96	19.73	19.48	19.41	<.001	0.082	0.061
Lactose (%)	4.93	4.48	4.67	4.84	4.98	5.00	4.64	4.54	4.54	<.001	0.230	0.019
Casein (%)	4.67	5.02	4.98	4.62	4.75	4.74	5.00	4.96	4.92	0.001	0.142	0.205
Ash	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.313	<.001	0.671
Urea (mg/l)	462.70	387.06	445.28	423.78	401.27	382.40	426.05	374.86	362.79	0.006	0.002	0.105
Cas/Prot	0.818	0.813	0.819	0.813	0.823	0.828	0.823	0.824	0.817	0.2738	0.5524	0.0449

PR = Primiparous ewes; M1 = Multiparous ewes with no previous udder infection; M2 = Multiparous ewes with a previous udder infection.
SCCs = Somatic cell count score; UHS = Category according to udder health status.

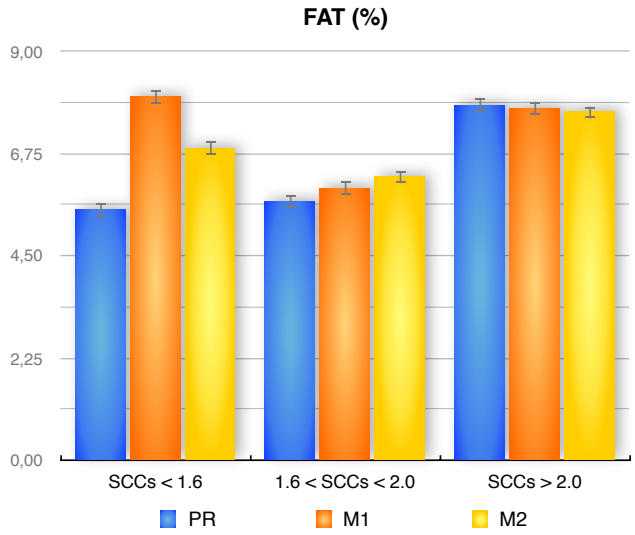


Figure 10.1: Fat content according to SCCs.

10.2.2. Crude protein

In PR, protein content was significantly higher in HSCCs. M1 presented significantly higher protein content in LSCCs than in MSCCs, but differed of values observed in HSCCs. In M2, protein content in MSCCs was significantly lower than in LSCCs and HSCCs.

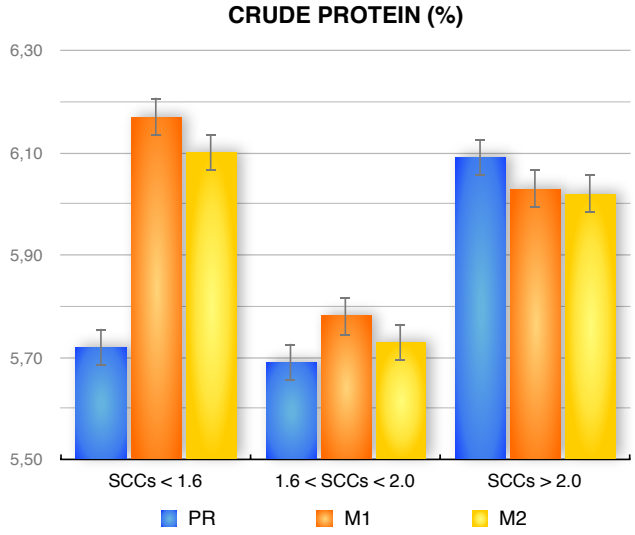


Figure 10.2: Crude protein according to SCCs.

The effects of SCC over protein in sheep milk are sometimes conflicting (Raynal-Ljutovac et al., 2007). Most authors did not find differences in the protein

content due to SCCs (Albenzio et al., 2005; Pérez-Ramos, 2006; Revilla et al., 2007). However, Nudda et al. (2003), Albenzio et al. (2004) and Bianchi et al. (2004) reported that protein concentration increased with SCC. In contrast, Jaeggi et al. (2003) found the lowest protein concentration in milk with the highest SCC levels.

Only in LSCC, PR presented a significantly lower protein than multiparous ewes (figure 10.2).

10.2.3. Lactose

No differences due to SCCs were found in lactose content in PR, though its values tend to decrease. In multiparous ewes (both M1 and M2), lactose was significantly lower in LSCCs and, especially, in HSCCs. Pirisi et al. (2000) and Revilla et al. (2007) found that, as SCC increased, the lactose content tend to decrease. Albenzio et al. (2004) and Leitner et al. (2004) reported a reduction of lactose in animals with SCC over 600×10^3 and 500×10^3 cells/ml, respectively.

The fact that no evident variation in lactose content was observed in PR from the present study, might be due to the low SCC measured in all samples, with average values below 300×10^3 cells/ml (as shown in table 8.1).

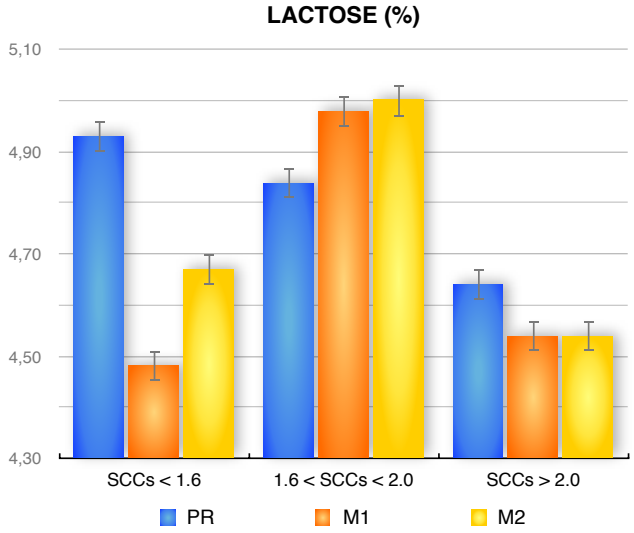


Figure 10.3: Lactose content according to SCCs.

Some authors associated the negative effect of SCC over lactose (mostly during late lactation) with a reduction of the synthesis capability of the mammary gland

Table 10.2: Contrast analysis comparing milk composition means among different SCCs ranges in PR.

	LOW×MID	LOW×HIGH	MID×HIGH
Fat	0.746	0.001	0.001
Protein	0.834	0.051	0.025
Lactose	0.469	0.052	0.159
Total solids	0.935	0.002	0.001
Casein	0.687	0.023	0.006
Ash	0.388	0.389	0.101
Urea	0.106	0.196	0.932

Values colored in grey are not significant (P>0.05).

Table 10.3: Contrast analysis comparing milk composition means among different SCCs ranges in M1.

	LOW×MID	LOW×HIGH	MID×HIGH
Fat	0.003	0.688	0.010
Protein	0.031	0.386	0.185
Lactose	0.001	0.665	0.003
Total solids	0.004	0.507	0.028
Casein	0.055	0.683	0.133
Ash	0.739	0.781	0.585
Urea	0.604	0.630	0.348

Values colored in grey are not significant (P>0.05).

Table 10.4: Contrast analysis comparing milk composition means among different SCCs ranges in M2.

	LOW×MID	LOW×HIGH	MID×HIGH
Fat	0.182	0.078	0.002
Protein	0.005	0.521	0.021
Lactose	0.001	0.183	<0.001
Total solids	0.132	0.185	0.004
Casein	0.014	0.465	0.060
Ash	0.302	0.314	0.929
Urea	0.002	<0.001	0.300

Values colored in grey are not significant (P>0.05).

due to the damage on the epithelial tissue (Bianchi et al., 2004). This reduction of the secretory activity seems to induce a progressive replacement of lactose by other active osmotic compounds, such as chlorides (Albenzio et al., 2004). Some researchers found that, after the rupture of the epithelial barrier of the mammary gland, lactose passes to blood. Thus, high concentrations of lactose have been reported in blood and urine of mastitic cows (Pérez-Ramos, 2006). However, other authors also suggested that the cause of a lactose drop in high SCC milk, may be a lower glucose availability due to the energy competition between secretory cells and cells with phagocytic functions (Martí De Olives et al., 2013).

Regarding UHS, significant differences were only found in LSCCs, where lactose percentage in PR was higher than in multiparous (M1 and M2).

10.2.4. Total solids

In PR and M2, total solids were significantly higher with HSCCs (tables 10.2 and 10.4). In M1, the TS values were significantly lower with MSCCs (table 10.3). These results differ from those obtained by Pirisi et al. (2000) and Pérez-Ramos (2006), who did not found that SCC affected total solids. However, other authors did report some effect of SCC over this variable. Jaeggi et al. (2003) measured the lowest TS values in high SCC milks. Revilla et al. (2007) also reported some effects of SCC over TS, though it followed different patterns that seemed to depend mainly on the breed.

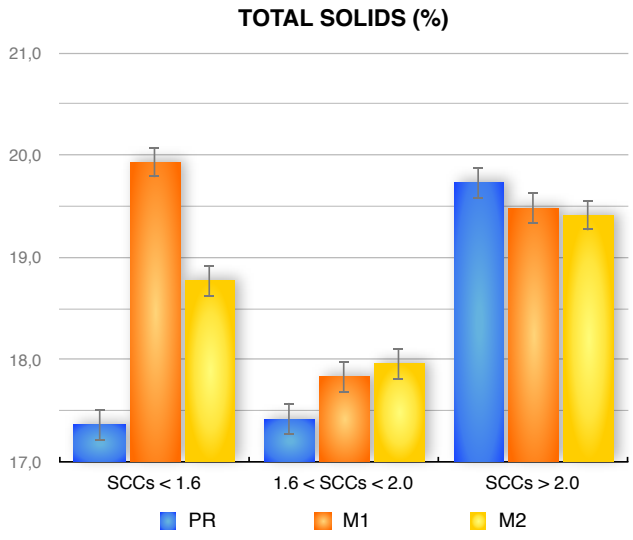


Figure 10.4: Total solids (TS) according to SCCs.

Based on UHS, only in LSCCs previous udder infections seemed to have a significant impact on total solids: M1 presented the highest TS content, followed by M2 and, finally, PR.

10.2.5. Casein

In PR with HSCCs, casein was significantly higher than in the rest of SCC levels. M1 and M2 presented a lower casein content in MSCCs, although values were quite similar to those found in HSCCs. Generally, studies concerning caseins report conflicting results. Some authors as Duranti and Casoli (1991) or Summer et al. (2012) reported a decrease in casein percentage as SCC increased. This could be related to an increase of plasmin activity caused by high SCC, which would produce a greater level of β-CN hydrolysis and, consequently, a decrease in crude casein. Similar patterns were found in sheep Jaeggi et al. (2003) and cow milk Politis and Ng Kwai Hang (1989). Contrastingly, other authors reported an opposite trend: Bianchi et al. (2004) measured a higher casein content as SCC increased.

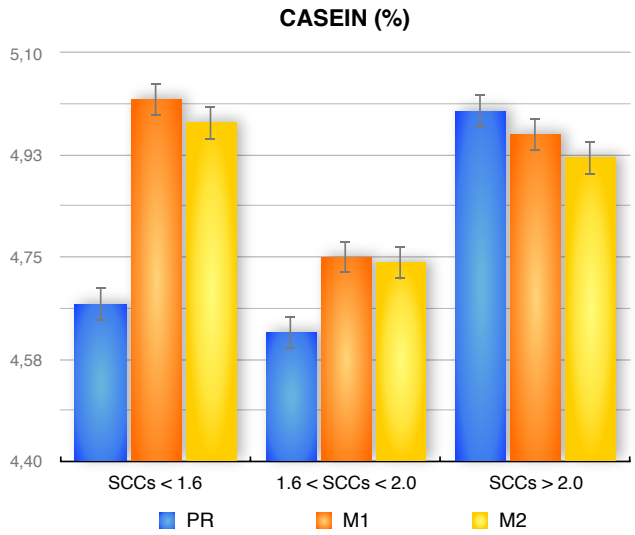


Figure 10.5: Casein content according to SCCs.

Finally, several authors did not find a significant impact of SCC over casein content (Pirisi et al., 1996, 2000; Pellegrini et al., 1997; Nudda et al., 2003; Albenzio et al., 2004, 2005). However, some researchers studied the casein/protein ratio instead of the casein content, as it does not depend on the volume of milk produced. In most cases, this ratio decreased in milk with high SCC. Nevertheless, in the present study no significant effect of SCCs over

the casein/protein ratio were found (table 10.1).

Only in LSCCs, casein content was significantly lower in PR than in multiparous (M1 and M2). This agrees with Jaramillo et al. (2008), who found that casein percentage increased with parity. In addition, Bianchi et al. (2004) reported lower casein content in healthy udders than in infected udders, what could explain the significantly lower values measured in PR.

10.2.6. Ash

Ash content decreased in PR with HSCCs. There were no difference due to SCCs in M1 and M2. Even though references in literature associating SCC with this variable are scarce, Revilla et al. (2007) reported no significant effect of somatic cells on the ash content.

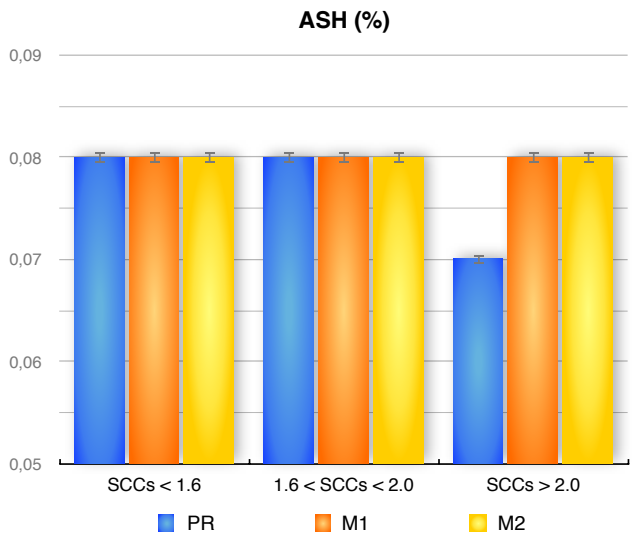


Figure 10.6: Ash content according to SCCs.

Furthermore, in milk from other species, Ahmad et al. (2012) did not found changes in ash due to parity.

10.2.7. Urea

No significant differences due to SCCs were found for PR and M1, which agrees with Pirisi et al. (2000) y (Pérez-Ramos, 2006). However, in M2 milk urea concentration decreased as SCCs rose.

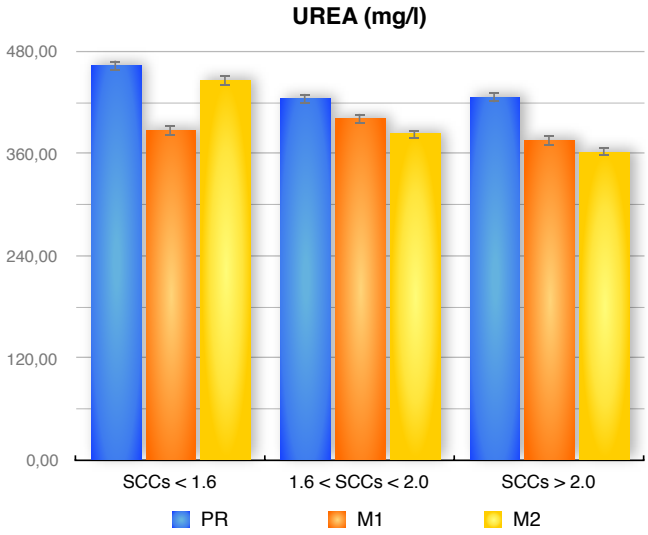


Figure 10.7: Milk urea concentration according to SCCs.

According to UHS, milk from M1 presented a higher concentration of urea with LSCCs. With HSCCs, urea was significantly higher in PR than in multiparous, what seems to agree with the results obtained in chapter 9.2.7. Either way, in experiments performed on dairy cows, no significant impact of parity or SCC over urea milk concentration was reported (Henao-Velásquez et al., 2014).

SUMMARY

In low SCCs: Urea decreases in M2. Crude protein and lactose concentration are higher in M1 and M2 than in PR. The highest fat and TS concentrations are found in M1, followed by M2 and, finally, PR. Casein content is higher in PR than in multiparous ewes. Urea milk concentration is lower in M1 than in the rest of UHS categories.

In mid SCCs: Fat diminishes in M1. Protein diminishes in M2. Lactose concentration increases and casein content decreases in M1 and M2. Total solids are lower in M1.

In high SCCs: Fat and total solids increase in PR and M2. In PR, protein and casein increase and ash content decreases. Urea milk concentration is higher in PR than in the rest of UHS categories.

10.3. Effect over renneting properties

Table 10.5 shows the least squares means of renneting parameters, as affected by SCCs and udder health status. Tables 10.6, 10.7 and 10.8 compare renneting parameters means between different SCCs ranges in PR, M1 and M2, respectively.

10.3.1. Rennet clotting time (RCT)

RCT remained constant regardless of SCCs. This agrees with the results obtained by Revilla et al. (2009), but differs from the results obtained by Duranti and Casoli (1991), Pirisi et al. (2000), Albenzio et al. (2004, 2005) and Giaccone et al. (2005), who reported that RCT increased as somatic cell counts were higher. Jaeggi et al. (2003) suggested that the increase of RCT in milk with high SCC was probably an effect of the enhanced proteolysis. However, the lack of effects of somatic counts over RCT in the present study, could be explained by the good health conditions of the sampled animals.

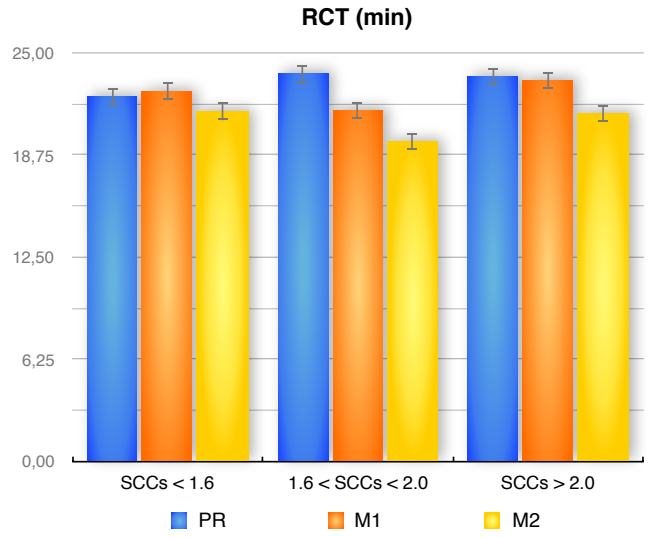


Figure 10.8: Rennet clotting time according to SCCs.

Concerning UHS categories, in MSCCs rennet clotting time was significantly higher in PR than in M2, but quite similar to values found in M1.

Table 10.5: Renneting parameters (least squares means) as affected by SCCs and udder health status.

	LOW SCCs			MID SCCs			HIGH SCCs			P		
	PR	**M1**	**M2**	**PR**	**M1**	**M2**	**PR**	**M1**	**M2**	**SCCs**	**UHS**	**SCCs×UHS**
RCT (min)	22.32	22.66	21.44	23.68	21.52	19.55	23.57	23.33	21.30	0.697	0.124	0.855
k_{20} (min)	3.95	3.87	3.74	4.26	3.33	3.98	5.37	5.04	5.00	0.011	0.680	0.955
RCT+k_{20}	26.26	26.53	25.18	27.94	24.85	23.53	28.94	28.37	26.30	0.274	0.169	0.877
A_{30} (mm)	27.89	27.08	31.58	25.68	31.12	32.65	27.96	26.06	29.05	0.672	0.163	0.737
A_{60} (mm)	39.75	45.22	45.10	36.73	44.75	43.06	38.50	41.61	43.03	0.197	<.001	0.775
Curd yield (g)	2.45	2.91	2.79	2.50	2.76	2.67	2.78	2.94	2.94	0.008	<.001	0.614

PR = Primiparous ewes; M1 = Multiparous ewes with no previous udder infection; M2 = Multiparous ewes with a previous udder infection.
SCCs = Somatic cell count score; UHS = Category according to udder health status.

10.3.2. Curd firming time (k_{20})

Curd firming time remained more or less constant but tended to be higher with HSCCs, regardless of previous udder infections. This agrees with the results obtained by Pirisi et al. (2000), Pirisi et al. (1996, 2000), Pellegrini et al. (1997), Nudda et al. (2001) and Albenzio et al. (2004, 2005).

Additionaly, previous udder health status did not affect k_{20}.

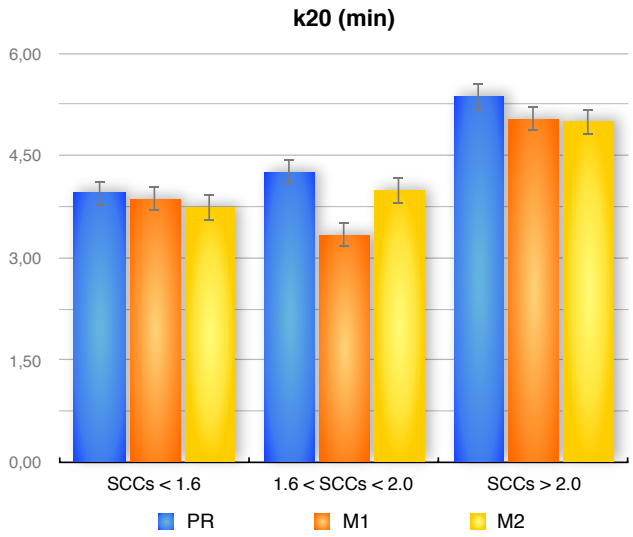


Figure 10.9: Curd firming time according to SCCs.

10.3.3. Curd firmness at 30 and 60 minutes (A_{30} and A_{60})

In M1 and M2, no significant impact of SCCs over A_{30} was found, although it seemed to slightly increase in MSCCs. Contrastingly, in PR, values for A_{30} were similar regardless of SCCs, but gently decreased in MSCCs, though not significantly. Regarding UHS, significant differences in A_{30} were only evident with MSCCs, when values in M2 were higher than in PR. Contrastingly, in multiparous ewes (M1 and M2), A_{60} seemed to decrease as SCCs was higher. In PR, the lowest values were found with MSCCs. This agrees, in part, with (Pirisi et al., 2000), who reported a decrease in curd firmness as SCC were higher.

No differences between UHS categories were found in A_{30} according to SCCs. However, values for A_{60} in PR were significantly lower than in the remaining categories with LSCCs and MSCCs.

Table 10.6: Contrast analysis comparing renneting parameters means among different SCCs ranges in PR.

	LOW×MID	**LOW×HIGH**	**MID×HIGH**
RCT	0.538	0.636	0.965
k_{20}	0.695	0.139	0.224
RCT+k_{20}	0.515	0.385	0.733
A_{30}	0.572	0.988	0.607
A_{60}	0.190	0.649	0.498
Curd yield	0.655	0.033	0.063

Values colored in grey are not significant (P>0.05).

Table 10.7: Contrast analysis comparing renneting parameters means among different SCCs ranges in M1.

	LOW×MID	**LOW×HIGH**	**MID×HIGH**
RCT	0.653	0.773	0.485
k_{20}	0.553	0.167	0.069
RCT+k_{20}	0.568	0.497	0.243
A_{30}	0.364	0.803	0.268
A_{60}	0.855	0.137	0.245
Curd yield	0.304	0.826	0.231

Values colored in grey are not significant (P>0.05).

Table 10.8: Contrast analysis comparing renneting parameters means among different SCCs ranges in M2.

	LOW×MID	**LOW×HIGH**	**MID×HIGH**
RCT	0.329	0.939	0.348
k_{20}	0.730	0.060	0.132
RCT+k_{20}	0.464	0.601	0.202
A_{30}	0.755	0.435	0.274
A_{60}	0.309	0.279	0.989
Curd yield	0.278	0.161	0.012

Values colored in grey are not significant (P>0.05).

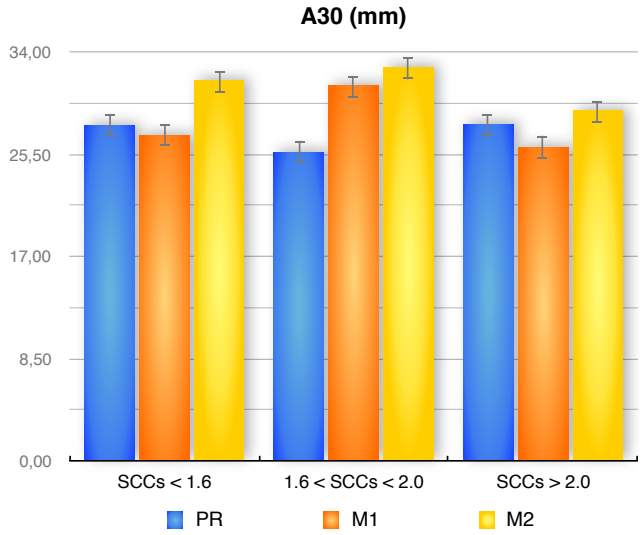


Figure 10.10: Curd firmness at 30 minutes according to SCCs.

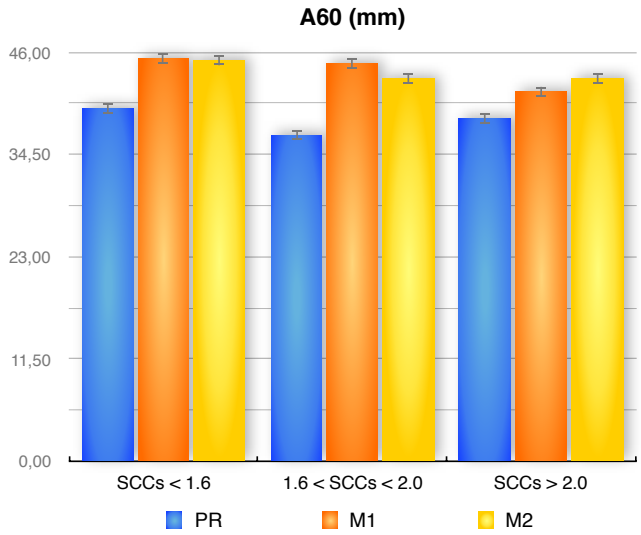


Figure 10.11: Curd firmness at 60 minutes according to SCCs.

10.3.4. Curd yield

Curd yield after draining was quite similar in all groups regardless of SCCs. Nevertheless, HSCCs had an increasing effect on curd yield in PR and M2, but only in the latter it was significant. Galina et al. (1996) and, afterwards, Revilla et al. (2009) established that SCC did not affect curd yield, what fairly supports the results obtained in this thesis. However, other authors did find a greater loss of protein in whey with high somatic cell counts. Similar results were reported in dairy cows by Barbano et al. (1991) and Rogers and Mitchell (1994). Consequently, milk with low SCC seems to be more suitable for cheesemaking, as it is possible to recover up to 4 % more protein in curd than with milk with high somatic cell counts (Pirisi et al., 2000).

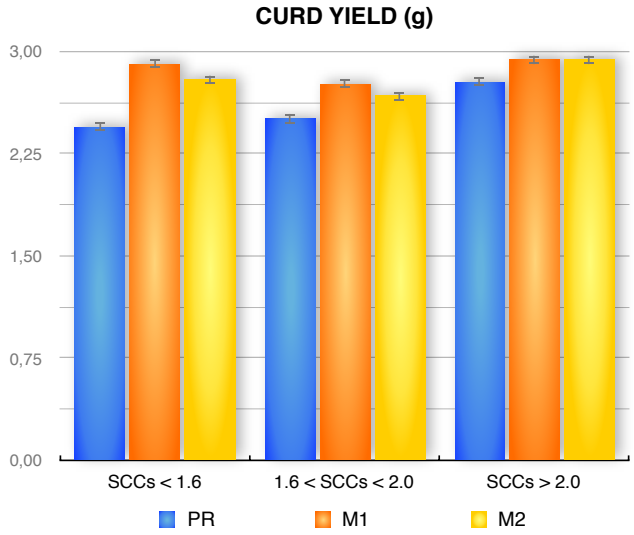


Figure 10.12: Curd yield according to SCCs.

Rogers and Mitchell (1994) reported that, in milk with high SCC, an increase of moisture in the curd might distort yield calculation, and higher values than expected may be measured. This was confirmed by Jaeggi et al. (2003) and Albenzio et al. (2004), who found an increase of the curd yield caused by an insufficient draining in cheeses made from high somatic cell count milk. In addition, Raynal-Ljutovac et al. (2007) suggested that this impairment in whey draining may induce a subsequent increase of proteolysis and lipolysis in cheese.

According to the udder health status category, only with LSCCs the curd yield in PR was significantly lower than in M1 and M2.

SUMMARY

As SCCs rises, curd firming time increases, and curd firmness at 60 minutes decreases. Rennet clotting time, curd firmness at 30 minutes and curd yield were not affected by this parameter.

Previous lesions of the udder do not clearly affect milk renneting properties, although A_{60} seems to be lower in PR than in M1 and M2.

10.4. Effect over initial pH

Table 10.9 shows the least squares means values of pH as affected by SCCs and UHS. Table 10.10 compares initial pH means between different SCCs ranges.

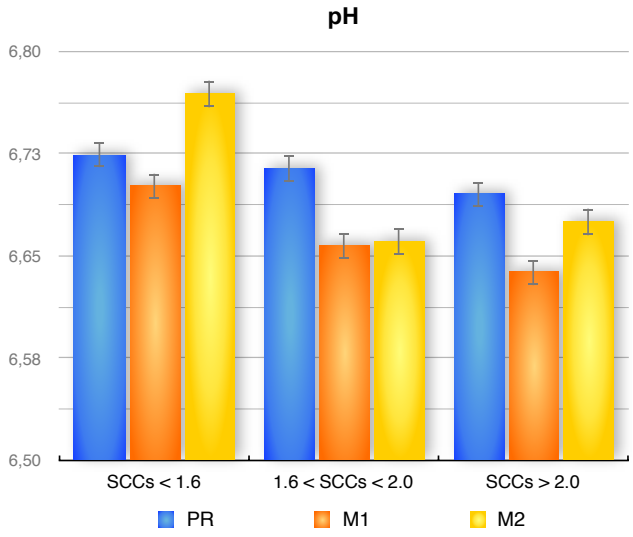


Figure 10.13: Initial pH according to SCCs.

In PR and M1, pH was not affected by SCCs. Meanwhile, in M2, pH was significantly higher with LSCCs. These results are opposite to those found by most authors, who usually reported an increase of pH as SCCs were higher (Pirisi et al., 2000; Nudda et al., 2001; Albenzio et al., 2004; Bianchi et al., 2004; Giaccone et al., 2005; Raynal-Ljutovac et al., 2007). However, as previously stated, the good sanitary conditions of the animals of the present study might explain the inconsistency of pH values when compared with other results found in literature.

Table 10.9: Initial pH (least squares means) as affected by SCCs and UHS.

	LOW SCCs			MID SCCs			HIGH SCCs			P		
	PR	**M1**	**M2**	**PR**	**M1**	**M2**	**PR**	**M1**	**M2**	**SRCS**	**G**	**SRCS × G**
Initial pH	6.72	6.70	6.77	6.71	6.66	6.66	6.70	6.64	6.67	0.017	0.178	0.441

PR = Primiparous ewes; M1 = Multiparous ewes with no previous udder infection; M2 = Multiparous ewes with a previous udder infection.
SCCs = Somatic cell count score; UHS = Category according to udder health status.

Table 10.10: Contrast analysis comparing initial pH means among different SCCs ranges.

	LOW×MID	**LOW × HIGH**	**MID × HIGH**
PR	0.806	0.549	0.680
M1	0.349	0.143	0.683
M2	0.002	0.003	0.652

Values colored in grey are not significant (P>0.05).

Concerning the udder health status category, with LSCCs milk from M2 tend to present higher pH values than the remaining categories, although differences were not statistically significant. No further differences were found in the rest of SCCs ranges.

SUMMARY

SCCs only affects M2, which present higher pH values in LSCCs.

UHS does not seem to affect the initial pH of milk

Chapter 11

Plasmin activity

CONTENTS

11.1. Verification of the plasmin activity assay

As explained in chapter 7, to verify the plasmin activity assay, Urea-PAGE was performed. Samples with the highest and the lowest plasmin activity values recorded according to the method developed by Richardson and Pierce (1981) were selected for this purpose. After performing electrophoresis and the subsequent staining of the gels, differences between high and low plasmin activity samples were clearly evidenced (figure 12.1). In the latters, a thickening of the γ-CN bands in the top of the gel can be observed, due to the proteolytic action of plasmin over β-CN. Similarly, in the bottom part of the gel, samples with high plasmin activity showed multiple diffuse bands of proteose-peptones as a result of proteolysis.

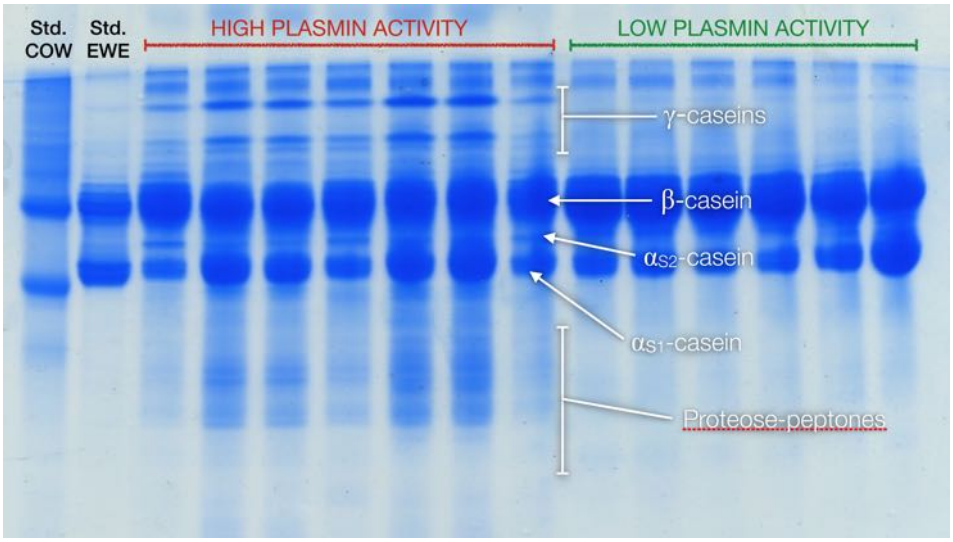


Figure 11.1: Electrophoresis of high and low plasmin activity milk samples.

11.2. Effect of stage of lactation and parity over plasmin

Figure 11.2 represents the global average of plasmin activity throughout lactation. As lactation advanced, plasmin activity in the flock decreased, and was significantly lower in the last month of lactation than in the beginning.

Considering 3 stages in lactation (early, mid, and late) and after performing the GLM procedure (table 11.1) and contrast analysis (table 11.2), a gradual

Table 11.1: Plasmin activity (least squares means) according to stage of lactation and parity.

	EARLY LACT		**MID LACT**		**LATE LACT**		**P**		
	PRIM	MULTI	PRIM	MULTI	PRIM	MULTI	SL	PAR	SL×PAR
Plasmin activity (u/ml)	0.76	1.69	0.71	1.08	0.46	0.83	0.3128	0.0764	0.6917

LACT = lactation; PRIM = primiparous; MULTI = multiparous; SL = stage of lactation; PAR = parity.

decrease in plasmin activity with the course of lactation was observed in all animals. The lowest values of plasmin activity were found in late lactation, although this drop was only significant in multiparous ewes (table 11.2).

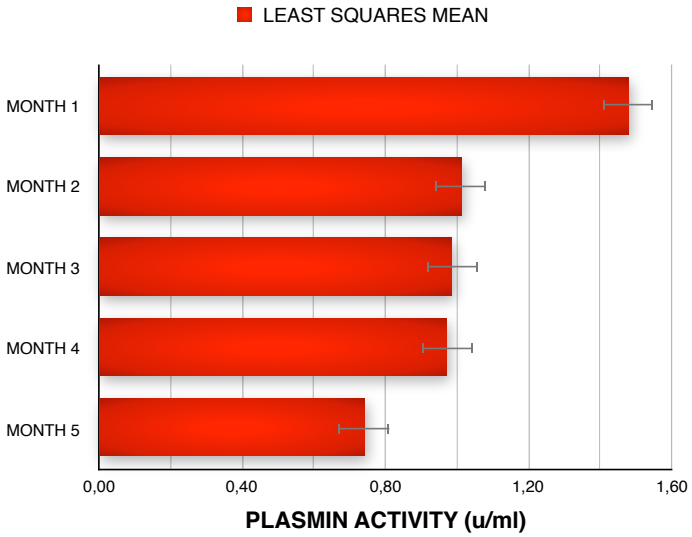


Figure 11.2: Plasmin activity global means during lactation.

Table 11.2: Contrast analysis comparing plasmin activity among different stages of lactation.

	Early×Mid	**Early×Late**	**Mid×Late**
Primiparous	0.935	0.640	0.699
Multiparous	0.097	0.036	0.545

Values colored in grey are not significant (P>0.05).

Although no big differences were found, the high plasmin activity recorded at the beginning of lactation, as well as its decreasing trend, agree with the results published by Albenzio et al. (2004, 2005, 2009), Theodorou et al. (2007), Caroprese et al. (2007) and Koutsouli et al. (2015), who measured the lowest values in late lactation. Contrastingly, several studies performed on bovine milk (Bastian and Brown, 1996; Ismail and Nielsen, 2010) and ewe milk (Bianchi et al., 2004; Sevi et al., 2004) reported a different pattern in plasmin activity, where the highest values were generally recorded in the end of lactation. Richardson (1983) suggested that the increase of plasmin activity observed in dairy cows during late lactation is due to a higher amount of plasmin entering the mammary gland, rather than to an increase of the activation rate of plasminogen to plasmin. In any case, Theodorou et al. (2007) found that the

activation of plasminogen to plasmin seems to be more effective in ovine than in bovine. Other authors relate the increase of plasmin in late lactation with the involution of the udder (Koutsouli et al., 2015) or with an increase of milking interval (Kelly et al., 1998; Castillo et al., 2008).

The fact that other experiments performed with goat milk also reported an increase of plasmin activity throughout lactation (Cortellino et al., 2006), along with the fact that most of the studies performed in ewes agree with the results of the present thesis, suggests that the behavior of the PL-PG system might be different in sheep than it is in cow and goat.

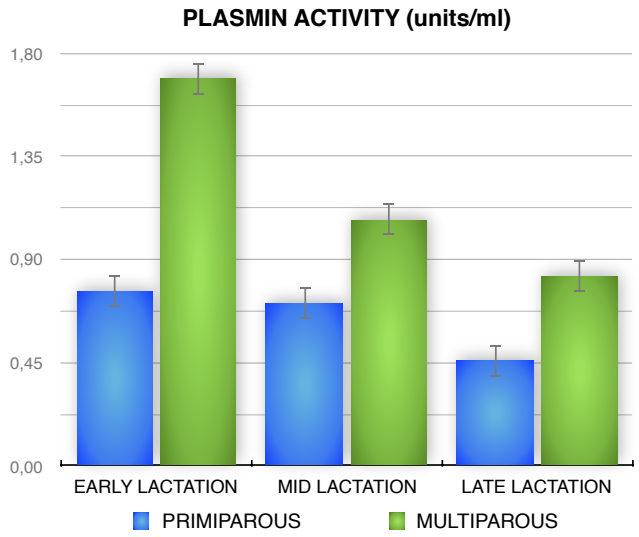


Figure 11.3: Plasmin activity throughout lactation.

No significant effect of parity was found, though plasmin activity seemed to be higher in multiparous ewes. Most of the studies performed in different species of domestic ruminants reported that plasmin activity increased with parity and age of the animals (Bastian et al., 1991a; Bastian and Brown, 1996; Battacone et al., 2005; Koutsouli et al., 2015).

SUMMARY

Plasmin activity followed a decreasing trend throughout lactation, with its highest values in early stages.

Parity did not affect significantly plasmin activity, though values were higher in multiparous ewes.

11.3. Effect of udder health status over plasmin

Figure 11.4 represents the evolution of plasmin activity throughout lactation in primiparous ewes (PR), multiparous ewes with no previous udder infection (M1), multiparous ewes with a previous minor udder infection ($M2_A$) and multiparous ewes with a previous severe udder infection ($M2_B$).

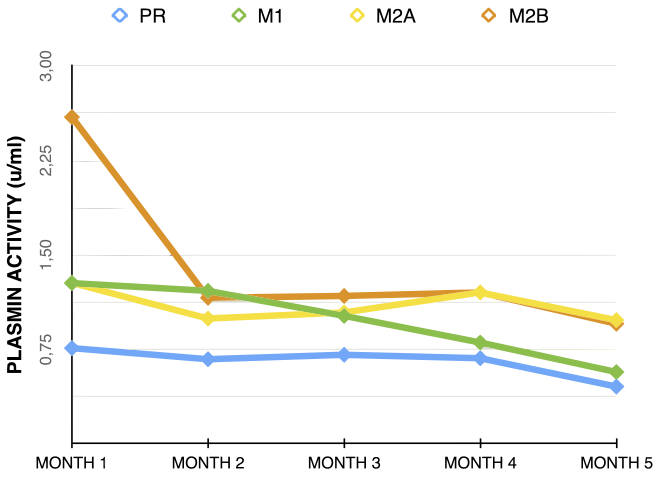


Figure 11.4: Plasmin activity throughout lactation according to UHS.

PR presented the lowest plasmin activity, followed by M1 and, lastly, $M2_A$ and $M2_B$, in which the highest values were recorded. Differences between PR and both $M2_A$ and $M2_B$ were statistically significant (figure 11.5).

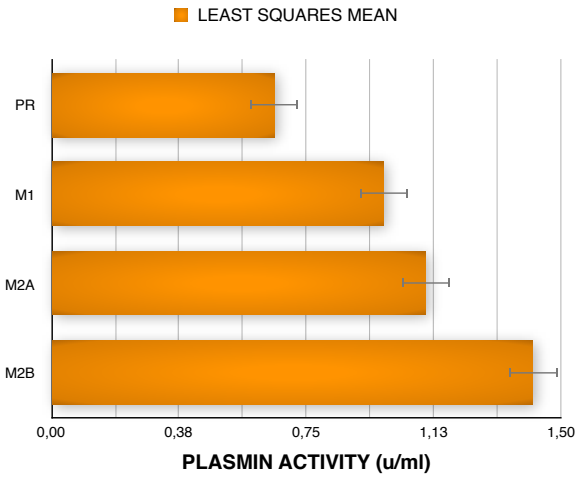


Figure 11.5: Plasmin activity in all sanitary categories.

Table 11.3: Plasmin activity (least squares means) as affected by SCCs and udder health status.

	LOW SCCs			MID SCCs			HIGH SCCs			P		
	PR	**M1**	**M2**	**PR**	**M1**	**M2**	**PR**	**M1**	**M2**	**SCCs**	**UHS**	**SCCs×UHS**
Plasmin activity (u/ml)	0.24	0.22	0.18	0.20	0.27	0.19	0.28	0.24	0.16	0.562	0.003	0.761

PR = primiparous; M1 = multiparous without previous udder infection; M2 = multiparous with a previous udder infection.
SCCs = somatic cell count score; UHS = category according to udder health status.

To simplify statistical processing, as there were no significant differences between animals with previous minor or severe udder infections, both categories were grouped in a main category, hereafter called M2 (multiparous ewes with previous udder infection).

Table 11.3 shows the least squares means of plasmin activity as affected by SCCs and category according to udder health status (UHS). Table 11.4 compares plasmin activity means between different SCCs ranges.

Table 11.4: Contrast analysis comparing plasmin activity means among SCCs ranges.

	Low×Mid	**Low×High**	**Mid×High**
PR	0.628	0.747	0.925
M_1	0.913	0.320	0.430
M_2	0.151	0.517	0.376

Values colored in grey are not significant (P>0.05).

Plasmin activity was similar in all UHS categories, despite variations in SCCs. This agrees with the results published by Bianchi et al. (2004), Höök (2015) and Koutsouli et al. (2015), who reported that SCC did not seem to affect plasmin activity. Even so, this could be due to the low SCC measured throughout lactation in the present study, as Albenzio et al. (2009) reported that in healthy ewes with SCC $< 600\times10^3$, the plasmin system was not affected. Other authors found that plasmin activity increased with SCC (Leitner et al., 2004; Battacone et al., 2005). Furthermore, experiments performed in Manchega ewes Castillo et al. (2008) found a positive correlation between plasmin and somatic cells in milk samples with really low SCC (175×10^3 cells/ml), what supports the hypothesis that the influence of SCC on the plasmin-plasminogen system may not follow a specific pattern. However, SCC is not the only variable for predicting PL evolution in milk, as PL activity is affected by a complex network of molecular interaction between enzyme activators an inhibitors (Albenzio et al., 2009).

According to UHS categories, with low SCCs, plasmin activity in M2 is significantly higher than in PR, and also tends to be higher than in M1. With high SCCs, plasmin activity in M2 was also higher than in PR, but in this case, similar to M1.

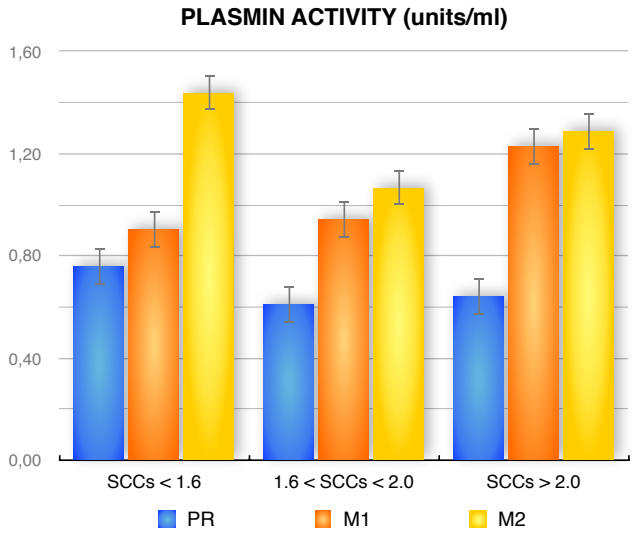


Figure 11.6: Plasming activity according to SCCs levels.

SUMMARY

Plasmin activity did not seem to be affected by SCCs.

M2 had, in general, higher plasmin activity than PR, and also a tendency to present higher activity than M1 in high SCCs.

11.4. Influence of plasmin on milk composition

Table 11.6 presents the correlation coefficients for plasmin activity and milk composition variables in PR, M1 and M2. Statistical signification was declared at $P<0.05$.

There was no existing correlation between plasmin activity and fat concentration, which agrees with Höök (2015) and Koutsouli et al. (2015), who reported that plasmin did not seem to affect fat content in milk.

Regarding milk proteins, there was a strong negative correlation between plasmin and casein content, which diminishes as plasmin activity increases, specially in multiparous ewes, regardless of their clinical history. Despite that some authors have not found an effect of plasmin over casein (Albenzio et al., 2004), and others have found a positive correlation (Baldi et al., 1996; Bianchi et al., 2004), most of the references found in literature have reported

similar results to those obtained in the present study (Politis and Ng Kwai Hang, 1989; Jaeggi et al., 2003; Leitner et al., 2004). Numerous authors have related this negative correlation with the proteolytic action of plasmin over β-CN, as described in chapter 5.3 (Grufferty and Fox, 1988; Nielsen, 2002; Leitner et al., 2004; Moatsou, 2010). Thus, this could also explain the negative correlation between plasmin activity and crude protein observed in PR and M2. In this case, the effect of proteolysis would not be so evident, as the measurement involves total protein and not only casein. This might prove why some recent works have not found a clear decrease in crude protein as plasmin activity increased (Höök, 2015; Koutsouli et al., 2015). Nevertheless, results found in literature are often controversial, as other authors have revealed a positive correlation between plasmin and crude protein. Anyhow, proteolysis in low SCC milk seems to be dominated by the action of plasmin, with a minor contribution from cathepsin D. In addition, as SCC increases, the relative significance of plasmin decreases, while the relative activity of other indigenous and microbial enzymes increases (Albenzio et al., 2005; Kelly et al., 2006; Santillo et al., 2009). Therefore, the further research of other endogenous enzymes should be considered to explain more clearly the effect of proteolysis in milk.

Table 11.5: Correlation between plasmin activity and milk composition.[1]

	PR	**M1**	**M2**
Fat	-0.1863	-0.0789	-0.1274
Crude protein	-0.2158*	-0.0056	-0.1593*
Total solids	-0.2054	-0.0942	-0.1527*
Lactose	0.1752	0.061	0.0979
Casein	-0.2539*	-0.4333***	-0.4442***
Ash	-0.1127	0.3237**	0.1024***
Urea	-0.0389	0.1674	0.0871
Initial pH	0.3845*	0.1974	0.4968

Values colored in grey are not significant (P>0.05).

Lactose concentration in milk did not seem to be significantly affected by plasmin, although some authors have reported a negative correlation of this parameter with plasmin activity (Leitner et al., 2004; Battacone et al., 2005).

There was a relatively slight correlation between plasmin activity and total solids, probably due to the already mentioned cleavage of casein. Meanwhile, ash content seemed to increase along with plasmin activity, but no references were found in literature that explained this fact.

[1] P<0.05 * , P<0.01 ** , P<0.001 ***

Lastly, there seems to be an increase of pH with plasmin activity in PR ewes. Some authors, such as Battacone et al. (2005) reported a correlation between plasmin activity and pH, although others did not find an effect of plasmin over the initial pH of milk (Höök, 2015; Koutsouli et al., 2015).

SUMMARY

Fat, lactose and urea were not affected by plasmin activity. Casein content decreased as plasmin activity increased in all UHS categories, and crude protein was similarly affected (except in M1 ewes). Total solids slightly decreased with high plasmin activity in M2. pH increased with plasmin activity only in PR.

11.5. Influence of plasmin on rennet coagulation

Table 11.6 presents the correlation coefficients for plasmin activity and the renneting parameters of milk in PR, M1 and M2 ewes. Statistical signification was established at $P<0.05$.

In the light of the results showed in the table, in general, rennet coagulation of milk worsens as plasmin activity increases. This is evident specially in milk from M2, since plasmin activity in this category is significantly higher than in the rest, as described in section 11.3. Therefore, in M2, rennet clotting time increased and curd firmness diminished, which agrees with the results obtained by Mara et al. (1998), Albenzio et al. (2004) and Battacone et al. (2005). According to Battacone et al. (2005), the worsening of rennet coagulation parameters is more related to disorders of permeability in the mammary gland than to casein breakdown by proteolysis. However, as mentioned in section 11.4, there were no effects of plasmin over lactose that could imply alterations of the milk-blood barrier. Due to all these reasons, the relationship between casein hydrolysis and rennet coagulation needs to be further investigated in greater detail.

On the other hand, Srinivasan and Lucey (2002) and Leiber et al. (2005) found that, normally, an increase of plasmin activity negatively affected renneting parameters and curd yield. Nevertheless, other authors did not find a clear correlation between plasmin and rennet coagulation. However, most of the experiments found in literature concerning plasmin activity describe addition of different plasmin concentrations to milk, to establish different levels or ranges of activity (Mara et al., 1998). Thereby, several authors have reported that high

levels of plasmin induce casein hydrolysis, affecting milk coagulation. However, in experiments performed on milk in natural conditions, native plasmin levels have been reported to be lower. This suggests that, although there was some impact of plasmin over renneting parameters in M2 ewes, in the rest of categories this effect might not be so evident at those concentrations of the enzyme.

Table 11.6: Correlation between plasmin activity and rennet coagulation variables.[2]

	PR	**M_1**	**M_2**
RCT	0.0914	0.0296	0.1685*
k_{20}	-0.0857	-0.0073	0.137
RCT+k_{20}	0.0587	0.0223	0.1928*
A_{30}	-0.1907	-0.0037	-0.1731*
A_{60}	-0.0136	-0.3006**	-0.214**
Curd yield	-0.2785**	0.26*	0.0429

Values colored in grey are not significant (P>0.05).

In addition, Bastian et al. (1991a) found that when SCC did not exceed 300×10^3 cells/ml (as in the animal population studied in this thesis), plasmin levels remain relatively low, and no further correlation between plasmin activity and renneting properties was found. This might certainly explain the low impact of plasmin on milk coagulation in PR and M1.

SUMMARY

Plasmin activity increased rennet clotting time in M2, and reduced curd firmness in M1 (A_{60}) and M2 (A_{30} and A_{60}). Curd yield decreased in PR but slightly increased in M1.

[2] P<0.05 * , P<0.01 ** , P<0.001 ***

Chapter 12

Discriminant function analysis

Five of the studied variables were selected as predictor variables in the discriminant model: SCC, urea, curd firmness after 60 minutes (A_{60}), curd yield and plasmin activity (tables 12.1 and 12.2). Wilk's test indicated that the complete model was significant ($P<0.001$) and explained the total variance shared between the variables.

Standardized canonical discriminant function coefficients show that the variables with greater discriminant ability in function 1 were A_{60} and urea concentration, being positive and negative, respectively. According to the values of the centroids, function 1 separated primiparous ewes from multiparous ewes.

The variables with greater discriminant ability in function 2 were SCC and A_{60}. The first one had the highest correlation with this function according to the structure matrix. Due to the value of the centroids, function 2 separated multiparous ewes with previous lesions of the udder from the rest of the animals (PR and M2).

Table 12.1: Canonical discriminant analysis results (I).

	Standardized coefficients		**Structure matrix**	
	1	**2**	**1**	**2**
SCC	0,346	0,863	0,268	0,919
Urea	-0,433	0,142	-0,428	0,147
A_{60}	0,689	-0,375	0,49	-0,464
Curd yield	0,449	0,091	0,508	0,136
Plasmin activity	0,453	-0,094	0,343	0,009

Table 12.2: Canonical discriminant analysis results (II).

	Función	
	1	**2**
Canonical correlation	0,438	0,109
Autovalues	0,237	0,012
% of variance explained	95,2	4,8
Centroids		
PR	-0,784	0,019
M1	0,374	0,156
M2	0,249	-0,106

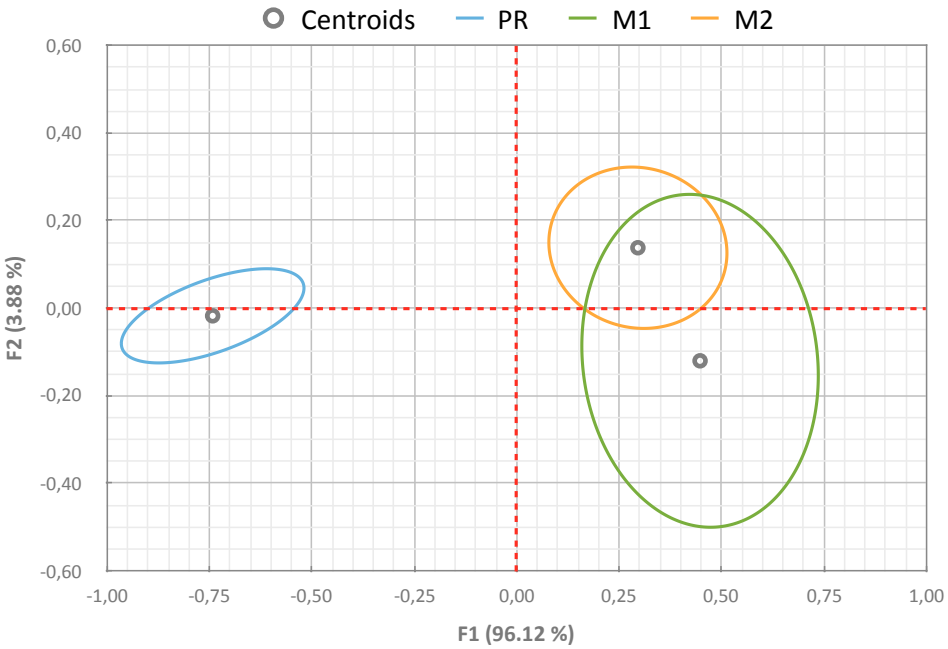


Figure 12.1: Discriminant function analysis

Part VI

CONCLUSIONS AND RECOMMENDATIONS

Conclusions and recomendations

CONCLUSION 1

Curd yield was not negatively affected by the health condition of the udder. This might be explained by an increasing amount of water trapped inside the curd as the sanitary conditions deteriorate.

CONCLUSION 2

Plasmin activity in Manchega ewes decreased throughout lactation, and its highest values were measured in animals that suffered udder infections in the previous lactation. Therefore, regardless the health condition of the ewes in the beginning of lactation, there seems to be a residual enzymatic activity persisting as a response to a previous infection.

CONCLUSION 3

Milk renneting parameters were not affected by parity although stage of lactation did have a negative impact on coagulation. This could be caused by a progressive deterioration of the udder and an increase of somatic cells in milk, what may induce the activity of several proteases other than plasmin.

CONCLUSION 4

In ewes with a previous udder infection, plasmin activity had a negative impact on coagulation, due to casein breakdown. In the rest of the animals this effect was lighter. However, the good health condition of the herd seems to camouflage the possible effects of plasmin on rennet coagulation.

CONCLUSION 5

According to the results obtained in this study, this field of research should be continued considering three aspects:

1. Total solids in the curd should be analyzed to verify if measured yield is really due to the richness in fat and protein, or caused by an increase of the moisture content trapped inside the curd.

2. Some effects of the sanitary conditions of the udder may have not been evidenced because of the low variability in somatic cell counts of the studied animals. Therefore, an animal population with a wider sanitary range should be chosen in further research.

3. Hydrolysis of casein may also be caused by endogenous enzymes other than plasmin, such as cathepsins or elastase. Thus, the activity of these proteases in milk, as well as the behavior of activators and inhibitors of the plasmin system should be studied in greater detail.

Part VII

APPENDICES

Published articles and contributions to congresses

Relationship of somatic cell count and composition and coagulation properties of ewe's milk

J. Caballero-Villalobos, A.I. Garzón, B. Oliete, R. Arias, L. Jiménez, N. Núñez-Sánchez, A.L. Martínez-Marín

Article published in the journal *Mljekarstvo* in early 2015. The results and experiments described served as a starting point for the experimental design of this PhD thesis.

Plasmin activity in Manchega ewe milk

J. Caballero-Villalobos, A.I. Garzón, A. Martínez, R. Arias, F. Ciocia, P.L.H. McSweeney

Poster submitted to the *IDF Parallel Dairy Science and Technology Symposia: Dairy products concentration and drying - Cheese science and technology*. Organized by the International Dairy Federation (IDF) y celebrated in Dublin (Ireland) from 11-13 April 2016.

Influencia del pH y del Recuento de Células Somáticas sobre la coagulación y las características tecnológicas en la leche de oveja Manchega

J. Caballero-Villalobos

Oral communication defended in *I Congreso de Veterinaria y Ciencia y Tecnología de los alimentos*. Organized by University of Córdoba and celebrated in Córdoba

(Spain), February the 12th, 2016. Awarded as second best oral communication of the congress.

Factores que afectan a la coagulación de la leche y la calidad del queso en ovino

J. Caballero-Villalobos, A. Figueroa, M. Sánchez-Rodríguez, A.I. Garzón

Poster submitted to *Creando Redes: III Congreso Científico de Investigadores en Formación en Agroalimentación*. Organized by Campus de Excelencia Internacional Agroalimentario (ceiA3) and celebrated in Córdoba (Spain) from 18-19 November 2014.

Nuevas herramientas en los sistemas de control de calidad de leche de oveja: evaluación de la composición, microbiología y propiedades tecnológicas para la producción de queso.

Jiménez, L., Oliete, B., Arias, R., Garzón, A., Caballero, J., Romero, J., Pérez-Guzmán, M.D., Arias, R.

Article published in the journal *Tierras Ovino* in 2013.

Preliminary communication - Prethodno priopćenje UDK: 637.112.2

Relationship of somatic cell count and composition and coagulation properties of ewe's milk

doi: 10.15567/mljekarstvo.2015.0208

Javier Caballero Villalobos[1], *Ana I. Garzón Sigler*[1], *Bonastre Oliete*[2], *Ramón Arias Sánchez*[2], *Lorena Jiménez*[2], *Nieves Núñez Sánchez*[1], *Andrés L. Martínez Marín*[1]*

[1]Departamento de Producción Animal, Universidad de Córdoba, Ctra. Madrid-Cádiz, km. 396, 14071 Córdoba, Spain
[2]Laboratorio de Lactología, Centro Regional de Selección y Reproducción Animal de la Junta de Comunidades de Castilla-La Mancha, Avenida del Vino, 10, 13300 Valdepeñas, Ciudad Real, Spain

Received - Prispjelo: 09.12.2014.
Accepted - Prihvaćeno: 02.03.2015.

Abstract

The relationship between somatic cell count (SCC) and raw milk composition and its coagulation properties measured at native or standardised pH values were investigated in Manchega ewes' milk. A total of 84 bulk tank milk samples from flocks included in the National Association of Manchega Sheep Breeders were used. According to their SCC, milk samples were divided into three terciles named low (562±138 cells/mL), medium (956±115 cells/mL) and high (1705±428 cells/mL) SCC groups. Within each SCC group, two pH treatments were applied before determining coagulation properties (rennet clotting time, curd firming time and curd firmness): no acidification of milk (coagulation at native pH) and acidification of milk at pH 6.5. Native milk pH significantly increased ($P<0.05$) as SCC rose. With respect to raw milk composition, fat contents were not affected ($P>0.05$) by SCC, protein content tended to be higher in the high SCC group ($P=0.05$) and lactose content was significantly lower ($P<0.05$) in that group. At native pH, the high SCC group had longer rennet clotting time, higher curd firming time and lower curd firmness after 30 min of rennet addition than the low and medium SCC groups ($P<0.05$). Standardising milk pH at 6.5 prior to rennet addition clearly cancelled out ($P<0.05$) the negative effects of high SCC on milk coagulation properties. In conclusion, despite the fact that acidification before renneting improved the coagulation properties of milk with high SCC, more research would be needed to determine the sensorial properties of cheese manufactured under such conditions.

Key words: sheep, somatic cell count, milk composition, milk clotting

Introduction

A major aspect of the quality of sheep milk is its capability to be transformed into high-quality cheeses and to produce high yields of cheese from each litre of milk (Bencini, 2002). The amount and quality of cheese that can be obtained mainly depends on the coagulation properties of the used milk (Bencini and Pulina, 1997) and its fat and casein contents (Politis and Ng-Kwai-Hang, 1988a). Coagulation properties are rennet clotting time, curd firming time, and curd firmness after 30 and 60 min of rennet addition. These parameters have been shown to be positively related to cheese yield (Ng-Kwai-Hang et al., 1989) and have been commonly used by researchers to assess milk processing performance (Ng-Kwai-Hang et al., 1989; Pirisi et al., 2000). Milk somatic cell count (SCC) is a widely used marker for both udder health and

*Corresponding author: e-mail: pa1martm@uco.es

milk quality. High SCC is either the consequence of an inflammatory process due to the presence of an intramammary infection or, under non-pathological conditions, the result of physiological processes such as advanced stage of lactation (Raynal-Ljutovac et al., 2007). Previous research indicate that high SCC in sheep milk alters milk pH and composition (Vivar-Quintana et al., 2006; Martí de Olives et al., 2013) as well as milk coagulation properties (Pirisi et al., 2000; Bianchi et al., 2004), which in turn reduces cheese yielding capacity of milk and cheese making efficiency (Politis and Ng-Kwai-Hang, 1988a, 1988b). On the other hand, pH before renneting affects milk coagulation properties (Balcones et al., 1996; Bencini, 2002). Since SCC is positively related to milk pH (Raynal-Ljutovac et al., 2007), lowering it would have positive effects on coagulation properties of milk with high SCC (Pirisi et al., 2000). These authors studied the effects of pH prior to renneting (native and standardised at 6.5) on milk coagulation properties, but they did not compare pH treatments within the SCC groups in their study.

Milk of the Manchega sheep breed is used to make Manchego cheese, which is one of the most important and well-known sheep's milk cheese in Spain and abroad. Manchego is a high-fat, hard, ripened cheese, manufactured at a factory level under controlled conditions using raw or pasteurized milk and with starter addition (Gonzalez-Viñas et al., 2001). Therefore, the objective of this study was to determine the effects of SCC on composition and clotting parameters, measured at native and standardised pH values, of Manchega ewe milk.

Materials and methods

A total of 84 bulk tank milk samples from flocks included in the National Association of Manchega Sheep Breeders were used. Milk samples were stored at 4 ºC until analysis within 3 days after collection. For each sample, pH (Crison Basic 20 pH-meter; Crison Instruments, Barcelona, Spain), SCC (Fossomatic; Foss Electric, Hillerød, Denmark), and fat, total protein, lactose and total solid contents (Milko Scan; Foss Electric, Hillerød, Denmark) were determined. According to their SCC, milk samples were divided into three terciles (i.e. 28 samples each group) named low (562±138 cells/mL), medium (956±115 cells/mL) and high (1705±428 cells/mL) SCC groups. Within each SCC group, two pH treatments were applied before determining coagulation properties: no acidification of milk (coagulation at native pH) and acidification of milk at pH 6.5 by adding a lactic acid solution (10 %) before renneting.

Coagulation properties were measured by using a Formagraph (Foss Electric, Hillerød, Denmark). The testing time of the analysis was set up at 60 min to investigate if milk that did not form a curd within the conventional threshold of 30 min showed coagulation aptitude after this time (De Marchi et al., 2012). The measured traits were rennet clotting time (named r, it is the interval in min from the addition of the rennet to the beginning of coagulation), curd firming time (named k20, it is the interval in min from the beginning of coagulation to the moment the width of the graph achieves a separation of 20 mm), and curd firmness (named A30 or A60, it is the width of the graph measured in mm after 30 or 60 min of rennet addition). For the samples that did not form a curd after 60 min, curd firmness values were arbitrarily assumed as 1 mm. Also, in samples that did not curdle after 60 min, coagulation time and curd firming time were attributed an arbitrary value of 60 min.

All data were analysed using the GLM procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). The statistical analysis of milk pH and composition data included SCC group as fixed effect in the model. The statistical analysis of milk coagulation properties included SCC group, pH and their interaction as fixed effects. Tukey's test was used to compare least squares means. Statistical significance was declared at $P<0.05$.

Results and discussion

Composition and pH values of bulk tank milk of Manchega ewes are shown in Table 1. The fact that native milk pH significantly increased ($P<0.05$) as SCC rose is commonly reported in the literature (Raynal-Ljutovac et al., 2007). This effect is attributed to increased permeability of the mammary epithelium, which can lead to the transfer of components from blood to milk, including citrates, bicarbonates, and Na and Cl ions. Higher levels of citrate and bicarbonate may be responsible for elevated pH levels (Harmon, 1994; Kitchen, 1981).

Table 1. Effects of somatic cell count (SCC, mean ± standard deviation in cells/mL) on the composition of bulk tank milk of Manchega ewes

	SCC group			
Traits	Low (562±138) n = 28	Medium (956±115) n = 28	High (1705±428) n = 28	S.E.M
Native pH	6.57^{c}	6.65^{b}	6.81^{a}	0.013
Fat, %	8.37	8.31	8.30	0.104
Total protein, %	6.25	6.21	6.41	0.051
Lactose, %	4.55^{a}	4.58^{a}	4.41^{b}	0.024
Total solids, %	20.01	19.93	19.99	0.141

S.E.M. Standard error of the mean
^{a,b,c}Means without a common superscript are statistically different at P<0.05

Table 2. Effects of somatic cell count (SCC, mean ± standard deviation in cells/mL) and pH prior to renneting on the coagulation properties of bulk tank milk of Manchega ewes

	SCC group						
	Low (562±138) n = 28		Medium (956±115) n = 28		High (1705±428) n = 28		
Traits[1]	N	L	N	L	N	L	S.E.M.
r, min	28.13^{b}	18.76^{c}	28.91^{b}	20.79^{c}	35.65^{a}	22.78^{bc}	0.760
k20, min	3.96^{b}	3.88^{b}	4.07^{b}	5.19^{ab}	11.62^{a}	4.07^{b}	0.714
A30, mm	15.25^{b}	37.80^{a}	15.09^{b}	35.42^{a}	6.01^{c}	28.90^{a}	1.458
A60, mm	42.43^{ab}	49.00^{a}	36.14^{b}	47.86^{a}	35.77^{b}	47.08^{a}	0.796

N = native pH, L = pH standardised at 6.5, S.E.M. Standard error of the mean
a,b,c - Means without a common superscript are statistically different at P<0.05
[1]r - rennet clotting time, k20 - curd firming time, A30 and A60 - curd firmness after 30 and 60 min of rennet addition, respectively

With respect to raw milk composition, fat contents were not affected (P>0.05) by SCC. Protein content tended to be higher in the high SCC group (P=0.05) while lactose content was lower (P<0.05) in that group. Our results are complementary with most previous published research on sheep milk, which indicate that fat content does not change, protein content either increases or does not change, and lactose content decreases with increasing SCC (Albenzio et al., 2004; Nudda et al., 2003; Pirisi et al., 2000; Vivar-Quintana et al., 2006). The negative effect of high SCC on the milk lactose content has been related to a decreased synthesis capacity of the mammary gland due to the damage of epithelial tissue, but also because of a lesser availability of its precursor, glucose, due to competition for energy between secretor cells and those with phagocyte functions (Martí de Olives et al., 2013). On the other hand, the absence of clear changes in protein content could be due to a reduced protein synthesis

by the udder and an increment of proteins coming from the bloodstream, both having opposite effects (Vivar-Quintana et al., 2006). Moreover, Martí de Olives et al. (2013) did not observe differences in casein content, but found lower casein to protein ratio due to high SCC in milk of Manchega ewes.

At native pH, the high SCC group had longer rennet clotting time, higher curd firming time and lower curd firmness after 30 min of rennet addition than the low and medium SCC groups ($P<0.05$, Table 2). Curd firmness after 60 min was slightly lower, but not statistically different, in the medium and high SCC groups compared with the low SCC group. Standardising milk pH at 6.5 decreased ($P<0.05$) rennet clotting time and stepped up ($P<0.05$) curd firmness at 30 min in all SCC groups. It also decreased ($P<0.05$) curd firming time in the high SCC group and increased curd firmness at 60 min in the medium and high SCC groups. Albenzio et al. (2004), Bianchi et al. (2004) and Pirisi et al. (2000) also reported poorer coagulation properties of ovine milk as SCC increased. The negative relationships between SCC and milk coagulation properties could to be related to increased casein breakdown due to a higher plasmin activity in the milk with high SCC (Albenzio et al., 2005; Bianchi et al., 2004; Leitner et al., 2004). In addition, the high SCC could negatively affect both the first phase of rennet coagulation due to elevated pH, as the optimum pH of chymosin activity is in the acidic range (Kumar et al., 2010), and the aggregation of paracasein micelle due to a lower Ca^{2+} activity, as suggested by Leitner et al. (2004). On the other hand, Ng-Kwai-Hang et al. (1989) observed that a shorter clotting time, a faster rate of firming, and a harder curd resulted in a higher cheese yield and efficiency due to lower losses of milk fat and protein in whey. Moreover, Pirisi et al. (2000) and Albenzio et al. (2004) reported a greater loss of protein in whey when ewe milk with high SCC was used for cheese manufacture. Therefore, higher yields of cheese might be expected in the milk with high SCC renneted at standardised pH compared with milk renneted at native pH.

Cheese producers can adjust pH value of milk to achieve the desired acidity by varying the percentage of inoculum of starter cultures (Bencini and Pulina, 1997) or by direct addition of food-grade acid (Lucey and Kelly, 1994). However, despite the fact that direct acid addition improved milk coagulation properties in ovine milk with high SCC in the present work, it should be noted that some authors have reported off-flavours and texture defects after ripening in cheese manufactured from high SCC ovine milk (Jaeggi et al., 2003; Revilla et al., 2007).

Conclusions

High SCC decreased milk lactose content and worsened clotting parameters (rennet clotting time, curd firming time and curd firmness). Standardising milk pH at 6.5 prior to rennet addition clearly cancelled out the negative effects of high SCC on coagulation properties. However, further studies should be performed to determine the sensorial properties of cheese manufactured under such conditions.

Acknowledgments

This work was financed by the PII10-0003 project of the Viceconsejería de Ciencia y Tecnología (Junta de Comunidades de Castilla-La Mancha, Spain). It was part of a research line continued in the RTA2011-00057-C02 project of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Ministerio de Economía y Competitividad, Madrid, Spain).

Odnos broja somatskih stanica i sastav i koagulacijska svojstva ovčjeg mlijeka

Sažetak

Cilj ovog rada bio je istražiti odnos između broja somatskih stanica, sastava sirovog mlijeka, i koagulacijskih svojstava sirovog mlijeka kod prirodne i standardizirane pH vrijednosti mlijeka mančego ovce. Analizirano je 84 uzoraka skupnog mlijeka stada Državnog udruženja uzgajivača mančego ovce. Prema broju somatskih stanica/mL (BSS/mL), uzorci mlijeka podijeljeni su u 3 skupine - niska (562±138 BSS/mL), srednja (956±115 BSS/mL) i visoka (1705±428 BSS/mL). U okviru svake skupine, pH je mjeren dva puta prije određivanja koagulacijskih osobina (vrijeme zgrušavanja sirilom, vrijeme učvršćivanja gruša i tvrdoća gruša): bez zakiseljavanja mlijeka (koagulacija kod prirodne pH vrijednosti)

i zakiseljavanje kod pH vrijednosti 6,5. pH vrijednost mlijeka signifikantno je povećana (P<0,05) povećanjem BSS. BSS nije utjecao (P>0,05) na udjel masti, dok je udjel proteina bio veći u grupi s visokim BSS (P=0,05), a udjel laktoze bio je signifikantno niži (P<0,05) u toj skupini. Kod prirodne pH vrijednosti mlijeka, visoki BSS utjecao je na duže vrijeme zgrušavanja mlijeka sirilom, sporije učvršćivanje gruša i na manju čvrstoću gruša nakon 30 min od dodatka sirila, u odnosu na skupinu u kojoj je BSS bio nizak i srednji (P<0,05). Standardizacija pH mlijeka na 6,5 prije dodavanja sirila anulirala je (P<0,05) negativan utjecaj visokog BSS na koagulacijska svojstva mlijeka. Može se zaključiti da su, unatoč činjenici kako je acidifikacija mlijeka prije zgrušavanja poboljšala koagulacijske osobine mlijeka s visokim BSS, daljnja istraživanja potrebna kako bi se utvrdila senzorska svojstva sira proizvedenog takvim postupkom.

Ključne riječi: ovce, broj somatskih stanica, sastav mlijeka, zgrušavanje mlijeka

References

1. Albenzio, M., Caroprese, M., Santillo, A., Marino, R., Muscio, A., Sevi, A. (2005): Proteolytic patterns and plasmin activity in ewes' milk as affected by somatic cell count and stage of lactation, *Journal of Dairy Research 72* (01), 86-92.
   doi: dx.doi.org/10.1017/S0022029904000676
2. Albenzio, M., Caroprese, M., Santillo, A., Marino, R., Taibi, L., Sevi, A. (2004): Effects of somatic cell count and stage of lactation on the plasmin activity and cheesemaking properties of ewe milk, *Journal of Dairy Science 87* (3), 533-542.
   doi: dx.doi.org/10.3168/jds.S0022-0302(04)73194-X
3. Balcones, E., Olano, A., Calvo, M.M. (1996): Factors affecting the rennet clotting properties of ewe's milk, *Journal of Agricultural and Food Chemistry 44* (8), 1993-1996.
   doi: dx.doi.org/10.1021/jf960138u
4. Bencini, R. (2002): Factors affecting the clotting properties of sheep milk, *Journal of the Science of Food and Agriculture 82* (7), 705-719.
   doi: dx.doi.org/10.1002/jsfa.1101
5. Bencini, R., Pulina, G. (1997). The quality of sheep milk: A review. *Animal Production Science 37* (4), 485-504. doi: dx.doi.org/10.1071/EA96014
6. Bianchi, L., Bolla, A., Budelli, E., Caroli, A., Casoli, C., Pauselli, M., Duranti, E. (2004): Effect of udder health status and lactation phase on the characteristics of Sardinian ewe milk, *Journal of Dairy Science 87* (8), 2401-2408.
   doi: dx.doi.org/10.3168/jds.S0022-0302(04)73362-7
7. De Marchi, M., Penasa, M., Tiezzi, F., Toffanin, V., Cassandro, M. (2012): Prediction of milk coagulation properties by Fourier Transform Mid-Infrared Spectroscopy (FTMIR) for genetic purposes, herd management and dairy profitability. In Proceedings of the 38th *International Committee for Animal Recording* (ICAR) Meeting (Vol. 28). http://www.icar.org/cork_2012/Presentations/RLN%20Cassandro.pdf (24.4.2014).
8. González-Viñas, M.A., Poveda, J., Ruiz, A.G., Cabezas, L. (2001): Changes in chemical, sensory and rheological characteristics of Manchego cheeses during ripening, *Journal of Sensory Studies 16* (4), 361-371.
   doi: dx.doi.org/10.1111/j.1745-459X.2001.tb00307.x
9. Harmon, R.J. (1994): Physiology of mastitis and factors affecting somatic cell counts, *Journal of Dairy Science 77* (7), 2103-2112.
   doi: dx.doi.org/10.3168/jds.S0022-0302(94)77153-8
10. Jaeggi, J.J., Govindasamy-Lucey, S., Berger, Y.M., Johnson, M.E., McKusick, B.C., Thomas, D.L., Wendorff, W.L. (2003): Hard ewe's milk cheese manufactured from milk of three different groups of somatic cell counts, *Journal of Dairy Science 86* (10), 3082-3089.
    doi: dx.doi.org/10.3168/jds.S0022-0302(03)73908-3
11. Kitchen, B.J. (1981): Bovine mastitis: milk compositional changes and related diagnostic tests, *Journal of Dairy Research 48* (01), 167-188.
    doi: dx.doi.org/10.1017/S0022029900021580
12. Kumar, A., Grover, S., Sharma, J., Batish, V.K. (2010): Chymosin and other milk coagulants: sources and biotechnological interventions, *Critical Reviews in Biotechnology 30* (4), 243-258.
    doi: dx.doi.org/10.3109/07388551.2010.483459
13. Leitner, G., Chaffer, M., Shamay, A., Shapiro, F., Merin, U., Ezra, E., Saran, A., Silanikove, N. (2004): Changes in milk composition as affected by subclinical mastitis in sheep, *Journal of Dairy Science 87* (1), 46-52.
    doi: dx.doi.org/10.3168/jds.S0022-0302(04)73140-9
14. Lucey, J., Kelly, J. (1994): Cheese yield, *International Journal of Dairy Technology 47* (1), 1-14.
    doi: dx.doi.org/10.1111/j.1471-0307.1994.tb01264.x
15. Martí De Olives, A., Díaz, J.R., Molina, M.P., Peris, C. (2013): Quantification of milk yield and composition changes as affected by subclinical mastitis during the current lactation in sheep, *Journal of Dairy Science 96* (12), 7698-7708.
    doi: dx.doi.org/10.3168/jds.2013-6998
16. Ng-Kwai-Hang, K.F., Politis, I., Cue, R.I., Marziali, A.S. (1989): Correlations between coagulation properties of milk and cheese yielding capacity and cheese composition, *Canadian Institute of Food Science and Technology Journal 22* (3), 291-294.
    doi: dx.doi.org/10.1016/S0315-5463(89)70397-7

17. Nudda, A., Feligini, M., Battacone, G., Macciotta, N.P.P., Pulina, G. (2003): Effects of lactation stage, parity, beta-lactoglobulin genotype and milk SCC on whey protein composition in Sarda dairy ewes, *Italian Journal of Animal Science 2* (1), 29-39.
doi: dx.doi.org/10.4081/ijas.2003.29

18. Pirisi, A., Piredda, G., Corona, M., Pes, M., Pintus, S., Ledda, A. (2000): Influence of somatic cell count on ewe's milk composition, cheese yield and cheese quality. In D.L. Thomas, S. Porter (Eds.) Proc. 6th Great Lakes Dairy Sheep Symposium *Proceedings of 6th Great Lakes Dairy Sheep Symposium*, Ontario, Canada, pp 47-59.

19. Politis, I., Ng-Kwai-Hang, K.F. (1988a): Effects of somatic cell count and milk composition on cheese composition and cheese making efficiency, *Journal of Dairy Science 71* (7), 1711-1719.
doi: dx.doi.org/10.3168/jds.S0022-0302(88)79737-4

20. Politis, I., Ng-Kwai-Hang, K.F. (1988b): Association between somatic cell count of milk and cheese-yielding capacity, *Journal of Dairy Science 71* (7), 1720-1727.
doi: dx.doi.org/10.3168/jds.S0022-0302(88)79738-6

21. Raynal-Ljutovac, K., Pirisi, A., De Cremoux, R., Gonzalo, C. (2007): Somatic cells of goat and sheep milk: Analytical, sanitary, productive and technological aspects, *Small Ruminant Research 68* (1), 126-144.
doi: dx.doi.org/10.1016/j.smallrumres.2006.09.012

22. Revilla, I., Rodríguez-Nogales, J.M., Vivar-Quintana, A.M. (2007): Proteolysis and texture of hard ewes' milk cheese during ripening as affected by somatic cell counts, *Journal of Dairy Research 74* (02), 127-136.
doi: dx.doi.org/10.1017/S0022029906002342

23. Vivar-Quintana, A.M., Beneitez De La Mano, E., Revilla, I. (2006): Relationship between somatic cell counts and the properties of yoghurt made from ewes' milk, *International Dairy Journal 16* (3), 262-267.
doi: dx.doi.org/10.1016/j.idairyj.2005.03.006

Plasmin activity in Manchega ewe milk

*J. Caballero-Villalobos[1], A.I. Garzón[1], A. Martínez[1], R. Arias[2], F. Ciocia[3], P.L.H. McSweeney[3]

[1] Departamento de Producción Animal (Universidad de Cordoba, Spain)
[2] Laboratorio de Lactología del CERSYRA (Castilla La Mancha, Spain)
[3] School of Food and Nutritional Sciences (University College Cork, Ireland)
* Corresponding author: v22cavij@uco.es

Introduction

Proteolysis of casein can affect milk renneting properties reducing cheese yield. Proteolytic enzymes like plasmin are known to modify milk composition, reducing its quality and producing bitter flavours. Thus, controlling the activity of endogenous enzymes at both farm and industrial levels could lead to improvement of the quality in dairy products, perhaps also reducing production costs.

Aims of the research

1. To evaluate the effects of stage of lactation (SL), parity (PA), somatic cell count (SCC) and previous udder health status on plasmin activity in Manchega ewe milk.

2. To establish the influence of plasmin activity over milk renneting properties and cheese yield.

Methodology

Forty Manchega ewes were distributed in 3 groups according to udder health status in the previous lactation:

- **PR**: primiparous.
- **M1**: multiparous with no previous udder infection.
- **M2**: multiparous with previous udder infection.

Milk samples from all animals were taken in early, mid and late lactation and analysed for:

- **Somatic Cell Count (SCC)**: using a Fossomatic Minor Cell Counter.
- **Renneting properties**: monitored with a Formagraph as described by McMahon and Brown (1982).
- **Plasmin activity**: as described by Richardson and Pierce (1981).

Plasmin and renneting

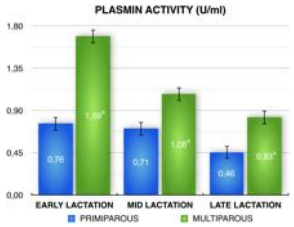
The following table represents correlation between plasmin activity and milk renneting parameters.

	PR	M1	M2
RCT	0,09	0,03	**0,17***
k_{20}	-0,09	-0,01	0,137
A_{30}	-0,19	-0,01	**-0,17***
A_{60}	-0,01	**-0,30****	**-0,21****
Curd yield	**-0,28****	**0,26***	0,04

P<0.05 * , P<0.01 ** , P<0.001 ***

In M2 animals, with higher values of plasmin activity, rennet clotting time (RCT) increases and curd firmness (A_{30} and A_{60}) diminishes, though no effect over rate of firming (k_{20}) or curd yield was observed. In M1 ewes curd firmness at 60 min (A_{60}) was negatively correlated with plasmin activity and curd yield was correlated positively. No correlations were found in PR but for curd yield, which decreased significantly with increasing plasmin activity.

Effect of SL and PA over Plasmin activity

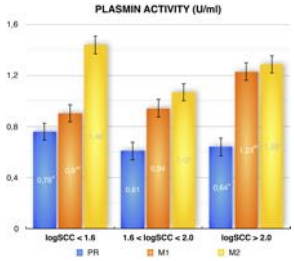


Plasmin activity diminishes throughout lactation in all animals, though this drop is only significant in multiparous ewes.

Parity does not affect plasmin activity as no significant differences were found due to this effect. Nevertheless, plasmin activity tends to present higher values in multiparous ewes.

Within each PA group means without a common superscript are statistically different at P<0.05

Effect of SCC and udder health over Plasmin activity



SCC did not have any effect over plasmin activity, as values were similar in all ranges.

According to the udder health status in the previous lactation, in low ranges of SCC, group M2 presents significantly higher plasmin activity than PR. In addition, there is an inclination in M2 to present higher plasmin activity than in M1. In high ranges of SCC, plasmin activity values M2 animals were also significantly higher than in PR, but quite similar to those measured in M1.

Within each logSCC group means without a common superscript are statistically different at P<0.05
logSCC = Somatic cell count (normalized)

Conclusions

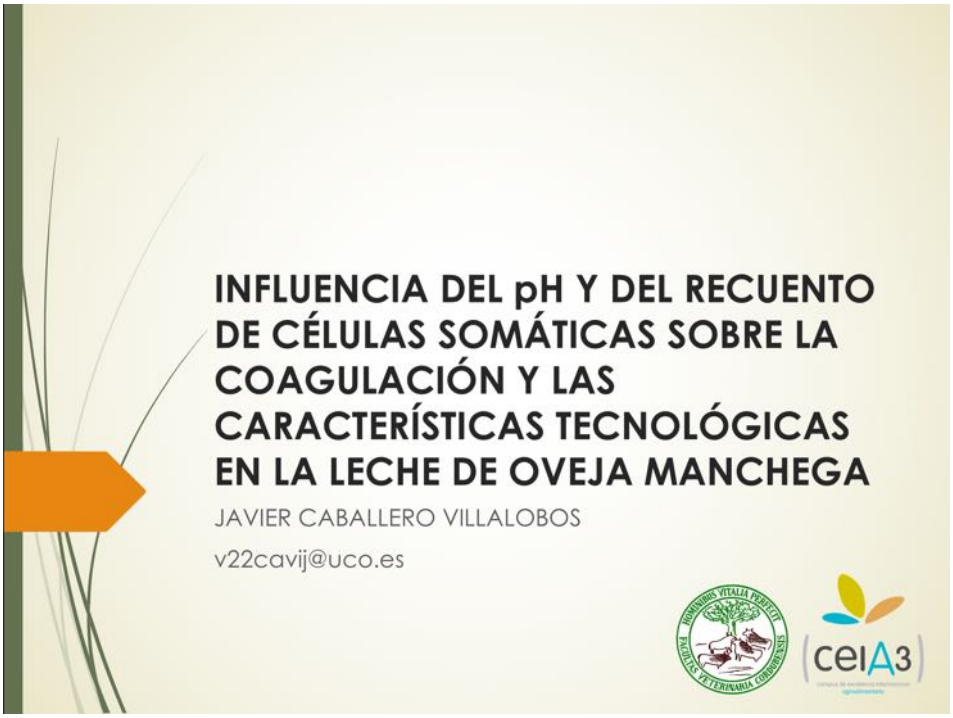
Plasmin activity decreased with lactation and changes were more evident in multiparous ewes. Plasmin did not seem to be affected by SCC, but its activity was higher in animals with previous udder infections (M2). Also in this group of animals renneting parameters were visibly worsened, though the low SCC measured in all ewes ($< 300 \times 10^3$) may have camouflaged the effect of plasmin over coagulation in PR and M1.

References

[1] Richardson, B. & Pierce, K. (1981). The determination of plasmin in dairy products. *N Z J Sci. Tech.* 16, 209- 220. [2] McMahon, D.J. & Brown, R.J. (1982) Evaluation of Formagraph for comparing rennet solutions. *J. Dairy Sci.* 65, 1639-1642.

Acknowledgments

GOBIERNO DE ESPAÑA · MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD · MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE · Santander UNIVERSIDADES · ceiA3 · AGROMETODOS, S.A.


INFLUENCIA DEL pH Y DEL RECUENTO DE CÉLULAS SOMÁTICAS SOBRE LA COAGULACIÓN Y LAS CARACTERÍSTICAS TECNOLÓGICAS EN LA LECHE DE OVEJA MANCHEGA
JAVIER CABALLERO VILLALOBOS
v22cavij@uco.es
ceiA3


Agradecimientos
ceiA3
campus de excelencia internacional
agroalimentario
Santander
UNIVERSIDADES
GOBIERNO DE ESPAÑA
MINISTERIO DE ECONOMIA Y COMPETITIVIDAD
MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE

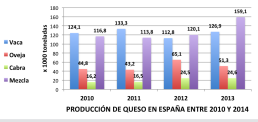
Factores que afectan a la coagulación de la leche y la calidad del queso en ovino

Caballero Villalobos, Javier*; Figueroa Sánchez, Antonio; Sánchez RodrÃguez, Manuel; Garzón Sígler, Ana Isabel

Laboratorio Lechero (Dpto. Producción Animal, Universidad de Córdoba) *v22cavij@uco.es

INTRODUCCIÓN

En España, la leche de ovino se utiliza casi en su totalidad para la elaboración de queso, ya sea de forma pura o mezclada con leche de caprino o vacuno. Por ello, es importante conocer cuáles son los factores (tanto genéticos, como fisiológicos, sanitarios o físico-químicos) que pueden afectar a este proceso de transformación de la materia prima y cómo influyen sobre la producción del queso. Así, podrían generarse patrones de control (tanto a nivel industrial como a nivel de manejo y de selección) que permitan reducir costes y generar un producto de calidad que sea ampliamente aceptado y valorado por el consumidor.



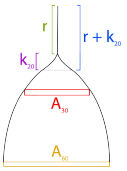
OBJETIVOS

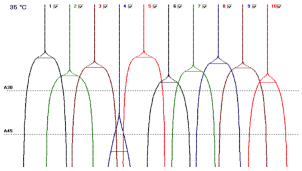
1. Estudiar la producción y las características tecnológicas de la leche de ovino, así como los factores que pueden influir sobre su rendimiento quesero.

2. Explicar los efectos de la actividad proteolítica derivada de la Plasmina y el Plasminógeno sobre la calidad higiénico-sanitaria de la leche y sobre su aptitud para la elaboración de queso.

MATERIAL Y MÉTODOS

Para el estudio se muestrearon durante una lactación completa 40 ovejas de raza Manchega procedentes de la ganadería La Nava Del Conejo, en Valdepeñas (Castilla La Mancha). En todos los muestreos se tomaron muestras separadas de ubre izquierda y derecha. Mediante un Formagraph se realizó la monitorización de la coagulación, obteniéndose valores para tiempo de coagulación (r), velocidad de endurecimiento (k_{20}) y durezas media y máxima (A_{30} y A_{60}).

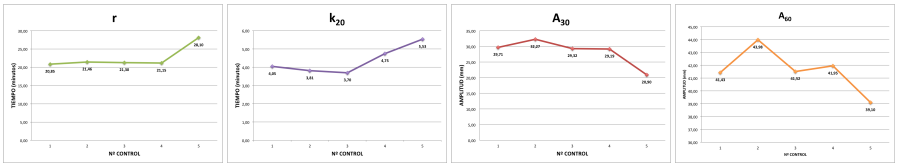




Se han obtenido también los valores de producción lechera por animal, los parámetros de calidad higiénico-sanitaria (recuento de células somáticas y unidades formadoras de colonias) y de composición físico-química (grasa, proteína, lactosa, sólidos totales, extracto seco, cenizas, caseínas y punto crioscópico). Asímismo, se ha aislado suero por ultracentrifugación de la leche con citrato sódico para estudiar mediante técnicas colorimétricas la actividad proteolítica derivada del sistema enzimático Plasmina-Plasminógeno y sus efectos sobre la coagulación.

RESULTADOS (ESTADO ACTUAL DEL TRABAJO)

Se han obtenido los estadísticos descriptivos para todas las variables estudiadas. El análisis de correlación mediante coeficientes de Pearson ha puesto de manifiesto altas correlaciones entre el recuento de células somáticas con el tiempo de coagulación (r) y con la velocidad de endurecimiento de la cuajada (k_{20}). Además, los datos sugieren que el tiempo de coagulación (r) es más corto a medida que descienden los valores de pH. También, a valores ácidos de pH se obtienen geles más firmes tras la coagulación.



Los valores medios obtenidos muestran un importante descenso de la dureza media (A_{30}) y la dureza máxima (A_{60}) de la cuajada conforme avanza la lactación, lo cual se traduce en una mayor pérdida de proteína durante el corte y el desuerado. Los valores para la velocidad de endurecimiento parecen disminuir suavemente hasta alcanzar su mínimo en el tercer mes de lactación, a partir del cual aumentarán de forma muy notable. Sin embargo, los valores obtenidos para el tiempo de coagulación (r) permanecen más o menos estables durante casi toda la lactación, siendo el último mes donde se observa un considerable enlentecimiento en la formación del gel.

BIBLIOGRAFÍA

|1| Albenzio, M., Caroprese, M., Santillo, A., Marino. R., Taili, L., Sevi, A., 2004. Effects of somatic cell count and stage of lactation on the plasmin activity and cheesemaking properties of ewe milk. J Dairy Sci 87, 533-542. |2| Bencini, R., 2002. Factors affecting the clotting properties of sheep milk. J Agr Food Chem 82, 705-719. |3| Fox, P.F., Kelly, A.L., 2006. Indigenous enzymes in milk. Int Dairy J 16, 500-516.

AGRADECIMIENTOS

Este proyecto ha sido cofinanciado por ceiA3, el Ministerio de Educación Cultura y Deporte/Ministerio de Economía y Competitividad, el Banco de Santander y Agrométodos S.A. a través del programa "Doctores en Empresas".

MONOGRAFÍA Herramientas en los sistemas de control de calidad de leche [tierras OVINO 32 nº 004

NUEVAS HERRAMIENTAS EN LOS SISTEMAS DE CONTROL DE CALIDAD DE LECHE DE OVEJA:

EVALUACIÓN DE LA COMPOSICIÓN, MICROBIOLOGÍA Y PROPIEDADES TECNOLÓGICAS PARA LA PRODUCCIÓN DE QUESO

Jiménez, L.[1]; Oliete, B.[1]; Arias, C.[2]; Garzón, A.[2]; Caballero, J.[2]; Romero, J.[3]; Pérez-Guzmán, M.D.[2]; Arias, R.[2]

[1]Centro Regional de Selección y Reproducción Animal (CERSYRA). Consejería de Agricultura de Castilla-La Mancha. Avda. del Vino, 10, 13.300 Valdepeñas, España. Mail: lorenaj@jccm.es

[2]Departamento de Producción Animal. Facultad de Veterinaria. Universidad de Córdoba. Campus de Rabanales, 14071.Córdoba, España. Mail: pa1gasia@uco.es

[3]Laboratorio Interprofesional Lácteo de Castilla-La Mancha, Avda. de Portugal, 42, 45600 Talavera de la Reina, España. Mail: mdperez@jccm.es

CALIDAD DE LA LECHE EN LA ACTUALIDAD DEL SECTOR OVINO LECHERO

La cuenca mediterránea es una de las zonas de implantación del ganado ovino lechero más importantes del mundo. Esta actividad, sobre todo en las regiones más desfavorecidas, tiene una gran importancia en el mantenimiento de la población rural y en la conservación del medio ambiente. El principal aprovechamiento de esta leche es la producción de una multitud de quesos de peculiares características (Manchego, Roquefort, Peccorino Sardo, Idiazábal, Serra da Estrella, etc), una herencia incalculable por su sello de calidad.

España contabiliza un censo de 2.481.119 ovejas de ordeño, en profunda regresión en los últimos años, que se concentra predominantemente en dos Comunidades Autónomas, Castilla y León y Castilla-La Mancha, con un 45% y 31% de las ovejas lecheras, respectivamente (MAGRAMA, 2011). La producción de leche se estima de 368.700 Tn (MAGRAMA, 2011b), siendo el segundo país en producción de la Unión Europea tras Grecia (FAOSTAT), y que es destinada prácticamente en su totalidad a la producción de quesos: 44.800 Tn de queso de oveja y 116.800 de queso mezcla (FENIL, 2010). Los quesos de oveja con calidad diferenciada en España alcanzan una especial importancia, con una producción estimada de 12.681.525 kilos, correspondiendo el 78% de esta producción al Queso Manchego con Denominación Origen Protegida, de especial importancia por su repercusión en el comercio con la Unión Europea y con terceros países, fundamentalmente con Estados Unidos (MAGRAMA, 2010).

El sector se encuentra en la actualidad en un periodo de profunda reestructuración habiéndose caracterizado en los últimos años por dos fenómenos que han condicionado su supervivencia y futuro: la necesidad de incrementar la sostenibilidad y competitividad de las explotaciones e industria transformadora, así como la obligación de cumplir con los requerimientos de calidad. Ambas directrices han precisado de un esfuerzo de organización sectorial, muy distinta según las zonas productivas; basta comparar por ejemplo la organización cooperativa de Castilla y León frente a la de Castilla-La Mancha. Por una parte los esfuerzos de los ganaderos han ido encaminados a conseguir sistemas más productivos, mejorando el manejo y el potencial genético, controlando los aspectos reproductivos, sanitarios y de alimentación, mientras que la industria se ha encaminado a la automatización y normalización de procesos.

Como se ha comentado uno de los objetivos del sector es la obligación de cumplir los requerimientos de calidad. El concepto de calidad es complejo de definir. La Real Academia Española la define como la propiedad o conjunto de características inherentes a algo que permite juzgar su valor. Aplicada a la calidad de un alimento, esta definición pone en evidencia cómo el concepto de calidad es un requisito complejo donde se combinan aspectos tanto de la propia naturaleza del producto, de su utilidad y su aceptación por parte del consumidor. Desde el punto de vista de la calidad de la leche, investigadores como Boyanzoglu y Morand-Fehr (2001) han definido tres aspectos:

- **Calidad nutricional:** Depende de la composición de los principales nutrientes de la leche, como la grasa y la proteína, y de la tipología de éstos (ácidos grasos y caseínas), sin olvidarnos de importantes componentes minoritarios como las vitaminas, minerales y otras sustancias de importancia en el ámbito dietético y sanitario (www.idfdairynutrition.org). En este sentido, es importante señalar aspectos relacionados con los productos lácteos o derivados de la leche, que como alimentos funcionales, están siendo muy demandados por parte del consumidor en los últimos años.

• **Calidad higiénico-sanitaria:** Ligada a la contaminación microbiológica ambiental y química (presencia de residuos de fármacos, pesticidas, micotoxinas, metales pesados, desinfectantes o detergentes), y a aspectos relacionados con enfermedades infectocontagiosas de los animales, particularmente de la mamitis.

• **Calidad sensorial:** Sobre todo en relación con el queso, como producto final, y se podría definir como aquella que es detectable por los órganos de los sentidos: color, olor, sabor, consistencia, que a su vez depende de multitud de factores, entre los que cabría destacar la alimentación o aspectos relacionados con la calidad higiénico-sanitaria.

Pero estos conceptos de calidad están en continua evolución, sobre todo por la propia evolución del mercado y de los requerimientos de los consumidores. Es innegable, que en los últimos años, los aspectos sociales relacionados con la alimentación han cambiado considerablemente. Prácticamente en una generación se ha pasado del uso de alimentos básicos y próximos al consumidor, al consumo de productos con un mayor grado de transformación, que requieren de procesos tecnológicos tanto en su elaboración, como en su conservación y propio uso. Estos aspectos han acuñado la acepción de **Calidad Tecnológica,** que se podría definir como aquellos aspectos relacionados con la capacidad de una materia prima para la fabricación de un producto que sea susceptible de ser aceptado por el propio mercado y valorado por el consumidor. De esta forma, en el momento actual, esta definición engloba muchos de los aspectos comentados anteriormente, por lo que su abordaje requiere de una visión global de la concepción de calidad.

Una vez definido el concepto de calidad es necesario llevarlo a cabo y vigilar su cumplimiento, con el objetivo de valorizar la leche. Para ello tanto las Instituciones como el propio mercado tratan de regularizarla desde distintos aspectos. En los últimos años han tomado especial relevancia los aspectos relacionados con la Seguridad Alimentaria, sobre todos tras la sucesión de diversas crisis alimentarias, entre las que desgraciadamente cabe citar la "enfermedad de las vacas locas" o la contaminación de distintos alimentos con dioxinas. En este sentido, la Unión Europea ha arbitrado el llamado "Paquete de Higiene", compuesto por una serie de reglamentaciones que tratan de asegurar la salud de los consumidores, entre las que es necesario destacar el Reglamento CE nº 178/2002, Reglamento CE nº 852/2004, Reglamento CE nº 853/2004. Estas normas propugnan la necesidad de detectar y controlar los peligros alimentarios presentes en la producción primaria, inspirando la aplicación de Guías de Prácticas Correctas de Higiene a este nivel. Así, los productores de leche deberán tener en cuenta no sólo los parámetros de composición de la leche sino que estarán obligados a controlar todos aquellos aspectos relacionados con la sanidad animal y la transmisión de agentes patógenos, la limpieza de instalaciones y equipos o la posible contaminación provocada por el agua, personal manipulador, plagas, residuos y sustancias peligrosas, realizando un correcto uso de aditivos para piensos y medicamentos para animales.

Concretamente, la producción primaría de leche cruda queda regulada en la Sección IX del Reglamento CE nº 853/2004, modificada por el Reglamento CE nº 1662/2006, que incluye requisitos sanitarios para su obtención, requisitos de higiene de las explotaciones productoras de leche en cuanto a locales y equipos, higiene durante las labores de ordeño, recogida y transporte, así como del personal encargado de estas labores. Esta legislación fija los límites para el recuento de gérmenes totales a 30ºC (RMT), señalando que los operadores de la empresa alimentaria deberán garantizar que la leche cruda de ovino y caprino contenga un número ≤1.500.000 ufc/ml, siendo ≤500.000 ufc/ml si la leche cruda está destinada a la fabricación de productos lácteos sin la aplicación de ningún tratamiento térmico (media geométrica rodante durante un periodo de 2 meses, con dos muestras, por lo menos al mes).

El recuento de células somáticas (RCS) ha sido fijado por la Unión Europea para leche de vaca, y no así para la leche de oveja o cabra, aunque es considerado como un buen método indirecto, sensible y específico para el diagnóstico de su calidad sanitaria (Gonzalo et al., 2002). Indica el número de células sanguíneas, sobre todo polimorfonucleares neutrófilos, que afluyen a la leche como respuesta a la agresión bacteriana. La infección intramamaria es el principal factor de variación del recuento de células somáticas (Bergonier et al., 2003). Sin embargo, la interpretación de los resultados analíticos de la leche en cuanto a su RCS es complicada, sobre todo por los distintos factores no infecciosos implicados en su variación: estado de lactación, edad, tipo de parto, ordeño manual o mecánico, etc (Arias et al., 2012a).

Los aspectos metodológicos por el que se desarrollan estos reglamentos comunitarios para realizar los controles obligatorios por los agentes productores, las condiciones exigibles a los laboratorios de análisis de leche cruda de oveja y cabra, y la actuación de los mismos ante la toma de muestras, análisis y comunicación al órgano competente, están regulados en el Real Decreto 752/2011.

Por otra parte, los quesos con Denominación de Origen Protegida, amparados por el Reglamento UE nº 1151/2012, incluyen en sus Pliegos de Condiciones las características que deben reunir tanto la leche como el queso elaborado. Disponen sus características físico-químicas mínimas, y sólo la DOP Queso Manchego (Resolución 13-10-2010) fija unas características microbiológicas (*Escherichia coli* ß-glucuronidasa+, Estafilococos coagulasa positivos, Salmonella y *Listeria monocytogenes*) más estrictas que las reglamentadas por la normativa comunitaria de obligado cumplimiento (Reglamento CE nº 2073/2005). Por el cumplimiento de estos requerimientos, la leche de oveja Manchega destinada a la elaboración de quesos con DOP tiene un mayor valor; las cotizaciones de la Lonja de Albacete para extracto seco útil fueron en la última semana del año 2012 entre 8,77-9,07 € para leche DOP y de 7,26-7,50 € para leche no DOP (Lonja Agropecuaria para la Mancha, 2013).

La situación de los sistemas de pago por calidad es muy dispar en los distintos países productores e incluso áreas geográficas. Pirisi et al. (2007) apuntan como principal razón de la aún limitada implantación de estos sistemas en muchos países la estructura del sector de los pequeños rumiantes lecheros, con ganaderías de pequeño tamaño, a menudo localizadas en áreas extensas y con una amplia diversidad de sistemas de producción. El sector presenta tal variabilidad de situaciones (sistemas de producción,

nivel productivo y recogida de la leche, criterios de calidad y umbrales aplicados), que se hace imposible establecer sistemas globales para la determinación de criterios de pago por calidad de la leche de los pequeños rumiantes lecheros (Dubeuf y Le Jaouen, 2005). Estos autores señalan que es más beneficioso para el sector que se establezca un sistema de incentivo real, en vez de penalización, sobre los umbrales fijados para cada uno de los parámetros. Estas medidas económicas deben ir acompañadas con acciones de tipo técnico con soluciones eficaces y económicamente viables para los ganaderos, sobre todo mediante la aplicación de programas de selección genética y acciones de asesoramiento en alimentación e higiene de la explotación. Además es necesario un sistema de valoración continua de los procedimientos óptimos y frecuencia de recogida y análisis, así como de los parámetros que deben ser considerados y su peso económico.

En España estos sistemas han estado tradicionalmente sometidos a competición entre las distintas empresas y queserías, estando muy supeditada su aplicación a la situación del mercado, con inciertas relaciones contractuales entre ganaderos y queserías (Pirisi et al., 2007). La Tabla 1 muestra ejemplos de los sistemas de pago por calidad llevados a cabo actualmente en distintas queserías de Castilla-La Mancha, en el que se puede apreciar que paradójicamente no hay uniformidad en los umbrales utilizados en industrias tan cercanas. Además, estos sistemas con distintos valores umbrales de penalización y bonificación podrían provocar situaciones un tanto peculiares. Un ejemplo, lo muestra Ramón et al. (2004) al estudiar un modelo establecido por la industria, en el que se fijaba un umbral de penalización para recuento de células somáticas por encima del 1.500.000 cel/ml y unos umbrales de bonificación por debajo de las 500.000 ó 750.000 cel/ml, que habrían dado lugar a una acomodación en aquellos casos con recuentos entre ambos umbrales, quizás porque el ganadero piense que no compensa el esfuerzo y la inversión económica para alcanzar los umbrales de bonificación.

Tabla 1. Sistemas de pago por calidad de leche de oveja de distintas queserías de Castilla-La Mancha.

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9
Composición físico-química									
Extracto seco útil	F	F	F	F	F	F	F	F	F
Recuento de Gérmenes Totales (x10³ ufc/ml)									
<100	B					B	B	B	B
100-200						B	B	B	B
200-300							B	B	
300-500	P							B	
500-1.000	P			P	P	P	P	P	
1.000-1.500	P		P	P	P	P	P	P	P
1.500-3.000	P	P	P	P	P	P	P	P	P
>3.000	P	P	P	P	P	P	P	P	P
Recuento de Gérmenes Totales (x10³ ufc/ml)									
<500	B			B	B	B	B	B	B
500-750	B				B		B		
750-1.000	B				B		B		
1.000-1.250	P			P			B		
1.250-1.500	P			P	P	P	B		
1.500-2.000	P	P	P	P	P	P		P	
>2.000	P	P	P	P	P	P	P	P	
Nuevos conceptos									
Recuento de E.coli (ufc/ml)									
<100	B								
>1.000	P								
Ganadero joven								B	
Camino									
En buen estado								B	
En mal estado								P	

F: Concepto fijo. B: Concepto variable-Bonificación. P: Concepto variable-Penalización

En otros países como Grecia, Italia y Francia también se han establecido sistemas de pago por calidad basados en criterios clásicos de composición físico-química y los higiénico-sanitarios (RMT y RCS). Además, en Francia, desde hace años se ha establecido un sistema de clasificación de la leche de oveja incluyendo nuevos criterios, siendo de destacar los umbrales de clasificación del índice combinado entre el RMT y el recuento de coliformes (Tabla 2) o la determinación del recuento de esporas butíricas causantes de la hinchazón tardía en quesos de pasta prensada (Tabla 3), que son un importante avance en la consideración de la calidad de la leche como un concepto más integral.

En este contexto, el sector ovino lechero español ha dispuesto entre otras herramientas, la constitución de la Interprofesional Láctea (INLAC). Uno de sus objetivos es establecer un mecanismo de arbitraje objetivo en las transacciones comerciales de compra-venta de leche, que en la actualidad toma una mayor relevancia a la vista de las disposiciones por las que se disponen la obligatoriedad de establecer relaciones contractuales en el sector de la leche y de los productos lácteos (Reglamento UE nº 261/2012 y Real Decreto 1363/2012), una vez que han sido reguladas las organizaciones de productores de leche y las organizaciones interprofesionales en el sector lácteo (Real Decreto 460/2011). Estos contratos pretenden crear un compromiso entre las partes, en el que se especifique entre otros aspectos un precio fijo para la leche o un precio que se calculará combinando varios factores establecidos en el contrato, que pueden incluir indicadores que reflejen los cambios en las condiciones del mercado, el volumen suministrado y la calidad o composición de la leche cruda suministrada. Para facilitar la puesta en práctica de este sistema, INLAC ha diseñado un modelo de contrato en el que se establecen los criterios de calidad obligatorios para gérmenes totales o residuos que establecen la legislación comunitaria, o unas condiciones mínimas para la leche en cuanto al extracto seco útil (≥10%), la acidez (≤23º Dornic), o el punto crioscópico. Tal como explicaba Molina et al. (2009) se trata de fijar un precio en función de una calidad mínima y un precio variable, con bonificaciones o penalizaciones en base al RMT, RCS u otros objetivos de calidad, así como a otros conceptos como por ejemplo el cumplimiento de los Códigos de Buenas Prácticas Ganaderas, la toma en

consideración del recuento de *E.coli* que ya se está empleando en alguna quesería de Castilla-La Mancha, etc (Tabla 1).

En definitiva, vista la evolución del sector en los últimos años, este podría ser un buen momento para realizar un análisis en profundidad de la problemática actual, en el que la aparición de nuevas organizaciones de productores podrían cambiar el *status quo* de la relación entre ganaderos e industria, estableciendo además de relaciones comerciales duraderas y satisfactorias para las partes, nuevos criterios para definir la calidad de la leche de oveja mucho más acordes con lo que las nuevas tendencias del mercado, velando en cualquier caso por la obtención de productos seguros para el consumidor.

Tabla 2. Sistema pago por calidad higiénico-sanitaria de la leche de oveja de las regiones francesas de Roquefort (Campaña 1998) y Pirineos Atlánticos (Campaña 2005).

CLASIFICACIÓN CALIDAD BACTERIOLÓGICA DE LA LECHE (Recuento de Coliformes/RMT)				
Recuento de coliformes (ufc/ml)	Recuento de gérmenes totales (RMT) (ufc/ml)			
	<100.000	100.000-250.000	>250.000	
≤500	3	2	1	
501-2.500	2	1	1	
>2.500	1	0	0	
Clasificación	Puntos mensuales totales			Penalizaciones
	3 controles	2 controles	1 control	
A	9-8	6-5	3	0
B	7-6	4	2	-0,03 €
C	≤5	≤3	1-0	-0,07 €
CALIFICACIÓN CALIDAD SANITARIA (RCST)				
<1.000.000				3
1.000.001-1.500.000				2
>1.500.001				1
Clasificación	Puntos mensuales totales			Penalizaciones
	3 controles	2 controles	1 control	
A	9-8	6-5	3	0
B	7-6	4	2	-0,03 €
C	≤5	≤3	1	-0,07 €

Fuente: (Pirisi et al., 2007)

Tabla 3. Sistema pago por calidad butírica de la leche de oveja de las regiones francesas de Roquefort y Pirineos Atlánticos.

Región de Roquefort. Campaña 1998		
Esporas/L		Puntos
≤ 1.300		3
1.301-2.400		2
≥ 2.400		1
Clasificación	Puntos totales para cuatro controles	Penalización
A	12-10	0
B	9-7	-0,03 €
C	6-7	-0,07 €
Región de los Pirineos Atlánticos. Campaña 2005		
Esporas / L		Puntos
≤ 1000		3
1000-2000		2
≥ 2000		1
Clasificación	Puntos totales para tres controles	Bonificación/ Penalización
Super A	9	+0,061 €
A	8	0
B	5-7	-0,0015 €
C	3-4	-0,0045 €
Super C	Tres análisis>5000	-0,015 €

Fuente: (Pirisi et al., 2007)

NUEVOS CRITERIOS DE CALIDAD DE LA LECHE DE OVEJA

A partir de la información presentada hasta el momento queda patente la falta de uniformidad en los criterios utilizados en los sistemas de pago por calidad de la leche de oveja. Además existe un gran desconocimiento de parámetros de calidad alternativos a los tradicionales que podrían y deberían utilizarse en estos sistemas con el objetivo de optimizar la calidad de la leche. Estos criterios resultan insuficientes en el concepto de calidad global de la leche que se maneja en la actualidad. Es necesario por tanto revisar los criterios de calidad con el fin de determinar los parámetros a incluir en los sistemas de control y pago por calidad de la leche. Sin embargo, es difícil establecer dichos parámetros de calidad ya que estos dependen del uso final que se dará a esa leche. Es decir, si la leche se destina a la elaboración de queso, deben primarse las características que aseguran una coagulación correcta; mientras que, si la leche se destina a leche líquida de consumo, la leche debe asegurar su estabilidad térmica. A continuación se comentan parámetros de calidad alternativos relativos a la composición, carga microbiana, propiedades tecnológicas, y características sensoriales que deberían ser considerados en los sistemas de pago por calidad para que la leche cumpla las expectativas de calidad requeridas. Se hace especial atención a la capacidad de elaborar queso de calidad, ya que la mayor parte de la leche de oveja se destina a elaborar este producto.

► Calidad físico-química

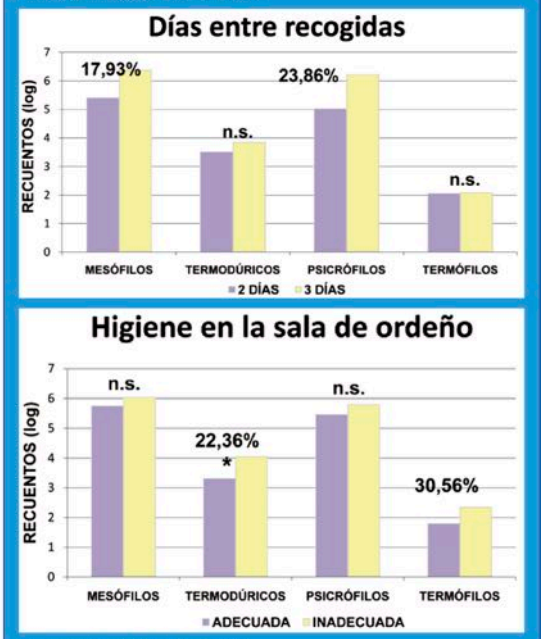
Los componentes mayoritarios de la leche, además de agua, son grasa, proteína y lactosa, y en menor medida, sales orgánicas e inorgánicas, iones, y vitaminas. Entre los componentes de la leche, la proteína y la grasa tienen una importancia fundamental en la producción (rendimiento), composición y características sensoriales de los productos lácteos (Scintu y Piredda, 2007), especialmente en el queso. Por ello estos componentes son los que se han considerado de forma tradicional en los sistemas de control de calidad de la leche de oveja. Además del contenido en estos com-

puestos, ciertos parámetros físico-químicos son importantes indicadores de la calidad de la leche como el punto de congelación, que permite detectar fraudes por adición de agua (Antunac et al., 2001), o el pH, que puede utilizarse como indicador de la calidad higiénica de la leche (Pirisi et al., 2007). Asimismo, existen otros componentes que no se han considerado tradicionalmente a pesar de su importancia en las características tecnológicas, sensoriales y nutricionales de los productos lácteos como son la composición en ácidos grasos, la cantidad y tipología de las caseínas, o la presencia de péptidos bioactivos.

Los ácidos grasos que componen la grasa de la leche tienen gran influencia sobre las propiedades nutricionales, sensoriales y tecnológicas del queso (Ha y Lindsay, 1993; Park et al., 2007). Es importante resaltar el alto contenido de la leche de pequeños rumiantes en ácidos grasos de cadena media, como caproico, caprílico y cáprico (Sanz Sampelayo, 2007), que presentan interés desde el punto de vista terapéutico por su aplicación en ciertas enfermedades metabólicas (Haenlein, 2001). La composición en ácidos grasos varía en función del tipo de alimentación suministrada a las ovejas de ordeño (Addis et al., 2005), demostrando que la variedad de los pastos de la zona mediterránea, contribuyen de forma importante en las características nutricionales de leche y queso. Un claro ejemplo es el ácido linoléico conjugado, ligado a sistemas de manejo semiextensivos, y con efectos beneficiosos para la salud humana (McGuire y McGuire, 2000), en particular por su efecto anticancerígeno y antiaterogénico.

En cuanto a las proteínas, que también se determinan de forma generalizada en los sistemas de control de calidad, hay que distinguir dos tipos: las caseínas y las proteínas del suero. Las caseínas constituyen el 76-83% de las proteínas totales de la leche (Park et al., 2007) y son las responsables de la formación de la cuajada a la hora de elaborar queso. Además hay que tener en cuenta que existen diferentes fracciones caseínicas (α_{s1}-caseína, α_{s2}-caseína, β-caseína y κ-caseína), que intervienen de distinta forma en la coagulación. En ovino los estudios sobre los polimorfismos genéticos de las proteínas lácteas han identificado seis variantes de la α_{s1}-caseína, la más heterogénea de ellas y la que más influye sobre el contenido de caseínas, diámetro de las micelas y propiedades de coagulación (Chianese et al., 1996). En raza Manchega, sus características han sido estudiadas por Garzón (1996). También se han descrito tres variantes (A-C) para α_{s2}-caseína y para β-caseína (Chianese et al., 1995). Dado que las caseínas son las proteínas que intervienen en la coagulación, los sistemas de control de calidad de la leche de oveja deberían determinar su contenido y polimorfismo en lugar del contenido proteico total como se realiza actualmente. Además se ha comprobado que la leche de oveja posee menor contenido en caseínas de tipo α_s que la leche de vaca lo que ocasiona menores sabores amargos en los quesos de oveja que en los de vaca (Assenat, 1991).

Figura 1. Efecto de las condiciones de manejo sobre diferentes grupos de microorganismos de la leche de oveja.



*n.s.: no significativo. *p<0,05. **p<0,01.*

Además de las caseínas, la leche contiene un 17-22% de proteínas que permanecen solubles en el suero cuando se elabora queso. Las más representativas son la α-lactoalbúmina y la β-lactoglobulina (Park et al., 2007). Las inmunoglobulinas, la seroalbúmina del suero, la lactoferrina o los productos de degradación de las caseínas por la acción de la plasmina, entre otras, se encuentran en concentraciones más pequeñas. Es muy importante la relación entre caseínas y proteínas del suero, que condiciona la calidad tecnológica de la leche. El incremento de proteínas del suero debido a infecciones intramamarias, presencia de calostro, etc., haría aumentar el contenido total de proteínas aunque las caseínas, que son las proteínas verdaderamente responsables de la coagulación, se mantendrían constantes.

Recientemente se ha comprobado la existencia en la leche de secuencias de aminoácidos con actividad biológica/fisiológica que provienen de la hidrólisis de las proteínas y que se denominan péptidos activos (Gobbetti et al. 2004). Entre los más estudiados están los fosfopéptidos, los inhibidores del la enzima de la conversión de angiotensina (capacidad antihipertensiva y antitrombótica), péptidos opiáceos o los péptidos inmunomoduladores. Debido a su gran versatilidad, estos compuestos presentan un futuro prometedor en la elaboración de alimentos funcionales y aplicaciones farmacéuticas, y que por consiguiente podrían valorizar la leche convirtiéndose en componentes a controlar en los sistemas de control y pago por calidad de la leche.

El contenido en minerales es otro de los factores que podrían tenerse en cuenta en el control de la calidad de leche debido a su influencia en las propiedades tecnológicas y nutricionales (Park et al., 2007). La leche de oveja, con aproximadamente un 0,9%, posee mayor contenido en minerales que la leche de vaca con un 0,7%. Los niveles de Ca, P, Mg, Zn, Fe y Cu son superiores en la leche de oveja que en la de vaca, aunque tiene menor contenido en K, Na y Mn. Algunos de estos minerales, como el Ca y el P, participan activamente en la coagulación (por acción del cuajo) por lo que su mayor contenido aumentará el rendimiento del proceso. En general el contenido en minerales de la leche de oveja parece fluctuar más que el de la leche de vaca (Rincón et al., 1994) debido fundamentalmente a diferencias en la alimentación.

Según lo expuesto queda claro que al considerar únicamente la cantidad de los componentes mayoritarios, los sistemas de control de la calidad trabajan con información incompleta y claramente insuficiente para asegurar los estándares de calidad necesarios actualmente. Consciente de esta situación, el grupo de investigación formado por el Laboratorio de Lactología del CERSYRA de Valdepeñas, el Laboratorio Lechero del Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad de Córdoba, y el Laboratorio Interprofesional Lácteo de Castilla-La Mancha, estamos estudiando la relación entre distintos parámetros de composición, microbiológicos y las características tecnológicas de la leche de oveja Manchega, que se explicarán a continuación. Estos trabajos se están realizando en el marco del Proyecto Regional PII10-0003 de la Consejería de Educación y Ciencia de la Junta de Comunidades de Castilla-La Mancha, y del Proyecto de Investigación Fundamental Orientados a los Recursos y Tecnologías Agrarias en Coordinación con las Comunidades Autónomas del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RTA2011-00057), cuyos resultados se esperan sean aplicativos para el sector ovino lechero.

► Calidad microbiológica diferencial

El Recuento de Mesófilos Totales (RMT) es el indicador tradicionalmente utilizado para determinar la calidad higiénica de la leche (Gonzalo et al., 2006; Elmoslemany et al., 2009). Sin embargo, este parámetro presenta el inconveniente de ser muy poco específico, ya que la contaminación microbiana de la leche puede provenir de muy diversas fuentes como el interior de la ubre, la suciedad en el ambiente, la maquinaria de ordeño, los tanque de refrigeración, etc. (Claps et al., 1999; Elmoslemany et al., 2009). Cada grupo de microorganismos puede afectar de diferente forma a la calidad de la leche, por lo que es necesario conocer sus efectos así como distinguir su procedencia para establecer las medidas necesarias para su control específico. Hay que tener en cuenta que los grupos de microorganismos presentes en la leche además varían en relación con otros factores como la estación del año, la raza, la producción de leche, el tipo de ordeño o el uso de terapias de secado (Garnica et al., 2013).

Además, el desarrollo de los diferentes grupos de microorganismos depende del tipo de queso a elaborar así como de los tratamientos a los que se somete la leche. Varios estudios (Pinna et al., 1999; Pirisi et al., 2000) han señalado que el uso de leche cruda tiene una influencia positiva en la composición y propiedades sensoriales en la maduración de los quesos. La microflora y enzimas específicos de la leche cruda de cada raza mantienen las características propias del queso, por lo que un RMT excesivamente bajo tras la práctica de tratamientos térmicos podría ser perjudicial para la microflora láctica específica de cada tipo de leche. En el caso de los quesos tradicionales, los principios de precaución de seguridad higiénica deben ser contemplados, aunque sin abandonar la utilización de los procesos tecnológicos tradicionales, lo que habilita el carácter típico y la originalidad tradicional de los quesos. Estos autores propugnan la definición de límites de RMT en función del tipo de queso a producir, ya que un queso fresco o suave con un tiempo corto de maduración es ciertamente más sensible desde el punto de vista microbiológico que un queso de media o larga maduración.

En esta línea, nuestro grupo de investigación está estudiando el efecto que tienen distintos grupos de microorganismos sobre las propiedades tecnológicas de la leche de raza Manchega, que se comentarán posteriormente. Además, para profundizar en el conocimiento de la microflora diferencial presente, se ha analizado la influencia de las condiciones de manejo sobre diferentes grupos de microorganismos clasificados en función de su temperatura de incubación (Oliete et al., 2011). Se ha comprobado, por ejemplo, que al disminuir la frecuencia de recogida de la leche, pasando de recoger cada 2 días a recoger cada 3 días, aumenta significativamente el recuento de mesófilos y de psicrófilos (Figura 1). Los microorganismos psicrófilos son aquellos que se desarrollan a bajas temperaturas, por lo que es normal pensar que cuanto más tiempo pase la leche en el tanque de frío, más se desarrollarán este tipo de microorganismos. Este incremento notable en los psicrófilos puede ocasionar retrasos en las características de coagulación de la leche ("leches perezosas"), y la necesidad del uso de cultivos comerciales que faciliten la coagulación de la leche a la hora de elaborar queso. Es importante recordar que mediante la refrigeración se reduce el desarrollo de los microorganismos de la leche, pero nunca se detiene. De hecho al mantener la leche un día más en el tanque de frío el recuento de mesófilos totales aumenta un 17,93% y el recuento de psicrófilos un 26,83%. Por tanto, elevados recuentos de psicrófilos informan sobre el detrimento de la calidad de conservación de la leche cruda.

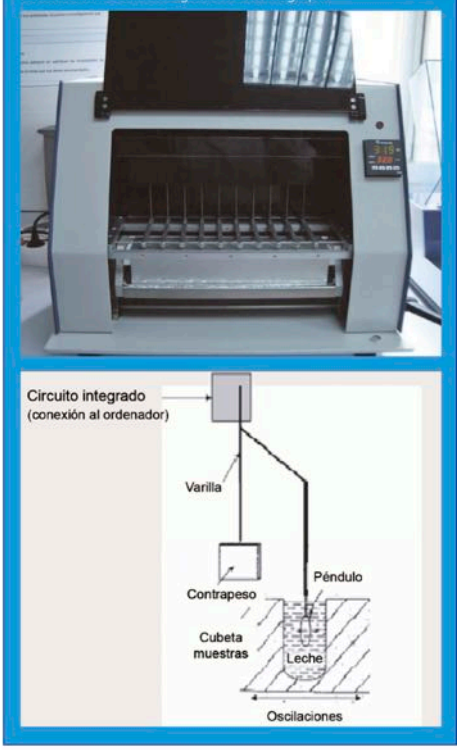
Al analizar las condiciones higiénicas de la sala de ordeño se ha comprobado que condiciones higiénicas deficientes aumentan significativamente el recuento de microorganismos termodúricos y termófilos (Figura 1). Los microorganismos termodúricos, es decir aquellos que resisten la pasteurización de la leche, aumentan un 22,36% cuando las condiciones higiénicas son deficientes. Por su parte, los microorganismos termófilos, aquellos que se desarrollan a temperaturas elevadas (55ºC), aumentan un 30,56%. Estos dos grupos de microorganismos están asociados a incorrectas prácticas de higiene en el ordeño, equipos sucios y depósitos en los circuitos de recogida. Garnica et al. (2013) también han observado que prácticas higiénicas pobres se relacionan con recuentos elevados de

microorganismos Gram negativos. Estos resultados confirman que las condiciones higiénicas de las ganaderías determinan el tipo de microorganismos presentes en la leche de oveja. Estos grupos de microorganismos presentan elevada carga enzimática que pueden afectar a las características finales del queso durante la maduración. No se observan sin embargo, cambios significativos en el RMT, poniendo de manifiesto el carácter inespecífico de este índice como indicador de calidad higiénica. Queda clara por tanto la necesidad de considerar otros grupos de microorganismos en el estudio de la calidad de la leche.

Otro aspecto a considerar en el estudio de la microflora diferencial es la presencia de microorganismos que pueden causar defectos en el queso una vez elaborado. Este es el caso de la contaminación de la leche por esporas butíricas, que como se ha comentado anteriormente, es controlado desde hace años, en algunas zonas productoras como por ejemplo en Roquefort o en Pirineos-Atlánticos. Las especies del género *Clostridium* spp. fermentadoras del lactato presentes en la leche cruda o pasteurizada son las responsables de un defecto del queso denominado hinchazón tardía característico de los quesos madurados de pasta dura o semi-dura (Ingham et al., 1998; Klijn et al., 1995), como por ejemplo el queso Manchego. Los quesos afectados por esta fermentación presentan defectos de textura y aspecto como grietas y cavernas, provocando incluso la ruptura de la masa (Gaggiotti et al., 2006; Inocente y Corradini, 1996). Este defecto tiene lugar durante la maduración del queso, cuando las esporas germinan y se multiplican las formas vegetativas liberando gas (H_2 y CO_2) y ácido butírico. Esta alteración reduce la calidad comercial del producto y, aunque no supone un riesgo para la salud, producen grandes pérdidas económicas.

Tradicionalmente el control de este problema tecnológico se ha llevado a cabo a nivel de quesería mediante la bactofugación, ultrafiltración, microfiltración (Stadhouders, 1990; Waes et al., 1990), o la utilización de aditivos como los nitratos o lisozima, que evitan el crecimiento de las bacterias butíricas (Lodi, 1990; Stadhouders, 1990; Van der Berg et al., 2004). Sin embargo, para evitar los inconvenientes que ocasionan estos métodos, la contaminación butírica de la leche debería empezar a controlarse en la propia ganadería. En los estudios realizados al respecto se ha comprobado que, en general, la leche de tanque muestra una calidad butírica mediocre, aunque se considere de buena calidad respecto a los habituales parámetros considerados en el control de calidad (composición físico-química, recuento de mesófilos totales y recuento de células somáticas) (Arias et al., 2010). Este resultado reafirma la ineficiencia de parámetros tradicionales como el RMT para asegurar la calidad de la leche en cuanto a problemáticas específicas como la hinchazón tardía. Para reducir la contaminación butírica de la leche se ha comprobado que debe vigilarse el recuento de esporas en el alimento, controlando el uso de forrajes ensilados en las mezclas unifeed o el uso de subproductos de la industria como el bagazo de cerveza. Pero además se ha constatado la importancia de extremar las medidas de higiene de la sala de ordeño, evitando la presencia de polvo (Arias et al., 2012b). Existen además otros factores que influyen en los

Figura 2. Lactodinamógrafo Formagraph.
Detalle de modulo de registro del Formagraph.



recuentos de esporas butíricas tales como la época del año o el grado de intensificación de la explotación en relación con su tamaño censal (Arias et al., 2011). Por otra parte se ha comprobado que los factores comentados determinan también las especies de *Clostridium* spp. presentes en la leche de oveja, observándose que las inadecuadas prácticas de manejo favorecen la presencia de *C.beijerinkii* y *C.tyrobutyricum* que además son los mayores productores de gas. El *C.sporogenes*, a pesar de estar presente en todas las muestras de leche, no destaca por su producción de gas (Arias et al., 2012b). Por ello, para evitar el peligro de la hinchazón tardía en queso, debería incluirse el recuento de bacterias butíricas en los sistemas de control de la calidad de la leche. De hecho, su determinación podría realizarse con la metodología actual, a partir de las muestras de leche conservadas con azidiol, ya que se ha comprobado que la presencia de este conservante no influye en el resultado de los recuentos de esporas butíricas de la leche.

A partir de lo expuesto es necesario realizar un estudio diferencial de la flora microbiana presente en la leche a la hora de establecer los sistemas de control y pago por calidad. Además de definir los grupos de microorganismos que deberían controlarse en función de la utilidad final de la leche, es necesario establecer los límites aceptables de cada uno de estos microorganismos. Por otro lado, habría

que estudiar dichos microorganismos no solo a nivel de especie, sino también a nivel de cepa ya que su peligrosidad puede variar. Asimismo hay que tener en cuenta que todos los microorganismos presentes en la leche forman un complejo sistema en equilibrio y que el desarrollo de un grupo, modificaría el crecimiento del resto, incluidas las bacterias beneficiosas como la flora láctica autóctona, que confiere unas características diferenciales a cada tipo de leche debido a su metabolismo enzimático específico que participa en la diferenciación del producto final. Todos estos aspectos ponen de manifiesto la necesidad de realizar estudios más profundos respecto a qué grupo específico de microorganismos debería incluirse por su interés en los sistemas de control de calidad de la leche.

Figura 3. Gráficos de coagulación de diferentes muestras de leche obtenidos del Formagraph.

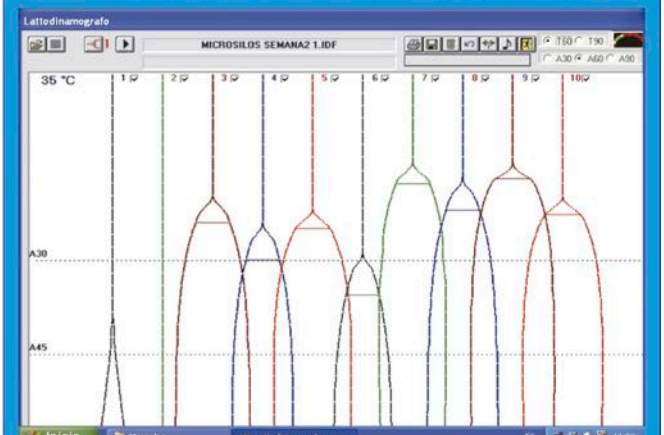
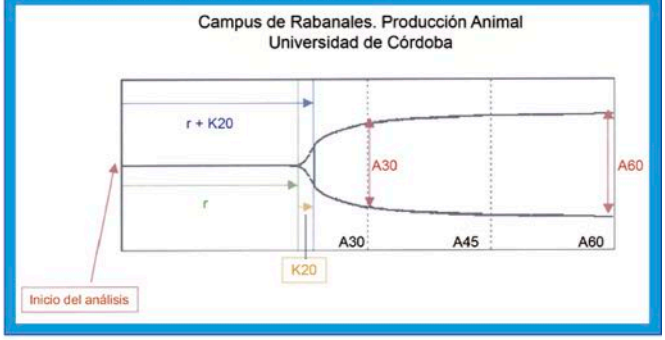


Figura 4. Diagrama de los parámetros de coagulación de la leche.



► Propiedades tecnológicas

Los criterios de composición y características microbiológicas deben verse ampliados con el análisis de las características tecnológicas de la leche, más aún si tenemos en cuenta que prácticamente la totalidad de la leche de oveja se destina a la elaboración de queso.

La calidad tecnológica de la leche puede ser valorada globalmente por su aptitud para obtener un buen queso, bajo condiciones particulares de trabajo y con rendimiento satisfactorio. El comportamiento de la leche frente al cuajo puede ser considerado como el carácter fundamental y definitorio de la calidad tecnológica (Lenoir y Schneid, 1990), aunque ello depende de múltiples factores (composición química de la leche, riqueza en caseínas, naturaleza y carga microbiana, etc).

Las propiedades de coagulación de la leche se han estudiado por diversos métodos (vidrio de cobalto, fotometría, Gelograph, Optigraph, Formagraph). En nuestro estudio hemos utilizado métodos lactodinamograficos, como el que utiliza el Formagraph (Foss-Electric, Hillerød, Dinamarca) (Figura 2), por sus satisfactorios resultados a la hora de caracterizar las propiedades de coagulación y eficiencia de la leche (Büeler, 2002), y que se basa en el movimiento oscilatorio de péndulos circulares inmersos en muestras de leche que se encuentran en proceso de coagulación por adición de cuajo. El registro del aparato muestra una línea vertical mientras la leche permanece líquida. Al dar comienzo la coagulación, por el aumento de la viscosidad de la leche, el aparato registra puntos laterales que forman el típico gráfico de coagulación en forma de campana (Figura 3).

De este análisis se recogen cuatro parámetros para definir la calidad tecnológica de la leche (Figura 4):

- r.- Representa el tiempo transcurrido hasta la formación del coágulo (tiempo de coagulación). Se determina midiendo la distancia desde el origen del análisis hasta el punto donde la curva comienza a abrirse (1 mm de apertura). El resultado viene expresado en minutos.
- K20.- Representa la velocidad de endurecimiento del coágulo y es la distancia media entre el inicio del análisis y el punto donde la campana presenta una amplitud de 20 mm. El resultado se expresa en minutos.
- A30 y A60.- Representan la dureza del coágulo a los 30 (dureza media) y a los 60 minutos (dureza máxima) y se expresan en milímetros, según la amplitud que presente la campana en esa línea.

Además de los parámetros de calidad de la cuajada que hemos comentado, también se ha considerado interesante en nuestras experiencias el cálculo del rendimiento de la cuajada de los distintos tipos de leche, es decir la cantidad de cuajada obtenida a partir de cada leche.

A pesar de la información que los parámetros tecnológicos suministran para la mejora proceso de elaboración

de queso, éstos no son utilizados en los sistemas de control de calidad, probablemente debido a la necesidad de disponer de un equipamiento específico y al elevado coste que supondría un análisis adicional de este tipo. Sin embargo, la información aportada es de gran valor para el proceso de producción quesera. Por ello, hemos considerado interesante estudiar las características tecnológicas y conocer como éstas se ven modificadas por otros parámetros de calidad lechera (físico-químicos o microbiológicos). Nuestros resultados indican que al aumentar el porcentaje en grasa, proteína, lactosa y extracto seco también aumenta la dureza media y máxima del coágulo y el rendimiento en cuajada (Jimenez et al., 2011), aunque otros autores (Bencini, 2002 Garzón, 1996; Jaramillo et al., 2008) han realizado estudios similares con resultados dispares. Las discrepancias podrían deberse a los distintos métodos empleados en su estudio, señalando la necesidad de uniformizar métodos de análisis. Además, las diferencias podrían indicar la existencia de una relación no lineal entre los parámetros tecnológicos y de composición de la leche que sería necesario determinar en futuras investigaciones.

Tabla 4. Estimación de los parámetros tecnológicos en relación con la composición y las características higiénico-sanitarias.

Ecuación de estimación	R^2
(r+K20) = 65,89-53,83*Cas/Prot-4,94*logRMT+9,97*Prot-2,42*Grasa	0,35
A60 = -18,91+11,15*Lac+ 11,51*Grasa+2,51*logRCS+10,09*Prot-9,00*ES	0,14 a pH=6,5
RC = -1,18+ 0,03*ES+0,18*Cas+0,26*logRCS+ 0,16*Grasa	0,54

Para profundizar en el tema se han realizado estudios estadísticos para relacionar las propiedades tecnológicas con el resto de parámetros físico-químicos e higiénico-sanitarios, para lo cual se han calculado una serie de ecuaciones de regresión (Jiménez et al., 2012), que se muestran en la Tabla 4. En ellas se observa que los parámetros tecnológicos dependen de los parámetros de composición así como del recuento de gérmenes totales y del recuento de células somáticas de la leche. La ecuación obtenida para estimar (r+K20) señala al parámetro caseína/proteína como el de mayor peso, incluyendo además el RMT y otros parámetros de composición como la grasa y la proteína. Bencini (2002) también señala la importancia de las proteínas sobre r y K20. La ecuación de regresión de la dureza máxima del coágulo (A60) de la leche tras normalizar el pH a 6,5 incluye parámetros de composición (grasa, proteína, extracto seco y lactosa) y el recuento de células somáticas (logRCS). La relación entre el recuento celular y la dureza del coágulo también ha sido señalada por Pirisi et al. (2000) en un estudio sobre la influencia de las células somáticas en la composición de leche y calidad del queso. La ecuación de regresión obtenida para estimar el rendimiento en cuajada incluye el extracto seco como el parámetro de mayor importancia, además de otras características de composición (caseína bruta, grasa) e higiénico-sanitarias (logRCS, logRMT). Jaramillo et al. (2008), en un estudio sobre la aptitud quesera de dos razas lecheras también destaca la influencia significativa de la grasa, la proteína, la caseína y los sólidos totales sobre el rendimiento de la cuajada.

Asimismo, es importante reflejar otros estudios que relacionan las propiedades tecnológicas y el tipo de proteínas presentes en la leche. La mayor parte de las que se refieren a caseínas se han centrado en el efecto del polimorfismo de la κ-caseína (κ-Cn) sobre el tiempo de coagulación (El Negoumy, 1972), la velocidad de endurecimiento y la dureza del coágulo (Feagan et al., 1970, 1972). Todos los autores coinciden, en general, en que la leche que posee la variante κ-Cn B es mejor que la que presenta la variante A, al tener la primera un tiempo de coagulación menor, una velocidad de endurecimiento mayor y producir un coágulo más firme (Mariani and Pecorani, 1991; Delacroix-Buchet et al., 1993). Con respecto a otras caseínas, parece que la leche que contiene α_{S1}-caseína BC es mejor, en términos de aptitud tecnológica, que la que presenta la variante BB (Mariani et al., 1988) y que la β-caseína BB es mejor también que la de tipo β-caseína AA (Pecorani and Mariani, 1990), estando este último fenotipo asociado a la formación de un coágulo extremadamente blando (Sandler et al., 1968). En la raza Merina, Serrano (1999) encuentra que aquellos animales con un fenogrupo caracterizado como β-L gBB, α_{S1}-Cn CC y β-Cn K- , son los que muestran los valores más favorables en cuanto a producción, composición y aptitud tecnológica de la leche en la población estudiada.

Además, se han realizado estudios que relacionan las características tecnológicas de la leche con las proteínas del lactosuero, fundamentalmente con la β-lactoglobulina. En raza Manchega, Garzón (1996), identifica al alelo β-lgA como el que confiere a la leche una mejor aptitud frente a la coagulación. Sin embargo, Nudda (2003), señala que el fenotipo de la β-lg afecta únicamente a la cantidad de leche ordeñada en la raza Sarda.

Los estudios realizados ponen de manifiesto la importancia de los parámetros tecnológicos de la leche a la hora de establecer los sistemas de control y pago por calidad. La tasa y la extensión de la coagulación de la leche por la acción del cuajo y las características del coágulo resultante son factores muy importantes a considerar durante el proceso de fabricación del queso. La reactividad de la leche con el cuajo determina el desarrollo completo del proceso de coagulación. Así, las leches anómalas, especialmente las de coagulación lenta no pueden emplearse de una forma útil en la coagulación, en especial en la fabricación de quesos con un largo período de maduración, dotados de características físico-químicas y estructurales peculiares, ya que determinan la formación de un coágulo con escasa masa caseínica y no uniformemente deshidratado, del que deriva un queso estructural y organolépticamente defectuoso (Mariano and Pecorari, 1991).

► Características sensoriales

Además de las metodologías empleadas para la caracterización de los alimentos tradicionales, no debemos obviar que el análisis sensorial realizado por un grupo de catadores expertos también podría ser considerado como criterio de los sistemas de control y pago por calidad. Estos análisis basados en la necesidad de evaluar los estímulos que afectan a los órganos de los sentidos, tiene la gran ventaja de facilitar un acercamiento entre el analista y el consumidor. Por esta razón, mientras las técnicas instrumentales permiten obtener información de la composición y de las características nutricionales del alimento, a veces difíciles de

entender por el público, los resultados obtenidos mediante análisis sensorial podrían permitir al consumidor asimilar el producto valorando sus características específicas.

En el ámbito lácteo, la cuestión que se plantea es si atender a las características sensoriales de la leche en sí (aspecto, textura, aroma, etc.) o a las características sensoriales del producto transformado (queso), que al fin y al cabo es el que va a llegar al consumidor. Como ya se ha comentado, la mayor parte de la leche de oveja se destina a la producción de queso y por lo tanto el análisis sensorial ideal sería aquel que evaluara las características del producto final y que permitiera relacionarlo con la leche usada para su producción.

Este análisis sensorial del queso no solo debe abordarse desde un punto de vista objetivo, sino también de manera consistente con la tradición del producto y con las percepciones del consumidor y las posibles diferencias culturales. En el marco del Programa AIR 2039 de la Comunidad Europea, se publicó la "Guía para la evaluación sensorial de la textura de quesos de pasta dura o semidura de leche de oveja". Para ello, se propone el análisis de variables de superficie (visuales y táctiles), mecánicas (elasticidad, firmeza, friabilidad y adherencia), geométricas (microestructura) y otras (solubilidad y humedad en boca). La conclusión a la que se llegó es que la metodología expuesta puede aplicarse a diversos campos: caracterización básica y específica de los diferentes tipos de queso dentro de los ámbitos de las Denominaciones de Origen Protegidas, validación de técnicas instrumentales para el control de la calidad y la defensa de las particularidades frente a las imitaciones, y finalmente la investigación de las preferencias de segmentos del mercado (elección, aceptación y consumo).

En la actualidad distintos quesos de calidad diferenciada con DOP, como es el caso del queso Manchego, valoran sus características sensoriales (consistencia, color, olor, sabor, aspecto, textura), que están perfectamente definidas en sus pliegos de condiciones, tanto para la Certificación del producto como en los sistemas de control de calidad interno de las propias queserías.

La inclusión de la calidad sensorial en los criterios de pago por calidad lechera podría resultar compleja en un principio, sin embargo debería ayudar a calificar la calidad integral de una leche de partida para obtener un queso con unas características finales esperadas, permitiendo el acercamiento entre la calidad de la leche y las expectativas del consumidor.

CONCLUSIÓN

La información aportada en los estudios presentados pone de manifiesto la necesidad de revisar los actuales sistemas de control y pago por calidad de la leche de oveja teniendo en cuenta nuevos criterios de calidad que complementen los actuales. Sin embargo, aún estamos lejos de poder definir claramente estos criterios de calidad, debido a la multitud de factores que determinan la calidad de la leche de oveja. Además, hay que tener en cuenta que los sistemas de control de calidad de la leche deberían estar adaptados a las diferentes zonas productivas, que determinan además de las condiciones de manejo, la utilidad ir orientados a evaluar las características de calidad en conjunto y establecer índices combinados a partir de estos criterios que permitan establecer la calidad integral de la leche de oveja.

REFERENCIAS BIBLIOGRÁFICAS

Addis, M., Cabiddu, A., Pinna, G., Decandia, M., Piredda, G., Pirisi, A., Molle, G., 2005. Milk and cheese fatty acid composition in sheep fed Mediterranean forages with reference to conjugated linoleic acid cis-9, trans-11. J. Dairy Sci., 88, 3443–3454.

Antunac, M., Havranek, J., Samarzua, D., 2001. Freezing point of goat milk. Milchwissensch 56, 14–16.

Arias, C., Oliete, B., Pérez-Guzmán, M.D., Arias, R., 2010. Problemática de la contaminación por esporas butíricas de la leche de oveja manchega. Relación con las condiciones higiénico-sanitarias y alimentación animal. Consorcio Queso Manchego, 17, 7-9.

Arias, C., Oliete, B., Pérez-Guzmán, M.D., Arias, R., 2011. Incidencia de la contaminación por esporas butíricas en la leche de tanque de oveja manchega y su relación con la alimentación animal y las condiciones higiénico-sanitarias de la ganadería. Tierras Ganadería, 184, 60-62.

Arias, R., Oliete, B., Ramón, M., Arias, C., Gallego, R., Montoso, V., Gonzalo, C, Pérez-Guzmán, M.D. 2012a. Long-term study of environmental effects on test-day somatic cell count and milk yield in Manchega sheep. Small Rumin. Res. 106, 92-97.

Arias, C., Oliete, B., Seseña, S., Jimenez, L., Pérez-Guzmán, M.D., Arias, R., 2012b. Importance of on-farm management practices on lactate-fermenting Clostridium spp. spore contamination of Manchega ewe milk: Determination of risk factors and characterization of Clostridium population. Small Rumin. Res. http://dx.doi.org/10.1016/j.smallrumres.2012.11.030

Assenat, L., 1991. Leche de oveja. Composición y propiedades. En: Leche y productos lácteos. Vaca, oveja, cabra. 1. La leche, de la mama a la lechería. F.M. Luquet (Ed.). Acribia, Zaragoza, 390pp.

Bencini, R., 2002. Factors affecting the clotting properties of sheep milk, J. Sci. Food Agric., 82, 705-719.

Bergonier, D., De Crémoux, R., Rupp, R., Lagriffoul, G, Berthelot, X. 2003. Mastitis of dairy small ruminants. Vet. Res. 34, 689-716.

Boyazoglu, J., Morand-Fehr, P. 2001. Mediterranean dairy sheep and goat products and their quality. A critical review. Small Rum. Res. 40, 1-11.

Büeler, T., 2002. Casein-Polymorphismus und gerinnungsrelevante Eigenschaften von Milch Schweizerischer Ziegenrassen. Tesis doctoral. Eidgenössischen Technischen Hochschule Zürich. 144 pp.

Claps, S., Rubino, R., Zarriello, G., Di Gennaro, P., Amato, D., Pizzillo, M., Petrizzi, N., Bochicchio, R., 1999. Situation des explotations laitières ovines et caprines vis á vis des dispositions de la directive UE 92/46. In: Barillet, F., Zervas, N.P. (Eds.), Proceedings of the Sixth International Symposium on the Milking of Small Ruminants. Milking and Milk Production of Dairy Sheep and Goats, Athens, Greece. Wageningen Pers, Netherlands: 286-291.

Chianese, L., Garro, G., Ferranti, P., Malorni, A., Addeo, F., Rabasco, A., Molina Pons, P., 1995. Discrete phosphorylation generates the electrophoretic heterogeneity of ovine β-caseína. J. Dairy Res., 62, 89-100.

Chianese, L., Garro, G., Mauriello, R., Laezza, P., Ferranti, P.N., Addeo, F., 1996. Occurrence of five αs1-caseína variants in ovine milk. J. Dairy Res., 63, 49-59.

Delacroix-Buchet, A., Lefier, D., Nuyts-Petit, V., 1993. Polymorphisme de la caseine-k de trois races bovines françaises et aptitude à la coagulation. Lait. ,73(1), 61-72.

Dubeuf, J.P., Le Jaouen, J.C. 2005. The sheep and goat dairy sectors in the European Union: present situation and stakes for the future. International Dairy Federation Bulletin 501, 1-7.

El Negoumy, A.M. 1972. Effect of polymorphic composition of calcium caseínate sol son their stability to rennin. J.Dairy Res., 39, 373-379.

Elmoslemany, A.M., Keefe, G.P., Dohoo, I.R., Jayarao, B.M., 2009. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1: Overall risk factors. J. Dairy Res. 92, 2634–2643.

FAOSTAT. Base de datos de la Organización de la Naciones Unidas para la Agricultura y la Alimentación.
http://faostat3.fao.org/home/index_es.html?locale=es#VISUALIZE. Consultada el día 19 de febrero de 2013.

Feagan, J.T., Hehir, A.F., Bailey, L.F.,1970. Factors influencing the heat stability and curd firmness of bovine milk. 28th Int. Dairy congress.

Feagan, J.T., Bailey, L.A., Hehir, A.F., Mclean, D.M., Ellis, N.J.S. 1972. Coagulation of milk proteins.I.Effect of genetic variants of milk proteins on rennet coagulation and heat stability of normal milk. Aust.J.Dairy Technol., 27, 129-134.

FENIL. 2010. Producción de leche y productos lácteos.
http://www.fenil.org/Documents/Sector/Produccion/LECHE%20Y%20P.%20LÁCTEOS%202010.doc. Consultada el día 19 de febrero de 2013.

Gaggioti, M., Calvinho, L.F., Reinheimer, J.A., 2006. Reducción de la contaminación por clostridios gasógenos. En: Avances en microbiología, bioquímica y tecnología de quesos. Reinheimer, J., Zalazar, C. (Eds.). Universidad Nacional del Litoral: Santa Fe, Argentina: 23-43

Garnica, M.L., Linage, B., Carriedo, J.A., De la Fuente, L.F., García-Jimeno, M.C., Santos, J.A., Gonzalo, C., 2013. Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk. J. Dairy Sci. 96, 1-9.

Garzón, A., 1996. Incidencia de las variantes genéticas de las proteínas lácteas sobre la aptitud tecnológica de la leche en ovejas de raza Manchega. Tesis Doctoral. Universidad de Córdoba. 301 pp.

Gobbetti M., Minervini F. & Rizzello C.G., 2004. Angiotensin I-converting-enzymeinhibitory and antimicrobial bioactive peptides. Int. J. Dairy Technol., 57,173–188.

Gonzalo, C., Ariznabarreta, A., Carriedo, J.A., San Primitivo, F. 2002. Mammary pathogens and their relationship wiht somatic cell count and milk yield losses in dairy ewes. J. Dairy Sci. 85, 1460-1467.

Gonzalo, C., Carriedo, J.A., Beneitez, E., Juárez, M.T., De la Fuente, L.F., San Primitivo, F., 2006. Bulk Tank Total Bacterial Count in Dairy Sheep: Factors of Variation and Relationship with Somatic Cell Count. Short Communication. J. Dairy Res 89, 549-552.

Ha, J.K., Lindsay, R.C., 1993. Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases. J. Dairy Sci. 76, 677-690.

Haenlein, G.F.W., 2001. The concept of milk quality in the USA. Int. J. Anim. Sci.16 (1), 5-8.

Ingham, S.C., Hassler, J.R., Tsai, Y., Ingham, B.H., 1998. Differentiation of lactate-fermenting, gas-producing Clostridium spp. Isolated from milk. Int. J. Food Microbiol. 43, (19), 173-183.

Inocente, N., Corradini, C., 1996. Use of low ripening temperature to control anomalous fermentations in Montasio cheese. Scienza e Tecnica Lattiero-Casearia, 47, (18), 89-102.

Jaramillo, D.P., Zamora, A., Guamis, B., Rodríguez, M., Trujillo, A.J., 2008. Cheesemaking aptitude of two Spanish dairy ewe breeds: Changes during lactation and relationship between physico-chemical and technological properties. Small Rumin. Res., 78, 48-55.

Jiménez, L., Oliete, B., Garzón, A., Romero, J. Calatayud, J.R., Pérez-Guzmán, M.D., Arias, R., 2012. Estimación de las características tecnológicas de la leche de tanque de oveja manchega a través de sus características cualitativas. XXXVII Congreso de la Sociedad Española de Ovinotecnia y Caprinotecnia (SEOC). Actas del congreso, 189-193. 19-21 de septiembre de 2012. Ciudad Real (España).

Jiménez, L., Oliete, B., Garzón, A., Romero, J., Arias, C, Calatayud, J.R., Pérez-Guzmán, M.D., Arias, R., 2011. Relación de los parámetros tecnológicos de leche de tanque de oveja manchega con su composición y carga microbioana. Resultados preliminares. XXXVI Congreso de la Sociedad Española de Ovinotecnia y Caprinotecnia (SEOC). Actas del congreso, 176-180. 6-7 de octubre de 2011. San Sebastián (España).

Klijn, N., Nieuwenhof, F.F.J. Hoolwerf, J.D., Waals, C.B.V.D., Weerkamp, A.H., 1995. Identification of Clostridium tyrobutyricum as the causative agent of late blowing in cheese by speciesspecific PCR amplification. Appl. Environ. Microbiol., 61 (18), 2919–2924.

Lenoir, J. and Scheneider. 1990. La aptitud de la leche a la coagulación por el cuajo. En: El Queso. A. Eck (Ed.). Editorial Omega (España).

Lodi, R. 1990. The use of lysozyme to control butyric acid fermentation. Bulletin of the International Dairy Federation, 251, 51-54.

Lonja Agropecuaria para la Mancha. Precios Indicativos de Origen. Albacete. Cotizaciones de 14.02.2013. Semana 7. 2013. Instituto Técnico Agronómico Provincial (ITAP). Diputación de Albacete.
http://www.itap.es/ITAP-LONJA/3publicaciones/Documentos/Periodicas/LONJA%2014-02-2013.pdf

MAGRAMA. 2010. Datos de las Denominaciones de Origen Protegidas (DOP) e Indicaciones Geográficas Protegidas (I.G.P.) de Productos Agroalimentarios.
http://www.magrama.gob.es/es/alimentacion/temas/calidad-agroalimentaria/DATOS_DOPs-IGPs_2010._tcm7-201461.pdf. Consultada el día 19 de febrero de 2013.

MAGRAMA. 2011. Efectivos ganaderos.
http://www.magrama.gob.es/es/estadistica/temas/estadisticas-agrarias/Ovino- caprino_web_Nov2011_tcm7-207830.pdf. Consultada el día 19 de febrero de 2013.

Mariani, P, Bonatti, P., Pecorari, M., 1988. Rennet coagulation properties of cow milk in relation to alpha s1-casein genotype. Sci. Tecn. Latt.-casearia., 39, 431-438.

Mariani, P. and Pecorari, M., 1991. Il ruolo delle variante genetiche Della k-caseína nella produzione del formaggio. Sci. Tec. Latt-Casearia. 42 (4), 255-285.

Mcguire, M.A., Mcguire, M.K., 2000. Conjugated linoleic acid (CLA): A ruminant fatty acid with beneficial effects on human health. Proc. Am. Soc. Anim. Sci. Annu. Mtg. 1999. Online. Available: http://www.asas.org/jas/symposia/proceedings/0938.pdf.

Molina, M.P., Berruga, M.I., Molina, A. 2009. Calidad de la leche de oveja. Ovinotecnia. Produccion y Economía en la especie ovina. Ed. Prensas Universitarias de Zaragoza, España, 355-367.

Nudda, A., Feligini, M., Battacone, G., Macciotta, N.P.P., Pulina, G. 2003. Effects of lactation stage, β-genotype and milk SCC on whey protein composition in Sarda dairy ewes. Ital. J. Anim. Sci. 2, 29-39.

Oliete, B., Arias, R., Jiménez-Sobrino, L., Garzón, A., Romero, J., Calatayud, J.R., Arias, C., Pérez-Guzmán, M.D., 2011. Presencia de Staphylococcus en leche de oveja de raza Manchega y su influencia sobre los parámetros de coagulación de la leche. Tierras Ganadería 182, 30-33.

Park, Y.W., Juárez, M., Ramos, M., Haenlein, G.F.W., 2007. Physico-chemical characteristics of goat and sheep milk. Small Rumin. Res. 68, 88-113.

Pecorari, M, Mariani, P.,1990. Caseína, attitudine alla coagulazione del latte, resa e qualitá del formaggio. Sci. Tec. Latt.-casearia., 41, 225-244.

Pinna, G., Pirisi, A., Piredda, G., Addis, M., Di Salvo, R. 1999. Effetto della termizzazione del latte sul formaggio DOP Fiore Sardo. 2. Andamento della lipolisi nel corso della maturazione. Effect of milk thermization on Fiore Sardo PDO cheese. 2. The progress of lipolysis during ripening. Sci. Tecn. Latt.-Cas. 50, 366-377.

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

Pirisi, A., Piredda, G., Corona, M., Pes, M., Pintus, S., Ledda, A., 2000. Influence of somatic cell count on ewe's milk composition, cheese yield and cheese quality. Pages 47–59 in Proc. 6th Great Lakes Dairy Sheep Symposium, Ontario, Canada. D. L. Thomas and S. Porter, ed.

Ramón, M., Román, M., Arias, R., Gallego, R., Pérez-Guzmán, M.D. 2004. Recuento de células somáticas (RCS) en leche de tanque en ganaderías de ovino manchego. En: XXI Jornadas del Grupo de Técnicos Especialistas en Mamitis y Calidad de la Leche. Oviedo 8 y 9 de octubre, 52-53.

Real Decreto 1363/2012, de 28 de septiembre, por el que se regula el reconocimiento de las organizaciones de productores de leche y de las organizaciones interprofesionales en el sector lácteo y se establecen sus condiciones de contratación (BOE nº 79, de 2 de abril de 2011, p. 34315-34323).

Real Decreto 460/2011, de 1 de abril, por el que se regula el reconocimiento de las organizaciones de productores de leche y de las organizaciones interprofesionales en el sector lácteo en relación a la normativa europea que modificara para el sector lácteo el Reglamento (CE) no 1234/2007 del Consejo (BOE nº 79, de 2 de abril de 2011, p. 34315-34323).

Real Decreto 752/2011, de 27 de mayo, por el que se establece la normativa básica de control que deben cumplir los agentes del sector de leche cruda de oveja y cabra (BOE nº 237, de 2 de octubre de 2012, p. 69843-69857).

Reglamento (CE) nº 178/2002 del Parlamento Europeo y del Consejo, de 28 de enero de 2002, por el que se establecen los principios y requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria. (DO L 31 de 1.2.2002, p. 1/24).

Reglamento (CE) nº 2073/2005 de la Comisión de 15 de noviembre de 2005 relativo a los criterios microbiológicos aplicables a los productos alimenticios (DO L 338 de 22.12.2005, p. 1/26).

Reglamento (CE) nº 852/2004 del Parlamento Europeo y del Consejo, de 29 de abril de 2004, relativo a la higiene de los productos alimenticios. (DO L 139 de 30.4.2004, p. 1/54).

Reglamento (CE) nº 853/2004 del Parlamento Europeo y del Consejo de 29 de abril de 2004, modificado por el Reglamento (CE) Nº 1662/2006, por el que se establecen normas específicas de higiene de los alimentos de origen animal. (DO L 139 de 30.4.2004, p. 55/205).

Reglamento (UE) nº 1151/2012 del Parlamento Europeo y del Consejo de 21 de noviembre de 2012 sobre los regímenes de calidad de los productos agrícolas y alimenticios (DO L 343 de 14.12.2012, p. 29)
Reglamento (UE) nº 261/2012 del Parlamento Europeo y del Consejo de 14 de marzo de 2012 que modifica el Reglamento (CE) n o 1234/2007 del Consejo en lo que atañe a las relaciones contractuales en el sector de la leche y de los productos lácteos (DO L 94 de 30.3.2012, p. 38/48)
Resolución 13-10-2010 de la Consejería de Agricultura y Medio Ambiente de la Junta de Comunidades de Castilla-La Mancha, por la que se emite decisión favorable en relación con la solicitud de modificación del pliego de condiciones de la Denominación de Origen Protegida Queso Manchego. [2010/17415] (DOCM nº 203 de 20-X-2010, p. 47583-47593). Corrección de errores DOCM nº 100 de 22-V-2012, p. 17719-17726.
Rincón, F., Moreno, R., Zurera, G., Amaro, M., 1994. Mineral composition as a characteristic for the identification of animal origin of raw milk. J. Dairy Res. 61, 151–154.
Sadler, A.M., Kiddy, C.A., McCann, R.E., Mattingly, W.A., 1968. Acid production and curd toughness in milks of different ⊠s1-Casein types. J.Dairy Sci., 51, 28-30.
Sanz Sampelayo, M.R., Chilliard, Y., Schmidely, P.H., Boza, J., 2007. Influence of type of diet on the fat constituents of goat and sheep milk. Small Rumin. Res. 68, 42-63.
Scintu, M.F., Piredda, G., 2007. Tipicity and biodiversity of goat and sheep milk products. Small Rumin. Res. 68, 221-231.
Serrano, B.; Garzón, A.I.; Garro, G.; Chianese, L.; Martínez, J.1999. Variabilidad genética de caseína en la raza ovina Merina. Arch. Zootec. 48, 197-206.
Stadhouders, J., 1990. Prevention of butyric acid fermentation by the use of nitrate. Bulletin of the International Dairy Federation 251, 40-46.
Van Der Berg, G., Meijer, W.C., Düsterhöft, E.M., Smit, G., 2004. Gouda and Related Cheese. En: Fox, P.F.; McSeeney, P.L.H.; Cogan, T.M.; Guinee, T.P. (Eds.). Cheese: Chemistry, Physics and Microbiology. Vol. 2: Major Cheese Groups, 3rd ed. Elsevier Academic Press, London, UK: 103-140.
Waes, G., Van Heddeghem, A., 1990. Prevention of butyric acid fermentation by bacterial centrifugation of cheese milk, FIL nº 251, 50.

References

ABILLEIRA, E., VIRTO, M., NÁJERA, A., SALMERÓN, J., ALBISU, M., PÉREZ-ELORTONDO, F. J., RUIZ DE GORDOA, J. C., DE RENOBALES, M. and BARRON, L. J. R. Effects of seasonal changes in feeding management under part-time grazing on the evolution of the composition and coagulation properties of raw milk from ewes. *Journal Of Dairy Science*, vol. 93, pages 3902–3909, 2010.

AGRAMA. *Reglamento del Libro Genealógico de la Raza Ovina Manchega*, 1977.

AHMAD, S., YAQOOB, M., BILAL, M., KHAN, M., MUHAMMAD, G., YANG, L. and TARIQ, M. Factors affecting yield and composition of camel milk kept under desert conditions of central Punjab, Pakistan. *Tropical Animal Health and Production*, vol. 44, pages 1403–1410, 2012.

ALBENZIO, M., CAROPRESE, M., SANTILLO, A., MARINO, R., MUSCIO, A. and SEVI, A. Proteolytic patterns and plasmin activity in ewes' milk as affected by somatic cell count and stage of lactation. *Journal Of Dairy Research*, vol. 72, pages 86–92, 2005.

ALBENZIO, M., CAROPRESE, M., SANTILLO, A., MARINO, R., TAILI, L. and SEVI, A. Effects of somatic cell count and stage of lactation on the plasmin activity and cheesemaking properties of ewe milk. *Journal Of Dairy Science*, vol. 87, pages 533–542, 2004.

ALBENZIO, M., SANTILLO, A., CAROPRESE, M., D'ANGELO, F., MARINO, R. and SEVI, A. Role of endogenous enzymes in proteolysis of sheep milk. *Journal Of Dairy Science*, vol. 92, pages 79–86, 2009.

ALI, S., MCGLENAGHAN, M., SIMONS, J. and CLARK, A. Characterization of the alleles encoding ovine β -lactoglobulin A and B. *Gene*, vol. 91, pages 202–207, 1990.

AMIGO, L., RECIO, I. and RAMOS, M. Genetic polymorphism of ovine milk proteins: its influence on technological properties of milk - a review. *International Dairy Journal*, vol. 10, pages 135–149, 2000.

ANDREWS, A. and ALICHANIDIS, E. Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal Of Dairy Research*, vol. 50, pages 275–290, 1983.

ARIAS, R. *Recuento de células somáticas y calidad de la leche de oveja en Castilla-La Mancha*. PhD thesis, Universidad de Castilla-La Mancha (Spain), 2009.

ASSENAT, L. Leche de oveja: composición y propiedades. In *Leche y Productos Lácteos: Vaca, Oveja, Cabra* (edited by F. Luquet), vol. I, La leche: de la mama a la lechería, pages 277–313. Ed. Acribia, Zaragoza (Spain), 1991.

BALCONES, E., OLANO, A. and CALVO, M. Factors affecting the rennet clotting properties of ewe's milk. *Journal Of Agricultural And Food Chemistry*, vol. 44, pages 1993–2006, 1996.

BALDI, A., SAVOINI, G., CHELI, F., FANTUZ, F., SENATORE, E., BERTOCCHI, L. and POLITIS, I. Changes in plasmin - plasminogen - plasminogen activator system in milk from italian friesian herds. *International Dairy Journal*, vol. 6, pages 1045–1053, 1996.

BALIGA, B. and BASU, K. Phospholipids in milk. Part 1: A method for the estimation of lecithin, cephalin and sphingomyelin in milk. *Indian Journal Of Dairy Science*, vol. 8, pages 119–128, 1955.

BALLESTEROS, C., POVEDA, J., GONZÁLEZ-VIÑAS, M. and CABEZAS, L. Microbiological, biochemical and sensory characteristics of artisanal and industrial Manchego cheeses. *Food Control*, vol. 17, pages 249–255, 2006.

BARBANO, D., RASMUSSEN, R. and LYNCH, J. Influence of milk somatic cell count and milk age on cheese yield. *Journal Of Dairy Research 74, 369-288.*, 1991.

BARBOSA, M., BARILLET, F., BERTHELOT, X., CASU, S., FOGLINI, A. and GABINA, D. Cellule somatiche e qualità del latte. In *International symposium: Somatic cells and milk of small ruminants - Conclusioni del comitato scientifico* (edited by R. Roberto). Europa Editrice, Bella (Italy), 1994.

BARILLET, F., ARRANZ, J. and CARTA, A. Mapping quantitative trait loci for milk production and genetic polymorphisms of milk proteins in dairy sheep. *Genetics Selection Evolution*, vol. 37(1), pages 109–123, 2004.

BASTIAN, E. and BROWN, R. Plasmin in milk and dairy products: an update. *International Dairy Journal*, vol. 6, pages 435–457, 1996.

BASTIAN, E. D., BROWN, R. and ERNSTORM, C. Plasmin activity and milk coagulation. *Journal Of Dairy Science*, vol. 74, pages 3677–3685, 1991a.

BASTIAN, E. D., BROWN, R. J. and ERNSTROM, C. A. Casein interference in bovine plasmin assays using a synthetic substrate. *Journal Of Dairy Science*, vol. 74, pages 4119–4124, 1991b.

BASTOS, E., AZEVEDO, J. and GUEDES-PINTO, H. Single strand conformation polymorphism (SSCP) detection in six genes in portuguese indigenous sheep breed Churra da Terra Quente. *Biotechnology, Agronomy, Society and Environment*, vol. 5, pages 7–15, 2001.

BATAVANI, R., MORTAZ, E., FALAHIAN, K. and DAWOODI, M. Study on frequency, etiology and some enzymatic activities of subclinical ovine mastitis in urmia, iran. *Small Ruminant Research*, vol. 50, pages 45–50, 2003.

BATTACONE, G., CANNAS, E., MAZZETTE, A., DIMAURO, C. and ENNE, G. Why does the increase of plasmin worsen the coagulation properties of milk in dairy sheep? *Italian Journal Of Animal Science*, vol. 4(2), pages 342–344, 2005.

BELL, K. and MCKENZIE, H. The whey proteins of ovine milk: β-lactoglobulins A and B. *Biochimica And Biophysica Acta*, vol. 147, pages 123–134, 1967.

BENCINI, R. Factors affecting the clotting properties of sheep milk. *Journal Of Agricultural And Food Chemistry*, vol. 82, pages 705–719, 2002.

BENCINI, R. and O AGBOOLA, S. Marketable products from sheep milk. Tech report, Rural Industries Research and Development Corporation, 2003.

BENCINI, R. and PULINA, G. The quality of sheep milk: a review. *Australian Journal Of Experimental Agriculture*, vol. 37, pages 485–504, 1997.

BENDELJA, D., ANTUNAC, N., MIKULEC, N., VNUCEC, I., MASEK, T., MIKULEC, Z. and HAVRANEK, J. Urea concentration in sheep's milk. *Mljekarstvo*, vol. 1(59), pages 3–10, 2009.

BERGONIER, D., DE CRÉMOUX, R., RUPP, R., LAGRIFFOUL, G. and BERTHELOT, X. Mastitis of dairy small ruminants. *Veterinary Research*, vol. 34, pages 689–716, 2003.

BIANCHI, L., BOLLA, A., BUDELLI, E., CAROLI, A., CASOLI, C., PAUSELLI, M. and DURANTI, E. Effect of udder health status and lactation phase on the characteristics of Sardinian ewe milk. *Journal Of Dairy Science*, vol. 87, pages 2401–2418, 2004.

BIKKER, F., KOOP, G., LEUSINK, N., NAZMI, K., KAMAN, W., BRAND, H. and VEERMAN, E. Tailor made plasmin substrates as potential diagnostic tool to test for mastitis. *Veterinary Research Communications*, 2014.

BLOWEY, R. and EDMONDSON, P. *Control de la mastitis en granjas de vacuno de leche*. Ed. Acribia, Zaragoza (Spain), 1995.

BOE. *Pliego de condiciones de la Denominación de Origen Protegida "Queso Manchego"*. 272. 13/11/2007.

BOUSSELMI, H. and OTHMANE, M. Milk composition and cheesemaking ability in ewes: Influence of major milk components and pH on individual cheese yield at a laboratory scale in ewes. *Greener Journal Of Biological Sciences*, vol. 5(1), pages 1–9, 2015.

BOYAZOGLU, J. and MORAND-FEHR, P. Mediterranean dairy sheep and goat products and their quality: a critical review. *Small Ruminant Research*, vol. 40, pages 1–11, 2001.

BRODERICK, A. and CLAYTON, M. A statistical evaluation of animal and nutritional factors influencing concentrations of milk urea nitrogen. *Journal Of Dairy Science*, vol. 80, pages 2964–2971, 1997.

BRULE, G. and LENOIR, J. La coagulación de la leche. In *El Queso* (edited by A. Eck), pages 3–20. Omega S.A., Barcelona (Spain), 1990.

BUCCIONI, A., MINIERI, S. and RAPACCINI, S. Effect of total proteose-peptone content on the variability of bovine milk foaming property. *Italian Journal Of Animal Science*, vol. 12(12), pages 72–76, 2013.

BUSETTI, M. La calidad en la leche de oveja. *IDIA XXI*, vol. 9, pages 150–156, 2007.

CABALLERO-VILLALOBOS, J., GARZÓN, A., MARTÍNEZ, A., ARIAS, R., CIOCIA, F. and MCSWEENEY, P. Plasmin activity in Manchega ewe milk. Poster in the IDF Parallel Symposia, Dublin, 2016.

CABALLERO-VILLALOBOS, J., GARZÓN, A., OLIETE, B., ARIAS, R., JIMÉNEZ, L., NÚÑEZ, N. and MARTÍNEZ, A. Relationship of somatic cell count and composition and coagulation properties of ewe's milk. *Mljekarstvo*, vol. 65(2), pages 138–143, 2015.

CAJA, G. Calidad de leche de ovino: el concepto en España. *Mundo Ganadero*, vol. 1, pages 34–38, 1991.

CAJA, G. and SUCH, X. Situación de la producción de leche de oveja en España. principales sistemas de producción. *Ovis*, vol. 15, pages 11–45, 1991.

CALLEJO RAMOS, A. Calidad higiénica de la leche. In *Ordeño mecánico*. 2015.

CANNAS, A., PES, A., MANCUSO, R., VODRET, B. and NUDDA, A. Effect of dietary energy and protein concentration on the concentration of milk urea nitrogen in dairy ewes. *Journal Of Dairy Science*, vol. 81, pages 499–508, 1998.

CARLSSON, J., BERGSTRÖM, J. and PEHRSON, B. Variations with breed, age, season, yield, stage of lactation and herd in the concentration of urea in bulk milk and individual cow's milk. *Acta Veterinaria Scandinavia*, vol. 36, pages 245–254, 1995.

CAROPRESE, M., SCHENA, L., MARZANO, A., MARINO, R., SEVI, A. and ALBENZIO, M. Contribution of macrophages to plasmin activity in ewe bulk milk. *Italian Journal Of Animal Science*, vol. 6(1), pages 545–547, 2007.

CARRIÓN, G. M. *Principios básicos para el control de la mastitis y el mejoramiento de la calidad de la leche*. Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación Para el Desarrollo Integral Regional de Michoacán, 2001.

CASOLI, C., DURANTI, E., MORBIDINI, L., PANELLA, F. and VIZIOLI, V. Quantitative and compositional variations of Massese sheep milk by parity and stage of lactation. *Small Ruminant Research*, vol. 2, pages 47–62, 1989.

CASTILLO, V., SUCH, X., CAJA, G., CASALS, R., ALBANELL, E. and SALAMA, A. A. K. Effect of milking interval on milk secretion and mammary tight junction permeability in dairy ewes. *Journal Of Dairy Science*, vol. 91, pages 2610–2619, 2008.

CERIOTTI, G., CHESSA, S., BOLLA, P., BUDELLI, E., BIANCHI, L. and DURANTI, E. Single nucleotide polymorphisms in the ovine casein genes detected by polymerase chain reaction - single strand conformation polymorphism. *Journal Of Dairy Science*, vol. 87, pages 2606–2613, 2004.

CHESSA, S., BOLLA, P., DARIO, C., PIERAGOSTINI, E. and CAROLI, A. Polimorfismi genetici lattoproteici nella razza ovina Gentile di Puglia: monitoraggio mediante focalizzazione isoelectrica. *Scienza E Tecnica Lattiero-Casearia*, vol. 54(3), pages 191–198, 2003.

CHESSA, S., RIGNANESE, D., BERBENNI, M., CERIOTTI, G., MARTINI, M. and PAGNACCO, G. New genetic polymorphisms within ovine β and α_{S2} caseins. *Small Ruminant Research*, vol. 88, pages 84–88, 2010.

CHIANESE, L. The casein variants of ovine milk and the relationships between the α_{S1}-casein variants and milk composition, micellar size and cheese yield. In *Caseins and Caseinates: Structures, Interactions, Networks*. Hannah Symposium, Scotland (UK), 1997.

CHIANESE, L., GARRO, G., ADDEO, F., LOPEZ-GALVEZ, G. and RAMOS, M. Discovery of an ovine α_{S2}-casein variant. *Journal Of Dairy Research*, vol. 60, pages 485–493, 1993.

CHIANESE, L., GARRO, G., MAURIELLO, R., LAEZZA, P., FERRANTI, P. and ADDEO, F. Occurrence of five α_{S1}-casein variants in ovine milk. *Journal Of Dairy Research*, vol. 63, pages 49–59, 1996.

CHIOFALO, L. and MICARI, P. Present knowledge on the variants of the milk protein in the sheep populations reared in Sicily. Experimental observations. *Scienza E Tecnica Lattiero-Casearia*, vol. 38, pages 104–114, 1987.

CHRISTENSEN, S., WIEGERS, T., HERMASEN, J. and SOTTRUP-JENSEN, L. Plasma-derived protease inhibitors in bovine milk. *International Dairy Journal*, vol. 5, pages 439–449, 1995.

CIPOLAT-GOTET, C., CECCHINATO, A., DE MARCHI, M., PENASA, M. and BITTANTE, G. Comparison between mechanical and near-infrared methods for assessing coagulation properties of bovine milk. *Journal Of Dairy Science*, vol. 95, pages 6806–6819, 2012.

CONSIDINE, T., HEALY, A., KELLY, A. and MCSWEENEY, P. Hydrolysis of bovine caseins by cathepsin B, a cysteine proteinase indigenous to milk. *International Dairy Journal*, vol. 14, pages 117–124, 2004.

CONSIDINE, T., HEALY, A., KELLY, A. L. and MCSWEENEY, P. L. H. Proteolytic specificity of elastase on bovine α_{S1}-casein. *Food Chemistry*, vol. 69, pages 19–26, (2000).

CORTELLINO, G., LOCCI, F. and RAMPILLI, M. An investigation of the plasmin-plasminogen system in caprine milk and cheese. *International Dairy Journal*, vol. 16, pages 619–622, 2006.

COULON, J., VERDIER, I., PRADEL, P. and ALMENA, M. Effect of lactation stage on the cheesemaking properties of milk and the quality of Saint-Nectaire-type cheese. *Journal Of Dairy Research*, vol. 65, pages 295–305, 1998.

CUNNINGHAM, J. *Textbook Of Veterinary Physiology*. WB Saunders Company, PA (USA), 3rd edition, 2002.

DE HAAS, Y., VEERKAMP, R., BARKEMA, H., GRÖHN, Y. and SCHUKKEN, Y. Associations between pathogen-specific cases of clinical mastitis and somatic cell count patterns. *Journal Of Dairy Science*, vol. 87, pages 95–105, 2004.

DE LA FUENTE, L., GAIÑA, D., CAROLINO, N. and UGARTE, E. *The Awassi and Assaf Breeds in Spain and Portugal*. European Association for Animal Production (EAAP), Antalya (Turkey), 2006.

DURANTI, E. and CASOLI, C. Variazione della composizione azotata e dei parametri lattodinamografici del latte di pecora in punzione del contenuto di cellule somatiche. *Zootecnia E Nutrizione Animale*, vol. 17, pages 99–105, 1991.

ERHARDT, G. Genetic polymorphisms of α-lactalbumin and β-lactoglobulin in sheep milk. *Animal Genetics*, vol. 20, pages 76–77, 1989.

ESROM. Memoria de actividades del esquema de selección de la raza ovina manchega. 2014.

ESTEBAN, J. Calidad de leche de ovino: Concepto CEE. *Mundo Ganadero*, (1), pages 27–33, 1991.

FAIRBAIRN, D. and LAW, B. Proteinases of psychrotrophic bacteria: their production, properties, effects and control. *Journal Of Dairy Research*, vol. 53, pages 139–177, 1986.

FAO. Norma general del Codex para el uso de términos lecheros. In *Codex Stan 206*. 1999.

FAO. *Manual sobre el uso de la lactoperoxidasa en la manipulación y la conservación de la leche*, 2000.

FAO-OMS. Beneficios y riesgos potenciales del sistema de la lactoperoxidasa en la conservación de la leche cruda. Tech report, FAO, Rome (Italy), 2005.

FARRELL, H., JIMENEZ-FLORES, R., BLECK, G., BROWN, E., BUTLER, J. and CREAMER, L. Nomenclature of the proteins of cows' milk, 6th revision. *Journal Of Dairy Science*, vol. 87, pages 1641–1674, 2004.

FEKADU, B., SORYAL, K., ZENG, S., VAN HEKKEN, D., BAH, B. and VILLAQUIRAN, M. Changes in goat milk composition during lactation and their effect on yield and quality of hard and semi-hard cheeses. *Small Ruminant Research*, vol. 59, pages 55–63, 2005.

FELIGINI, M., VLACO, S., CURIK, V., PARMA, P., GREPPI, G. and ENNE, G. A single nucleotide polymorphism in the sheep κ-casein coding region. *Journal Of Dairy Research*, vol. 72, pages 317–321, 2005.

FOX, P. and KELLY, A. Indigenous enzymes in milk: overview and historical aspects - part 1. *International Dairy Journal*, vol. 16, pages 500–516, 2006.

GALINA, M., MORALES, R., LÓPEZ, B. and CARMONA, M. Effect of somatic cell count on lactation and soft cheese yield by dairy goats. *Small Ruminant Research*, vol. 21, pages 251–257, 1996.

GALLEGO, L. La producción de leche. In *El Queso Manchego*. Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, 1991.

GALLEGO, L., BERNABÉU, R. and MOLINA, P. Producción de leche: Factores de Variación. In *Ganado Ovino Raza Manchega* (edited by L. Gallego, A. Torres and G. Caja), pages 173–189. Ediciones Mundi-Prensa, Madrid (Spain), 1994.

GALLEGO, L., CAJA, G., SUCH, X. and MOLINA, M. La produccion de leche de oveja en Castilla-La Mancha y el sistema de producción en la raza Manchega. *Ovis*, vol. 16, pages 29–54, 1991.

GARCÍA, A. *Células somáticas y alto recuento bacteriano - cómo controlarlos*. South Dakota State University, South Dakota (USA), 2004.

GARZÓN, A. I. *Incidencia de las variantes genéticas de las proteínas lácteas sobre la aptitud tecnológica de la leche en ovejas de la raza Manchega*. PhD thesis, Universidad de Córdoba (Spain), 1996.

GAYE, P., HUE-DELHAIE, D., MERCIER, J., SOULIERS, S., VILOTTE, J. and FURET, J. Ovine β-lactoglobulin messenger RNA: nucleotide sequence and mRNA levels during functional differentiation of the mammary gland. *Biochimie*, vol. 68, pages 1097–1107, 1986.

GAZI, I., VILALVA, I. and HUPPERTZ, T. Plasmin activity and proteolysis in milk protein ingredients. *International Dairy Journal*, vol. 38, pages 208–212, 2014.

GEERTS, N., DE BRABANDER, D., VANACKER, J., DE BOEVER, J. and BOTTERMAN, S. Milk urea concentration as affected by complete diet feeding and protein balance in the rumen of dairy cattle. *Livestock Production Science*, vol. 85(2-3), pages 263–273, 2004.

GIACCONE, P., SCATASSA, M. and TODARO, M. The influence of somatic cell count on sheep milk composition and cheese-making properties. *Italian Journal Of Animal Science*, vol. 4(2), pages 345–347, 2005.

GIAMBRA, I., CHIANESE, L., FERRANTI, P. and ERHARDT, G. Molecular genetic characterization of ovine α_{S1}-casein allele H caused by alternative splicing. *Journal Of Dairy Science*, vol. 93, pages 792–795, 2010.

GIAMBRA, I. and ERHARDT, G. Molecular genetic characterization of ovine CSN1S2 variants C and D reveal further important variability within CSN1S2. *Animal Genetics*, vol. 43, pages 642–645, 2012.

GINGER, M. R. and GRIGOR, M. R. Review comparative aspects of milk caseins. *Comparative Biochemistry And Physiology*, vol. 124, pages 133–145, 1999.

GIOVANETTI, V., DECANDIA, M., F. BOE, F., ZERBINI, E., CANNAS, A. and MOLLE, G. Effects of different dietary energy contents on milk urea concentration in stall-fed dairy sheep. In *Nutritional And Foraging Ecology Of Sheep And Goats* (edited by T. Papachristou, Z. Parissi, H. Ben Salem and P. Morand-Fehr), pages 399–403. CIHEAM / FAO / NAGREF, Zaragoza (Spain), 2009.

GIRARDET, J. and LINDEN, G. PP3 component of bovine milk: a phosphorylated whey glycoprotein. *Journal Of Dairy Research*, vol. 63, pages 333–350, 1996.

GONZALO, C., CARRIEDO, J., BARO, J. and SAN PRIMITIVO, F. Factors influencing variation of test day milk yield, somatic cell count, fat and protein in dairy sheep. *Journal Of Dairy Science*, vol. 77, pages 1537–1542, 1994.

GONZALO, C., TARDÁGUILA, A., ARIZNABARRETA, A., ROMEO, M., MONTORO, V. and PÉREZ-GUZMÁN, M. D. Recuentos de células somáticas en el ganado ovino

lechero y estrategias de control. Situación en España. *Ovis*, vol. 66, pages 21–27, 2000.

GORSAUD, J. Leche de vaca: composición y propiedades físico-químicas. In *Leche y Productos Lácteos: Vaca, Oveja, Cabra* (edited by F. Luquet), vol. I, La leche: de la mama a la lechería, pages 3–92. Ed. Acribia, Zaragoza (Spain), 1991.

GROVES, M. L., WICKHAM, E. and FARRELL, H. Environmental effects on disulfide bonding patterns of bovine kappa-casein. *Journal Of Protein Chemistry*, vol. 17, pages 73–84, 1998.

GRUFFERTY, M. B. and FOX, P. F. Milk alkaline proteinase. *Journal Of Dairy Research*, vol. 55, pages 609–630, 1988.

GUERRERO, L., ROMÁN, S. and PACHECO, L. Proteólisis de la leche cruda almacenada en frío. Efecto de las enzimas proteolíticas sobre la integridad de las caseínas. *Revista Científica FCV-LUZ*, vol. XIII(3), pages 187–192, 2003.

HABIBI-NAJAFI, M., LEE, B. and LAW, B. Bitterness in cheese: a review. *Critical Reviews In Food Science And Nutrition*, vol. 36, pages 397–411, 1996.

HAENLEIN, G. The concept of milk quality in the USA. *International Journal Of Animal Sciences*, vol. 16(1), pages 5–8, 2001.

HAYES, M., HURLEY, M., LARSEN, L. B., HEEGAARD, C. W., MAGBOUL, A., OLIVEIRA, J., MCSWEENEY, P. and KELLY, A. L. Thermal inactivation kinetics of bovine Cathepsin D. *Journal Of Dairy Research*, vol. 68, pages 267–276, 2001.

HENAO-VELÁSQUEZ, A., MÚNERA-BEDOYA, O., HERRERA, A., AGUDELO-TRUJILLO, J. and CERÓN-MUÑOZ, M. Lactose and milk urea nitrogen: fluctuations during lactation in Holstein cows. *Revista Brasileira De Zootecnia*, vol. 43(9), pages 479–484, 2014.

HERNANDEZ, J. and BEDOLLA, J. Importancia del conteo de células somáticas en la calidad de la leche. *Revista Electrónica De Veterinaria (REDVET)*, vol. IX(9), pages 1695–7504, 2008.

HÖÖK. *Effects of milking system on plasmin and plasminogen activity in bovine bulk milk*. Master thesis, Sweedish University of Agricultural Sciences, 2015.

HURLEY, M., LARSEN, L., KELLY, A. and MCSWEENEY, P. The milk acid proteinase Cathepsin D: a review. *International Dairy Journal*, vol. 10, pages 673–681, 2000.

IDF. Bovine mastitis - Definition and guidelines for diagnosis. In *IDF Bulletin*, vol. 211. International Dairy Federation, Brussels (Belgium), 1987.

ISMAIL, B., CHOI, L. H., WERE, L. M. and NIELSEN, S. S. Activity and nature of plasminogen activators associated with the casein micelle. *Journal Of Dairy Science*, vol. 89, pages 3285–3295, 2006.

ISMAIL, B. and NIELSEN, S. Invited review: Plasmin protease in milk. current knowledge and relevance to dairy industry. *Journal Of Dairy Science*, vol. 93, pages 4999–5009, 2010.

IUCCI, L., LANCIOTTI, R., KELLY, A. and HUPPERTZ, T. Plasmin activity in high-pressure homogenised bovine milk. *Milchwissenschaft*, vol. 63, pages 68–70, 2008.

JAEGGI, J., GOVINDASAMY-LUCEY, S., BERGER, Y., JOHNSON, M., MCKUSICK, B., THOMAS, D. and WENDORFF, W. Hard ewe's milk cheese manufactured from milk of three different groups of somatic cell counts. *Journal Of Dairy Science*, vol. 86, pages 3082–3089, 2003.

JARAMILLO, D. *Aptitud quesera de la leche de oveja Guirra y efecto de la dieta sobre las características tecnológicas de la leche y del madurado del queso*. PhD thesis, Universidad Autónoma de Barcelona (Spain), 2007.

JARAMILLO, D., ZAMORA, A., GUAMIS, B., RODRÍGUEZ, M. and TRUJILLO, A. J. Cheesemaking aptitude of two Spanish dairy ewe breeds: changes during lactation and relationship between physico-chemical and technological properties. *Small Ruminant Research*, vol. 78, pages 48–55, 2008.

JENNESS, R. Inter-species comparison of milk proteins. In *Developments in Dairy Chemistry,* (edited by P. Fox), vol. 1: Proteins, pages 87–114. Applied Science Publishers, New York (USA), 1982.

JENNESS, R. L. Composition and characteristics of goat milk: review 1968-1979. *Journal Of Dairy Science*, vol. 63, pages 1605–1630, 1985.

JOLLES, J., FIAT, A., SCHOENGTEN, F., ALAIS, C. and JOLLES, P. The amino-acid sequence of sheep κ-casein. II. sequence studies concerning the κ A-caseinoglycopeptide and establishment of the complete primary structure of the protein. *Biochimica And Biophysica Acta*, vol. 365, pages 335–343, 1974.

JOURNET, M., VERITE, R. and VIGNON, B. Non-protein nitrogen in milk: factors of variability. *Le Lait*, (543-544), pages 212–221, 1975.

KALANTZOPOULOS, G., DUBEUF, J., VALLERAND, F., PIRISI, A., CASALTA, E., LAURET, A. and TRUJILLO, T. Characteristics of sheep and goat milks: quality and hygienic factors for the sheep and goat dairy sectors. In *IDF Bulletin*, vol. 390, pages 17–28. 2004.

KAMINOGAWA, S. and YAMAUCHI, K. Acid protease of bovine milk. *Agricultural And Biological Research*, vol. 36, pages 2351–2356, 1972.

KELLY, A. and MCSWEENEY, P. Indigenous proteinases in milk. In *Advanced Dairy Chemistry* (edited by P. Fox and P. McSweeney), vol. A, Proteins, pages 495–521. Kluwer Academic/Plenum Publishers, New York, 2003.

KELLY, A., O'FLAHERTY, F. and FOX, P. Indigenous proteolytic enzymes in milk: a brief overview of the present state of knowledge. *International Dairy Journal*, vol. 6, pages 563–572, 2006.

KELLY, A., REID, S., JOYCE, P., MEANEY, W. and FOLEY, J. Effect of decreased milking frequency of cow in late lactation on milk somatic cell count, polymorphonuclear leukocyte numbers, composition and proteolytic activity. *Journal Of Dairy Research*, vol. 65(3), pages 365–373, 1998.

KING, J. The distribution of sheep β-lactoglobulins. *Animal Production*, vol. 11, pages 53–57, 1969.

KIRSCHKE, H., BARRETT, A. J. and RAWLINGS, N. D. *Lysosomal cysteine proteinases*. Oxford University Press, Oxford (UK), 2nd edition, 1998.

KOLDE, H. and BRAUNITZER, G. The primary structure of ovine β-lactoglobulin. *Milckwissenschaft*, vol. 38, pages 70–72, 1983.

KOLEV, K., OWEN, W. and MACHOVICH, R. Dual effect of synthetic plasmin substrates on plasminogen activation. *Biochimica And Biophysica Acta*, vol. 1247(2), pages 239–45, 1995.

KONIG, C., FIETEN, J. and GELLING, G. Le mastiti della pecora in Olanda. *Selecciones Veterinarias*, vol. XXVI(2), page 216, 1985.

KOUTSOULI, P., PATOUNASA, G., MASSOURASB, T., BIZELISA, I. and POLITIS, I. Plasmin-plasminogen system and milk coagulation properties of two Greek dairy sheep breeds. *Small Ruminant Research*, vol. 124, pages 89–94, 2015.

KRÁLÍCKOVÁ, S., POKORNÁ, M., KUCHTÍK, J. and FILIPCÍK, R. Effect of parity and stage of lactation on milk yield, composition and quality of organic sheep milk. *Acta Universitaris Agriculturae Et Silviculturae Mendelianae Brunensis*, vol. 1, pages 71–78, 2012.

KUCHTÍK, J., K. SUSTOVÁ, K., URBAN, T. and ZAPLETAL, D. Effect of the stage of lactation on milk composition, its properties and the quality of rennet curdling in East Friesian ewes. *Czech Journal Of Animal Science*, vol. 53(2), pages 55–63, 2008.

LARSEN, L. B., BOISEN, A. and PETERSEN, T. E. Procathepsin D cannot autoactivate to Cathepsin D at acid pH. *FEBS Letters*, vol. 319, pages 54–58, 1993.

LARSEN, L. B., WIUM, H., BENFELDT, C., HEEGAARD, C. W., ARDOK, Y., QVIST, K. B. and PETERSEN, T. E. Bovine milk Procathepsin D: presence and activity in heated milk and extracts of rennet free UF-feta. *International Dairy Journal*, vol. 10, pages 67–74, 2000.

LARSON, B. and SMITH, V. Lactation: a comprehensive treatise. vol. IV. Academic Press, New York (USA), 1974.

LE MARÉCHAL, C., THIÉRY, R., VAUTOR, E. and LE LOIR, Y. Mastitis impact on technological properties of milk and quality of milk products - a review. *Dairy Science And Technology*, vol. 91, pages 247–282, 2011.

LE MENS, P. La leche de cabra: Propiedades físico-químicas, nutricionales y químicas. In *Leche y Productos Lácteos: Vaca, Oveja, Cabra* (edited by F. Luquet), vol. I. La leche: De La Mama A La Lechería, pages 343–360. Ed. Acribia, Zaragoza (Spain), 1991.

LE ROUX, Y., COLIN, O. and LAURENT, F. Proteolysis in samples of quarter milk with somatic cell counts. 1. Comparison of somatic indicators of endogeneous proteolysis in milk. *Journal Of Dairy Science*, vol. 78, pages 1289–1297, 1995.

LEIBER, F., NIGG, D., KUNZ, C., SCHEEDER, M., WETTSTEIN, H. and KREUZER, M. Protein composition, plasmin activity and cheesemaking properties of cows' milk produced at two altitudes from hay of lowland and high-alpine origins. *Journal Of Dairy Research*, vol. 72, pages 65–74, 2005.

LEITNER, G., CHAFFER, M., SHAMAY, A., SHAPIRO, F., MERIN, U., EZRA, E., SARAN, A. and SILANIKOVE, N. Changes in milk composition as affected by subclinical mastitis in sheep. *Journal Of Dairy Science*, vol. 87, pages 46–52, 2004.

LINDERSTRØM-LANG, K. and KODAMA, S. Studies on casein (1): On the solubility of caseins in hydrochloric acid. *Comptes-Rendus des Travaux du Laboratoire Carlsberg*, vol. 16, pages 1–47, 1925.

LÓPEZ GALVEZ, G. *Estudio del polimorfismo de las proteínas de leche de oveja de las razas Manchega y Segureña. Aptitud a la coagulación y rendimiento quesero.*. PhD thesis, Universidad Complutense, Madrid (Spain), 1993.

LU, D. and NIELSEN, S. Isolation and characterization of native bovine-milk plasminogen activators. *Journal Of Dairy Science*, vol. 76, pages 3369–3383, 1993.

MARA, O., ROUPIE, C., DUFFY, A. and KELLY, A. L. The curd-forming properties of milk as affected by the action of plasmin. *International Dairy Journal*, vol. 8, pages 807–812, 1998.

MARENJAK, T., POLJICAK-MILAS, N. and STOJEVIC, Z. Svrha odredivanja koncentracije ureje u kravljem mlijeku. *Praxis Veterinaria*, vol. 52(3), pages 233–241, 2004.

MARTÍ DE OLIVES, A., DÍAZ, J., MOLINA, M. and PERIS, C. Quantification of milk yield and composition changes as affected by subclinical mastitis during the current lactation in sheep. *Journal Of Dairy Science*, vol. 96(12), pages 7698–7708, 2013.

MARTÍNEZ, O., AGUILERA, C. and GIL, A. Composición y calidad nutritiva de los alimentos. In *Tratado De Nutrición* (edited by A. Gil and M. Ruiz), vol. II, pages 367–396. Médica Panamericana, Madrid (Spain), 2010.

MARTINI, M. and CAROLI, A. Evaluation of ovine milk clotting aptitude. *Italian Journal Of Animal Science*, vol. 2, pages 89–95, 2003.

MATUTINOVIĆ, S., SALAJPAL, K. and KALIT, S. Variation in nitrogen components of sheep milk abstract in sub-Mediterranean area. *Mljekarstvo*, vol. 64(1), pages 27–33, 2014.

MCMAHON, D. and BROWN, R. Evaluation of Formagraph for comparing rennet solutions. *Journal Of Dairy Science*, vol. 65, pages 1639–1642, 1982.

MILERSKI, M., MARGETÍN, M., CAPISTRÁK, A., APOLEN, D., SPÁNIK, J. and ORAVCOVÁ, M. Relationships between external and internal udder measurements and the linear scores for udder morphology traits in dairy sheep. *Czech Journal Of Animal Science*, vol. 51(9), pages 383–390, 2006.

MOATSOU, G. Indigenous enzymatic activities in ovine and caprine milks. *International Journal Of Dairy Technology*, vol. 63(1), pages 16–31, 2010.

MOCQUOT, G., ALAIS, C. and CHEVALLIER, R. Étude sur les défauts de coagulation du lait par la présure. *Annales De Technologie Agricole (INRA)*, vol. 1, pages 1–44, 1954.

MOLINA, P. *Composición y factores de variación de la leche de ovejas de raza Manchega*. PhD thesis, Universidad Politécnica de Valencia (Spain), 1987.

MOLINA, P. and GALLEGO, L. Composición de la leche: factores de variación. In *Ganado Ovino Raza Manchega* (edited by L. Gallego, Torres, A. and G. Caja), pages 191–208. Ediciones Mundi-Prensa, Madrid (Spain), 1994.

MOUSSAOUI, F., MICHELUTTI, I., LE ROUX, Y. and LAURENT, F. Mechanisms involved in milk endogenous proteolysis induced by a lipopolysaccharide experimental mastitis. *Journal Of Dairy Science*, vol. 85, pages 2562–2570, 2003.

NABHAN, M. A., GIRARDET, J., CAMPAGNA, S., GAILLARD, J. and LE ROUX, Y. Isolation and characterisation of copolymers of β-lactoglobulin, α-lactalbumin, κ-casein and α_{S1}-casein generated by pressurisation and thermal treatment of raw milk. *Journal Of Dairy Science*, vol. 87, pages 3614–3622, 2004.

NÁJERA, A., DE RENOBALES, M. and BARRÓN, L. Effects of pH, temperature, CaCl2 and enzyme concentrations on the rennet-clotting properties of milk: a multifactorial study. *Food Chemistry*, vol. 80, pages 345–352, 2003.

NG-KWAI HANG, K. and GROSCLAUDE, F. Genetic polymorphisms of milk proteins. In *Advanced Dairy Chemistry* (edited by P. Fox), pages 405–455. Elsevier, London (UK), 1992.

NIELSEN, S. Plasmin system and microbial proteases in milk: Characteristics, roles, and relationship. *Journal Of Agricultural And Food Chemistry*, vol. 50, pages 6628–6634, 2002.

NUDDA, A., FELIGINI, M., BATTACONE, G., MACCIOTTA, N. and PULINA, G. Effects of lactation stage, parity, β-lactoglobulin genotype and milk SCC on whey protein composition in Sarda dairy ewes. *Italian Journal Of Animal Science*, vol. 2, pages 29–39, 2003.

NUDDA, A., FELIGINI, M., BATTACONE, G., MURGIA, P. and PULINA, G. Relationship between somatic cells count, whey protein and coagulation properties in sheep milk. In *Proceedings Of The ASPA XIV Congress*, pages 511–513. Firenze (Italy), 2001.

OCHOA-ALFARO, A., VEGA, L., OCHOA, M., BISSET, P. and TORRES, G. Physicochemical characteristics of Rambouillet ewes' milk under intensive management. *Revista Científica FCV-LUZ*, vol. XIX(2), pages 196–200, 2009.

OGSTON, D. Biochemistry of the plasmin system. *Journal Of Clinical Pathology*, vol. 33 (Suppl. Roy Coll Path 14), pages 5–9, 1980.

OHNO, F., SUGAHARA, T., KANDA, K. and NISHIMOTO, S. Proteose-Peptone fraction of bovine milk depressed IgE production in vitro and in vivo. *Bioscience, Biotechnology And Biochemistry*, vol. 74(7), pages 1332–1337, 2010.

PAAPE, M., BANNERMAN, D., ZHAO, X. and LEE, J. The bovine neutrophil: structure and function in blood and milk. *Veterinary Research*, vol. 34, pages 597–627, 2003.

PAAPE, M., WIGGANS, G., BANNERMAN, D., THOMAS, D., SANDERS, A. and CONTRERAS, A. Monitoring goat and sheep milk somatic cell counts. *Small Ruminant Research*, vol. 68, pages 114–25, 2007.

PACCARD, P. and LAGRIFFOUL, G. Synthèse bibliographique sur la composition du lait de brebis en composés d'intérêt nutritionnel. Personal communication, 2006.

PARISET, L., CAPPUCCIO, I., AJMONE-MARSAN, P., BRUFORD, M., DUNNER, S. and CORTES, O. Characterization of 37 breed-specific single-nucleotide polymorphisms in sheep. *Journal Of Heredity*, vol. 97, pages 531–534, 2006.

PARK, Y. Hypo-allergenic and therapeutic significance of goat milk. *Small Ruminant Research*, vol. 14, pages 151–159, 1994.

PARK, Y. Rheological characteristics of goat and sheep milk. *Small Ruminant Research*, vol. 68, pages 73–87, 2007.

PARK, Y., JUÁREZ, M., RAMOS, M. and HAENLEIN, G. Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research*, vol. 68, pages 88–113, 2007.

PAVIC, V., ANTUNAC, N., MIOC, B., IVANKOVIC, A. and HAVRANEK, J. Influence of stage of lactation on the chemical composition and physical properties of sheep milk. *Czech Journal Of Animal Science*, vol. 47, pages 80–84, 2002.

PAZZOLA, M., DETTORI, M., CARCANGIU, V., LURIDIANA, S., MURA, M. and VACCA, G. Relationship between milk urea, blood plasma urea and body condition score in primiparous browsing goats with different milk yield level. *Archiv Tierzucht*, vol. 54(5), 2011.

PAZZOLA, M., DETTORI, M. L., CIPOLAT-GOTET, C., CECCHINATO, A., BITTANTE, G. and VACCA, G. M. Phenotypic factors affecting coagulation properties of milk from Sarda ewes. *Journal Of Dairy Science*, vol. 97, pages 7247–7257, 2014.

PEARSE, M., LINKLATER, P., HALL, R. and MACIUNLAY, A. T. The effect of casein composition and casein dephosphorylation on the coagulation and syneresis of artificial micelle milk. *Journal Of Dairy Research*, vol. 53, pages 381–390, 1986.

PELLEGRINI, O., AUREL, M., LAGRIFFOUL, G., MARIE, C., REMEUF, F., RIVEMALE, M. and BARILLET, F. Relations entre les comptages de cellules somatiques, les caractéristiques physicochimiques et l'aptitude á la coagulation par la présure de laits individuéis de brebis de race Lacaune. In *Somatic Cells And Milk Of Small Ruminants*, pages 253–258. Wageningen Pers, Wageningen (The Netherlands), 1996.

PELLEGRINI, O., REMEUF, F., RIVEMALE, M. and BARILLET, F. Renneting properties of milk from individual ewes: influence of genetic and non-genetic variables, and relationship with physico-chemical characteristics. *Journal Of Dairy Research*, vol. 64, pages 355–366, 1997.

PÉREZ-RAMOS, A. *Efecto de las células somáticas sobre variables físicoquímicas y microbiológicas en leche cruda*. PhD thesis, Facultad de Zootecnia, Universidad Autónoma de Chihuahua (Mexico), 2006.

PERMYAKOV, E. and BERLINER, L. α-lactalbumin: structure and function. *FEBS Letters*, vol. 473, pages 269–274, 2000.

PICARIELLO, G., RIGNANESE, D., CHESSA, S., CERIOTTI, G., TRANI, A. and CAROLI, A. Characterization and genetic study of the ovine α_{S2}-casein (CSN1S2) allele B. *The Protein Journal*, vol. 28, pages 333–340, 2009.

PINTO, G., CAIRA, S., NICOLAI, M., MAURIELLO, R., CUOLLO, M., PIRISI, A., PIREDDA, G., CHIANESE, L. and ADDEO, F. Proteolysis and partial dephosphorylation of casein are affected by high somatic cell counts in sheep milk. *Food Research International*, vol. 53, pages 510–521, 2013.

PIRISI, A., PIREDDA, G., CORONA, M., PES, M., PINTUS, S. and LEDDA, A. Influence of somatic cell count on ewe's milk composition, cheese yield and cheese quality. In *6th Great Lake Dairy Sheep Symposium*. Guelph, Ontario (Canada), 2000.

PIRISI, A., PIREDDA, G., PAPOFF, C., DI SALVO, R., PINTUS, S. and GARRO, G. Effects of sheep alpha S1-casein CC, CD and DD genotypes on milk composition and cheesemaking properties. *Journal Of Dairy Research*, vol. 66, pages 409–419, 1999.

PIRISI, A., PIREDDA, G., PODDA, F. and PINTUS, S. Effect of somatic cell count on sheep milk composition and cheesemaking properties. In *Proceedings Of The International Symposium On Somatic Cells And Milk Of Small Ruminants*, pages 245–251. Bella (Italy), 1994.

PIRISI, A., PIREDDA, G., PODDA, F. and PINTUS, S. Effect of somatic cell count on sheep milk composition and cheesemaking properties. In *Somatic Cells and Milk of Small Ruminants*, EAAP Publication No. 77, pages 245–251. Wageningen Pers (The Netherlands), 1996.

POLITIS, I. Plasminogen activator system: implication for mamary cell growth and involution. *Journal Of Dairy Science*, vol. 79, pages 1097–1107, 1996.

POLITIS, I. and NG KWAI HANG, K. F. Environmental factors affecting plasmin activity in milk. *Journal Of Dairy Science*, vol. 72, pages 1713–1718, 1989.

POLITIS, I., ZAVIZION, B., BARBANO, D. M. and GOREWIT, R. C. Enzymatic assay for the combined determination of plasmin plus plasminogen in milk: revisited. *Journal Of Dairy Science*, vol. 76, pages 1260–1267, 1993.

PRADO, B. M., SOMBERS, S. E., ISMAIL, B. and HAYES, K. D. Effect of heat treatment on the activity of inhibitors of plasmin and plasminogen activators in milk. *International Dairy Journal*, vol. 16, pages 593–599, 2006.

PRIN-MATHIEU, C., LE ROUX, Y., FAURE, G. C., LAURENT, F., BÉNÉ, M. and MOUSSAOUI, F. Enzymatic activities of bovine peripheral blood leucocytes and milk polymorphonuclear neutrophils during intramammary inflammation caused by lipopolysaccharide. *Clinical And Diagnostic Laboratory Immunology*, vol. 9(4), pages 812–817, 2002.

PUGLIESE, C., ACCIAIOLI, A., RAPACCINI, S., PARISI, G. and FRANCI, O. Evolution of chemical composition, somatic cell count and renetting properties of the milk of Massese ewes. *Small Ruminant Research*, vol. 35, pages 71–80, 2000.

PULINA, G. Influence of feeding on ewes' milk quality. *I' Informatore Agrario*, vol. 37, pages 31–39, 1990.

PULINA, G. and NUDDA, A. Milk production. In *Dairy Sheep Nutrition* (edited by G. Pulina and R. Bencini), pages 1–12. CABI Publishing, Wallinford (UK), 2004.

PULINA, G., NUDDA, A., BATTACONE, G. and CANNAS, A. Effects of nutrition on the contents of fat, protein somatic cells, aromatic compounds, and undesirable substances in sheep milk. *Animal Feed Science And Technology*, vol. 131, pages 255–291, 2006.

PURROY, A. Producción de leche de oveja. Instituto Nacional de Investigaciones Agrarias (INIA), 1982.

PYÖRÄLÄ, S. Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research*, vol. 34, pages 565–578, 2003.

RAMÍREZ, B., SALAMA, A. A. K., CAJA, G., CASTILLO, V., ALBANELL, E. and SUCH, X. Response to lactation induction differs by season of year and breed of dairy ewes. *Journal Of Dairy Science*, vol. 91, pages 2299–2306, 2008.

RAMOS, A., MATOS, C., RUSSO-ALMEIDA, P., BETTENCOURT, C., MATOS, J. and MARTINS, A. Candidate genes for milk production traits in Portuguese dairy sheep. *Small Ruminant Research*, vol. 82, pages 117–121, 2009.

RAMOS, M. and JUAREZ, M. Sheep milk. In *Encyclopedia of Dairy Sciences* (edited by H. Roginski, J. Fuquay and P. Fox), vol. 4, pages 2539–2545. Academic Press, Amsterdam (The Netherlands), 2003.

RAUH, V. M. *Impact of plasmin activity on the shelf life and stability of UHT milk*. PhD thesis, Aarhus University (Denmark), 2014.

RAUH, V. M., BAKMAN, M., IPSEN, R., PAULSSON, M., KELLY, A. L., LARSEN, L. B. and HAMMERSHØJ, M. The determination of plasmin and plasminogen-derived activity in turbid samples from various dairy products using an optimised spectrophotometric method. *International Dairy Journal*, vol. 38, pages 74–80, 2014.

RAYNAL-LJUTOVAC, K., GABORIC, P. and LAURET, A. The relationship between quality criteria of goat milk, its technological properties and the quality of the final products. *Small Ruminant Research*, vol. 60, pages 167–177, 2005.

RAYNAL-LJUTOVAC, K., PIRISI, A., DE CRÉMOUX, R. and GONZALO, C. Somatic cells of goat and sheep milk: analytical, sanitary, productive and technological aspects. *Small Ruminant Research*, vol. 68, pages 126–144, 2007.

REAL ORTELLADO, M. *Caracterización productiva de la raza ovina Pampinta en la Región Semiárida Pampeana*. PhD thesis, Universidad de Córdoba (Spain), 1999.

RECIO, I., FERNÁNDEZ-FOURNIER, A., MARTÍN-ÁLVAREZ, P. and RAMOS, M. β-lactoglobulin polymorphism in ovine breeds: influence on cheese-making properties and milk composition. *Lait*, vol. 77, pages 259–265, 1997.

RECIO, I. and VISSER, S. Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. *International Dairy Journal*, vol. 10, pages 597–605, 2000.

RECIO, M., FERNÁNDEZ-FOURNIER, A. and RAMOS, M. Genetic polymorphism of the whey proteins for two Spanish ovine breeds. Influence of genetic polymorphism on renneting properties. In *IDF Seminar on the implications of genetic polymorphism of milk proteins on production and processing of milk*. Zurich (Switzerland), 1995.

RÈGLEMENT. 1107/96 de la Commission des Communautés Européennes, relatif à l'enregistrement des indications géographiques et des appellations d'origine au titre de la procédure prévue à l'article 17 du règlement (CEE) no 2081/92 du Conseil. 1996.

REMEUF, F., COSSIN, V., DERVIN, C., LENOIR, J. and TOMASSONE, R. Relations entre les caractéristiques physico-chimiques des laits et leur aptitude fromagère. *Lait*, vol. 71, pages 397–421, 1991.

REVILLA, I., RODRÍGUEZ-NOGALES, J. and VIVAR-QUINTANA, A. Proteolysis and texture of hard ewes' milk cheese during ripening as affected by somatic cell counts. *Journal Of Dairy Research*, vol. 74, pages 127–136, 2007.

REVILLA, I., RODRÍGUEZ-NOGALES, J. and VIVAR-QUINTANA, A. Effect of somatic cell counts on ewes'milk protein profile and cheese-making properties in different sheep breeds reared in spain. *Journal Of Dairy Research (2009)*, vol. 76, pages 210–215, 2009.

RICHARDSON, B. Variation of the concentration of plasmin and plasminogen in bovine milk with lactation. *New Zealand Journal Of Dairy Science And Technology*, vol. 18, pages 247–252, 1983.

RICHARDSON, B. and PIERCE, K. The determination of plasmin in dairy products. *New Zealand Journal Of Dairy Science And Technology*, vol. 16, pages 209–220, 1981.

RODRÍGUEZ-FRANCO, D., VÁZQUEZ-MORENO, L. and RAMOS-CLAMONT, G. Actividad antimicrobiana de la lactoferrina: mecanismos y aplicaciones clínicas potenciales. *Revista Latinoamericana De Microbiología*, vol. 47(3-4), pages 102–11, 2005.

ROGERS, S. and MITCHELL, G. The relationship between somatic cell count, composition and manufacturing properties of bulk milk. Cheedar cheese and skim milk-yoghurt. *Australian Journal Of Dairy Technology*, vol. 49, pages 70–74, 1994.

ROLLEMA, H., S., V. and J.K., P. Spectrophotometric assay of plasmin and plasminogen in bovine milk. *Milchwissenschaft*, vol. 38, pages 214– 217, 1983.

ROUTABOUL, M. Communic. Inter. Lab. Societé des Caves. Roquefort (France), 1981.

ROY, B., MEHLA, R. and SIROHI, S. Influence of milk yield, parity, stage of lactation and body weight on urea and protein concentration in milk of Murrah buffaloes. *Asian-Australasian Journal Of Animal Sciences*, vol. 16(9), pages 1285–1290, 2003.

SAHOO, B. and WALLI, T. Effect of feeding undegradable protein with energy on nutrient utilization, milk yield and milk composition of crossbred goats. *Small Ruminant Research*, vol. 75, pages 36–42, 2008.

SAINT-DENIS, T., HUMBERT, G. and GAILLARD, J.-L. Enzymatic assays for native plasmin, plasminogen and plasminogen activators in bovine milk. *Journal Of Dairy Research*, vol. 69, pages 437–449, 2001a.

SAINT-DENIS, T., HUMBERT, G. and GAILLARD, J. L. Heat inactivation of native plasmin, plasminogen and plasminogen activators in bovine milk: a revisited study. *Lait*, vol. 81, pages 715– 729, 2001b.

SÁNCHEZ-BELDA, A. *Razas Ovinas Españolas*. Publicaciones de Extensión Agraria, Madrid (Spain), 1979.

SANTILLO, A., KELLY, A., PALERMO, C., SEVI, A. and ALBENZIO, M. Role of indigenous enzymes in proteolysis of casein in caprine milk. *International Dairy Journal*, vol. 19, pages 655–660, 2009.

SARAN, A. and CHAFFER, M. *Mastitis y calidad de la leche*. Ed. Inter-Médica, Buenos Aires (Argentina), 2000.

SCHLIMME, E. and BUCHHEIM, W. *La leche y sus componentes: propiedades físicas y químicas*. Ed. Acribia, Zaragoza (Spain), 2002.

SELVAGGI, M., LAUDADIO, V., DARIO, C. and TUFARELLI, V. Investigating the genetic polymorphism of sheep milk proteins: a useful tool for dairy production. *Journal Of The Science Of Food And Agriculture*, vol. 94(15), pages 3090–3099, 2014.

SERRANO, B. *Caracterización de la estructura, producción lechera y aptitud tecnológica de la raza Merina en la comarca del Valle De Los Pedroches*. PhD thesis, Universidad de Córdoba (Spain), 1999.

SEVI, A., ALBENZIO, M., MARINO, R., SANTILLO, A. and MUSCIO, A. Effects of lambing season and stage of lactation on ewe milk quality. *Small Ruminant Research*, vol. 51, pages 251–259, 2004.

SEVI, A., TAIBI, L., ALBENZIO, M., MUSCIO, A. and ANNICCHIARICO, G. Effect of parity on milk yield, composition, somatic cell count, renneting parameters and bacteria counts of Comisana ewes. *Small Ruminant Research*, vol. 37, pages 99–107, 2000.

SIDELMANN, J. J., GRAM, J., JESPERSEN, J. and KLUFT, C. Fibrin clot formation and lysis: basic mechanisms. *Seminars In Thrombosis And Hemostasis*, vol. 26, pages 605–618, 2000.

SMITH, K. Standards for somatic cells in milk: physiological and regulatory. In *Newsletters Of The International Dairy Federation*, vol. 144, pages 7–9. IDF, 1996.

SOUSA, M., ARDÖ, Y. and MCSWEENEY, P. Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, vol. 11, pages 327–345, 2001.

SRINIVASAN, M. and LUCEY, J. Effects of added plasmin on the formation and rheological properties of rennet-induced skim milk gels. *Journal Of Dairy Science*, vol. 85, pages 1070–1078, 2002.

SUÁREZ, V. Mastitis en ovejas lecheras. *IDIA XXI*, vol. 9, pages 186–190, 2007.

SUMMER, A., MALACARNE, M., SANDRI, S., FORMAGGIONI, P., MARIANI, P. and FRANCESCHI, P. Effects of somatic cell count on the gross composition, protein fractions and mineral content of individual ewe's milk. *African Journal Of Biotechnology*, vol. 11(97), pages 16377–16381, 2012.

TARDÁGUILA, J. *Control de mastitis y producción de leche de alta calidad higio-sanitaria en el ganado ovino lechero de raza Churra*. PhD thesis, Facultad de Veterinaria, Universidad de León (Spain), 1999.

THEODOROU, G., KOMINAKIS, A., ROGDAKIS, E. and POLITIS, I. Factors affecting the plasmin-plasminogen system in milk obtained from three Greek dairy sheep breeds with major differences in milk production capacity. *Journal Of Dairy Science*, vol. 90, pages 3263–3269, 2007.

TOLEDO, J. *Factores que afectan a la producción, a la composición y a los parámetros tecnológicos de la oveja Merina de Grazalema*. PhD thesis, Universidad de Córdoba (Spain), 2013.

TORRES MAÑAS, E. Tratamiento y control de mamitis ovinas. *Mundo Ganadero*, vol. 155, pages 56–60, 2003.

VEISSEYRE, R. *Lactología Técnica*. Ed. Acribia, Zaragoza (Spain), 1988.

WATSON, D. and BUSWELL, J. Modern aspeets of sheep mastitis. *British Veterinary Journal*, vol. 140, pages 529–534, 1984.

WESSELS, G., HAMANN, H., ERHARDT, G. and DISTL, O. Genotypeffekte von milchprotein-polymorphismen auf die milchleistung beim ost- friesischen milchschaf. *Berliner Und Münchener Tierärztliche Wochenschrift*, vol. 117, pages 414–419, 2004.

WHITE, D., WALMSLEY, M., LIEW, A., CLAYCOMB, R. and MEIN, G. Chemical and rheological aspects of gel formation in the California Mastitis Test. *Journal Of Dairy Research*, vol. 72, pages 115–121, 2005.

WOHLT, J., KLEYN, D., VANDERNOOT, G., SELFRIDGE, D. and NOVOTNEY, C. Effect of stage of lactation, age of ewe, sibling status and sex of lamb on gross and minor constituents of Dorset ewe milk. *Journal Of Dairy Science*, vol. 64, pages 2175–2184, 1981.

ZHANG, Z. and GOFF, H. Protein distribution at air interface in dairy foams and ice cream as affected by casein dissociation and emulsifiers. *International Dairy Journal*, vol. 14, pages 647–657, 2004.

THIS PhD THESIS HAS BEEN FUNDED BY AGROMÉTODOS S.A., CAMPUS DE EXCELENCIA INTERNACIONAL AGROALIMENTARIO (ceiA3), MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE / MINISTERIO DE ECONOMIA Y COMPETITIVIDAD AND BANCO SANTANDER.

AGROMÉTODOS, S. A.