UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Ph.D. Thesis

Effect of antibiotics in goat milk on the manufacture and characteristics of cheese

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Supervisors:

Dr. Mª Pilar Molina Pons
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Efecto de antibióticos en leche de cabra sobre la fabricación y características del queso

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Departamento de Ciencia Animal
Instituto de Ciencia y Tecnología Animal
Universitat Politècnica de València

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INFORMAN:

Que la Tesis Doctoral titulada “Effect of antibiotics in goat milk on the manufacture and characteristics of cheese” ha sido realizada por Dña. Paloma G. Quintanilla Vázquez en el Departamento de Ciencia Animal bajo su dirección y que, una vez revisado y comprobado el trabajo, consideran que reúne los requisitos necesarios para la obtención del grado de Doctor con Mención Internacional por lo que autorizan su presentación.

Y para que así conste firman el presente informe en Valencia, a tres de abril de 2019.

Dra. Mª Pilar Molina Pons

Dr. Mª Isabel Escriche Roberto

Effect of antibiotics in goat milk on the manufacture and characteristics of cheese

This Thesis has been submitted in fulfilment of the requirements for the degree of Doctor with International Mention at the Universitat Politècnica de València.

Esta tesis ha sido escrita y presentada como uno de los requisitos para optar al grado de Doctor con Mención Internacional por la Universitat Politècnica de València.

By: Paloma G. Quintanilla Vázquez

Valencia, July 2019

Science knows no country,
because knowledge belongs to humanity,
and is the torch which illuminates the world.

Louis Pasteur

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Summary

Antibiotic residues in milk and other foodstuffs of animal origin are a great concern for public health, as they could lead to toxicological effects in sensitive consumers and contribute to the generation of bioresistance. Moreover, drug residues could also have negative implications for the dairy industry, affecting the fermentation processes required to make related products such as yogurt and cheese, the main destination of goat milk production. To guarantee the safety of milk and derivates, Maximum Residues Limits (EU-MRL) for different veterinary drugs in raw milk have been established by European legislation. However, the transfer of these substances from milk to cheese, has been poorly studied and, therefore, the impact of the use of raw milk containing admissible amounts of antibiotics on the cheese-making and the cheese safety is thus far unknown.

The aim of this thesis was to evaluate the transfer of the most widely used antibiotics used in dairy goats, from milk to fresh and matured cheese, as well as their effect on the cheese-making process and the quality characteristics of the cheeses during ripening. To this end, several studies were carried out using the experimental herd of Murciano-Granadina breed goats, and the pilot plant of Universitat Politècnica de València (Spain).

In the first study, antibiotic-free raw goat milk spiked individually with seven antibiotics (amoxicillin, benzylpenicillin, cloxacillin, erythromycin, ciprofloxacin, enrofloxacin and oxytetracycline) at EU-MRL equivalent antibiotic concentration, was used to make mature Tronchón cheeses, which were analyzed at different ripening times (0, 30, and 60 days) to determine antibiotic residues, pH, chemical composition, proteolytic and lipolytic activities, color, and textural properties. A sensory evaluation of 60 days ripened cheeses was also carried out. The cheese-making process was unaffected by the presence of most antibiotics in milk. Only erythromycin and oxytetracycline significantly increased the time required for cheese production (122±29 and 108±25 min, respectively). Regarding cheese characteristics, the few differences found were related to the free fatty acid concentration, color and textural properties, which remained mostly undetected by the sensory analysis panelists. However, variable amounts of antibiotics, ranging from 7.4 to 68 %, were transferred from milk spiked with antibiotics at concentrations equivalent to the MRL, to cheese. Oxytetracycline and quinolones presented the highest retention rates as well as persistence along maturation, with high concentrations of quinolones (enrofloxacin: 148±12 µg/kg, ciprofloxacin: 253±24 µg/kg) and oxytetracycline residues (20±5.7 µg/kg) in cheeses after 60 days of ripening.

Given that oxytetracycline is one of the most employed antibiotics in dairy goats, a similar study was carried out using different antibiotic concentrations closely related to the MRL (0, 50, 100, and 200 µg/kg). As described above, the presence of the oxytetracycline increased the time required for cheese production, the delay in acidification being antibiotic dose-dependent (26±7.2; 117±23.6; 217±28.4 min, respectively). The presence of this antibiotic hardly modified the organoleptic characteristics of the ripened cheeses such as texture, color and microstructure, whose differences were almost undetectable, even though the milk initially used contained twice the MRL allowed. Yet, the oxytetracycline concentration did not affect the volatile profile of the Tronchón cheeses, which was compared on a fortnightly basis during a 60-day period being was modified by the ripening time only. However, oxytetracycline was widely transferred from milk to cheese, with residue concentrations being 3.5-4.3 times higher than the drug concentration in raw milk used for cheese production. Oxytetracycline residues diminished along ripening in all cases, however, variable amounts of this substance remained in the ripened 60-day cheeses (<10-79 µg/kg), which could be of concern for public health.

On the other hand, the off-label use of antibiotics with a legally established minimum safety period of seven days is a common practice in dairy goats due to the limited availability of drugs registered for this species. Macrolide antibiotics are widely applied in an off-label manner to treat mastitis and other infectious diseases like contagious agalactia in endemic areas, thus increasing the risk of drug residues in milk, as the required elimination period is not always known. An in vivo experiment to verify if the exceptional use of macrolides (erythromycin, tylosin, and spiramycin) in dairy goats generates residues in milk and cheeses was carried out. Ripened cheeses were made from bulk milk obtained before drug administration, 24 hours after treatment, and at the end of the legal withdrawal period. Residual amounts of erythromycin (234.9±52.7 µg/kg), tylosin (198.7±57.8 µg/kg) and spiramycin (1,539.8±469.4 µg/kg), widely exceeding their legal EU-MRLs established were found in milk collected 24 hours after treatment, making the cheese production in most cases impossible. After the seven-day period, only spiramycin was detected in goat milk (79.6±19.2 µg/kg) although no antibiotic residues were found in the cheeses. Results herein suggest that a withdrawal period of seven days seems suitable to guarantee milk safety after the off-label administration of erythromycin and tylosin without any negative effects neither on the milk nor on the cheese properties. For spiramycin applications, an extended withdrawal period should be evaluated.

Finally, in the last study, the objective was to evaluate the presence of drug residues in pasteurized fluid milk and fresh cheeses obtained from goat milk containing antibiotics (amoxicillin, benzylpenicillin, cloxacillin, neomycin, erythromycin, ciprofloxacin, enrofloxacin and oxytetracycline) at safety levels (EU-MRLs). The safety margin of these dairy products for consumers was also evaluated. Results showed that high amounts of antibiotics, between 71-100% of the initial concentration in raw milk, remained in pasteurized goat milk and were transferred to cheese to a high extent, with retention percentages ranging from 37.5 to 75%. Regarding the safety margin of goat milk products, calculated taking into account different age groups (children, teenagers and adults), and the published negative effects of such antibiotics on consumer health, results indicate that the minimum safety margin of pasteurized milk was obtained for ciprofloxacin, enrofloxacin and erythromycin in the group of children. Regarding fresh cheese, an elevated safety margin was obtained for all antibiotics and age groups considered.

In summary, from the studies carried out, it can be concluded that the cheese-making process and the quality properties of the 60-days ripened Tronchón cheeses were slightly affected by the presence of antibiotics in goat milk at equivalent EU-MRL concentration. However, large amounts of highly stable substances such as quinolones could remain in the final products. Similarly, it is important to emphasize that relatively high concentrations of antibiotics could remain in pasteurized fluid goat milk and related products such as fresh cheese and cheeses of a short ripening period. The presence of these antibiotics in dairy products might contribute to the development and spread of antimicrobial resistance, which is considered an important public health concern worldwide.

The result of this research could serve the public health authorities to assess if current control systems of antibiotics in milk and dairy products are adequate or have to be revised. Considering the differences in the milk composition from different species, and the great variety of existing cheeses, it would be advisable to continue the study of traceability of antibiotics in order to increase the safety margin of dairy products and to guarantee public health.

Resumen

Los residuos de antibióticos en la leche y otros productos de origen animal constituyen un aspecto de gran importancia para la salud pública, ya que pueden causar problemas toxicológicos en consumidores sensibles y contribuir a la generación de resistencias antimicrobianas. Además, la presencia de estos residuos puede tener un efecto negativo en la industria láctea, afectando a los procesos de fermentación necesarios para elaborar determinados productos lácteos, como son el yogur y el queso, que son el principal destino de la producción de leche de cabra. Para proteger al consumidor la Unión Europea ha establecido los Límites Máximos de Residuos (UE-LMR) para diferentes medicamentos veterinarios en la leche cruda. Sin embargo, la transferencia de estas sustancias de la leche al queso ha sido poco estudiada y, por lo tanto, actualmente se desconoce el posible impacto de la presencia en la leche de cantidades admisibles de antibióticos sobre el proceso de elaboración y la seguridad del queso.

El objetivo de la tesis ha sido evaluar la transferencia de los antibióticos más empleados en el ganado caprino lechero, desde la leche a quesos frescos y curados, así como el efecto sobre el proceso de fabricación y la calidad de los quesos durante la maduración. Para alcanzar este objetivo diversos estudios han sido realizados utilizando el rebaño experimental de cabras de raza Murciano-Granadina y la planta piloto de la Universitat Politècnica de València (España).

En el primer estudio, leche cruda de cabra libre de antibiótico fue fortificada individualmente con siete antibióticos (amoxicilina, bencilpenicilina, cloxacilina, eritromicina, ciprofloxacina, enrofloxacina y oxitetraciclina) a una concentración equivalente de UE-LMR que fue utilizada para la fabricación de queso Tronchón curado. Los análisis se realizaron a diferentes tiempos de maduración (0, 30 y 60 días) para determinar el antibiótico residual, pH composición química, actividad proteolítica y lipolítica, parámetros de textura y color, así como la evaluación sensorial del producto final a 60 días. La mayor parte de los antibióticos en la leche no afectaron al proceso de fabricación. Solamente la eritromicina y oxitetraciclina incrementaron significativamente el tiempo requerido en el proceso de elaboración del queso (122±29 y 108±25 min, respectivamente). Respecto a las características del queso, las pocas diferencias encontradas se refirieron a la concentración de ácidos grasos libres, el color y las propiedades de textura, pero en su mayoría no fueron detectadas en la evaluación sensorial. Sin embargo, cantidades variables de antibióticos, de 7,4 a 68%, se transfirieron de la leche que contenía concentraciones equivalentes al MRL, al queso. Las quinolonas y oxitetraciclina presentaron las más elevadas tasas

de retención, así como persistencia a lo largo de la maduración, con altas concentraciones de quinolonas (enrofloxacina: 148±12 µg/kg; ciprofloxacina: 253±24 µg/kg) y de oxitetraciclina (20±5,7 µg/kg) después de los 60 días de curado.

Dado que la oxitetraciclina es uno de los antibióticos más empleados en el ganado caprino lechero, un estudio similar fue realizado usando diferentes concentraciones de oxitetraciclina cercanas al LMR (0, 50, 100, and 200 µg/kg) en la leche de cabra destinada a la fabricación de queso. Al igual que en el estudio anterior, la presencia de oxitetraciclina aumentó el tiempo requerido para la fabricación queso, el tiempo fue mayor conforme la concentración de antibiótico aumentaba (26±7,2; 117±23,6; 217±28,4 min, respectivamente). La presencia de oxitetraciclina apenas modificó las características organolépticas de los quesos madurados, como la textura, el color y la microestructura, cuyas diferencias fueron prácticamente indetectables, aunque la concentración de antibiótico en la leche fuera el doble del LMR establecido. Por otro lado, la concentración de oxitetraciclina no afectó al perfil de compuestos volátiles de los quesos Tronchón, los cuales fueron comparados cada quince días durante un período de maduración de 60 días, aunque dicho perfil si se modificó debido al tiempo de maduración. Sin embargo, la oxitetraciclina se transfirió ampliamente de la leche al queso, con concentraciones de residuos de 3,5 a 4,3 veces más altas que la concentración del fármaco en la leche cruda. Los residuos de oxitetraciclina disminuyeron a lo largo de la maduración del queso en todas las concentraciones estudiadas, aunque, cantidades variables de esta sustancia (<10-79 µg/kg), se encontraron en los quesos curados de 60 días lo que podría ser un problema para la salud pública.

Por otro lado, el uso excepcional (extra-label) de antibióticos registrados para otras especies en ganado caprino es una práctica común debido a la limitada disponibilidad de medicamentos registrados para esta especie. En estos casos el período de supresión legalmente establecido es de siete días como mínimo. Los antibióticos macrólidos se aplican ampliamente de esta forma extra-label para tratar la mastitis y otras enfermedades infecciosas como la agalactia contagiosa en áreas endémicas, lo que aumenta el riesgo de residuos de medicamentos en la leche, ya que no siempre se conoce el período de eliminación requerido. Para verificar si el uso excepcional de macrólidos (eritromicina, tilosina y espiramicina) en cabras lecheras generaba residuos en la leche y los quesos se llevó a cabo un estudio in vivo. Los quesos se elaboraron a partir de leche de mezcla de animales tratados, antes de la administración del fármaco, 24 horas después del tratamiento y al final del período de supresión recomendado. Concentraciones residuales de eritromicina (234,9±52,7

µg/kg), tilosina (198,7±57,8 µg/kg) y espiramicina (1539,8±469,4 µg/kg) que superaban ampliamente los LMR establecidos se encontraron en la leche a las 24 horas después del tratamiento, haciendo imposible la elaboración de queso en la mayoría de los casos. Después del período de siete días, solo se detectó espiramicina en la leche de cabra (79,6±19,2 µg/kg) aunque no se encontraron residuos de antibióticos en los quesos. Los resultados en este estudio sugieren que el período de supresión de siete días parece adecuado para garantizar la seguridad de la leche después de la administración de eritromicina y tilosina de forma extra-label, sin ningún efecto negativo sobre la leche ni sobre las propiedades del queso. Aunque para los tratamientos con espiramicina se recomienda evaluar un período de supresión más prolongado.

Finalmente, el último estudio tuvo como objetivo evaluar la presencia de residuos de medicamentos en la leche pasteurizada y en los quesos frescos a partir de leche de cabra con antibióticos (amoxicilina, bencilpenicilina, cloxacilina, neomicina, eritromicina, ciprofloxacina, enrofloxacina y oxitetraciclina) a niveles de seguridad (UE-LMR). También se evaluó el margen de seguridad de estos productos lácteos para los consumidores. Los resultados mostraron que altas cantidades de antibióticos, entre el 71 y el 100% de la concentración inicial en la leche cruda, permanecieron en la leche de cabra pasteurizada y que fueron transferidas al queso en gran medida, con porcentajes de retención que oscilaron entre 37,5 y 75%. Con respecto a los márgenes de seguridad de estos productos lácteos, se calcularon teniendo en cuenta diferentes grupos de edades (niños, adolescentes y adultos), y los efectos negativos de estos antibióticos sobre la salud, los resultados indicaron que el mínimo margen de seguridad en la leche pasteurizada lo presentaron la ciprofloxacina, enrofloxacina, y eritromicina para el grupo de niños. En relación al queso fresco, se obtuvo un elevado margen de seguridad para todos los antibióticos y grupos de edad considerados, lo que sugiere que este producto probablemente no tenga efectos negativos en la salud del consumidor.

En resumen, de los estudios realizados se puede concluir que el proceso de elaboración y las propiedades de calidad de los quesos Tronchón curados 60 días solamente se vieron ligeramente afectados por la presencia de antibióticos en la leche de cabra a una concentración equivalente de UE-LMR. Sin embargo, elevadas cantidades de antibióticos altamente estables, como las quinolonas, permanecen en el producto final. De manera similar, es importante enfatizar que concentraciones relativamente altas de antibióticos podrían permanecer en la leche de cabra pasteurizada y productos relacionados, como los quesos frescos y de corta

maduración. La presencia de estos antibióticos podría contribuir al desarrollo y propagación de la resistencia a los antimicrobianos que actualmente constituye un grave problema a nivel mundial.

Los resultados de este trabajo podrían servir a las autoridades de salud pública para evaluar si los sistemas actuales de control de antibióticos en la leche y los productos lácteos son adecuados o deberían ser revisados. Considerando las diferencias en la composición de la leche de las diferentes especies y la gran variedad de quesos existentes, sería aconsejable continuar el estudio de la transferencia de los antibióticos durante los procesos de elaboración para aumentar el margen de seguridad de los productos lácteos y garantizar la seguridad alimentaria.

Resum

Els residus d'antibiòtics en la llet i altres productes d'origen animal constitueixen un aspecte de gran importància per a la salut pública, ja que poden causar problemes toxicològics en consumidors sensibles i contribuir a la generació de resistències antimicrobianes. A més, la presència d'estos residus pot tindre un efecte negatiu en la indústria làctia, afectant els processos de fermentació necessaris per a elaborar determinats productes lactis, com són el iogurt i el formatge, que són el principal destí de la producció de llet de cabra. Per a protegir al consumidor la Unió Europea ha establit els Límits Màxims de Residus (UE-LMR) per a diferents medicaments veterinaris en la llet crua. No obstant això, la transferència d'estes substàncies de la llet al formatge ha sigut poc estudiada i, per tant, actualment es desconeix el possible impacte de la presència en la llet de quantitats admissibles d'antibiòtics sobre el procés d'elaboració i la seguretat del formatge.

L'objectiu de la tesi ha sigut avaluar la transferència dels antibiòtics més empleats en el bestiar caprí lleter, des de la llet a formatges frescos i curats, així com l'efecte sobre el procés de fabricació i la qualitat dels formatges durant la maduració. Per a aconseguir este objectiu diversos estudis han sigut realitzats utilitzant el ramat experimental de cabres de raça Murciano-Granadina i la planta pilot de la Universitat Politècnica de València (Espanya).

En el primer estudi, llet crua de cabra lliure d'antibiòtic va ser fortificada individualment amb set antibiòtics (amoxicilina, bencilpenicilina, cloxacilina, eritromicina, ciprofloxacina, enrofloxacina i oxitetraciclina) a una concentració equivalent d'UE-LMR que va ser utilitzada per a la fabricació de formatge Tronchón curat. Els anàlisis es van realitzar a diferents temps de maduració (0, 30 i 60 dies) per a determinar l'antibiòtic residual, pH composició química, activitat proteolítica i lipolítica, paràmetres de textura i color, així com l'avaluació sensorial del producte final a 60 dies. La major part dels antibiòtics en la llet no van afectar al procés de fabricació. Només l'eritromicina i l'oxitetraciclina van incrementar significativament el temps requerit en el procés d'elaboració del formatge (122±29 i 108±25 min, respectivament). Respecte a les característiques del formatge, les poques diferències trobades es van referir a la concentració d'àcids greixos lliures, el color i les propietats de textura, però majoritàriament no van ser detectades en l'avaluació sensorial. No obstant això, quantitats variables d'antibiòtics, de 7,4 a 68%, es van transferir de la llet que contenia concentracions equivalents al MRL, al formatge. Les quinolones i l'oxitetraciclina van presentar les més elevades taxes de retenció, així com persistència al llarg de la maduració, amb altes concentracions de quinolones

(enrofloxacina: 148±12 µg/kg; ciprofloxacina: 253±24 µg/kg) i d'oxitetraciclina (20±5,7 µg / kg) després dels 60 dies de curació.

Atés que l'oxitetraciclina és un dels antibiòtics més empleats en el bestiar caprí lleter, un estudi semblant va ser realitzat usant diferents concentracions d'oxitetraciclina pròximes al LMR (0, 50, 100, and 200 µg/kg) en la llet de cabra destinada a la fabricació de formatge. Igual que en l'estudi anterior, la presència d'oxitetraciclina va augmentar el temps requerit per a la fabricació formatge, el temps va ser major conforme la concentració d'antibiòtic augmentava (26±7,2; 117±23,6; 217±28,4 min, respectivament). La presència d'oxitetraciclina a penes va modificar les característiques organolèptiques dels formatges madurats, com la textura, el color i la microestructura, les diferències de la qual van ser pràcticament indetectables, encara que la concentració d'antibiòtic en la llet fora el doble del LMR establit. D'altra banda, la concentració d'oxitetraciclina no va afectar el perfil de compostos volàtils dels formatges Tronchón, els quals van ser comparats cada quinze dies durant un període de maduració de 60 dies, encara que el dit perfil sí es va modificar a causa del temps de maduració. No obstant això, l'oxitetraciclina es va transferir àmpliament de la llet al formatge, amb concentracions de residus de 3,5 a 4,3 vegades més altes que la concentració del fàrmac en la llet crua. Els residus d'oxitetraciclina van disminuir al llarg de la maduració del formatge en totes les concentracions estudiades, encara que, quantitats variables d'esta substància (<10-79 µg/kg), es van trobar en els formatges curats de 60 dies el que podria ser un problema per a la salut pública.

D'altra banda, l'ús excepcional (extralabel) d'antibiòtics registrats per a altres espècies en ramat caprí és una pràctica comuna a causa de la limitada disponibilitat de medicaments registrats per a esta espècie. En estos casos el període de supressió legalment establit és de set dies com a mínim. Els antibiòtics macròlids s'apliquen àmpliament d'esta manera extralabel per a tractar la mastitis i altres malalties infeccioses com l'agalàctia contagiosa en àrees endèmiques, la qual cosa augmenta el risc de residus de medicaments en la llet, ja que no sempre es coneix el període d'eliminació requerit. Per a verificar si l'ús excepcional de macròlids (eritromicina, tilosina i espiramicina) en cabres lleteres generava residus en la llet i els formatges es va dur a terme un estudi in vivo. Els formatges es van elaborar a partir de llet de mescla d'animals tractats, abans de l'administració del fàrmac, 24 hores després del tractament i al final del període de supressió recomanat. Concentracions residuals d'eritromicina (234,9±52,7 µg/kg), tilosina (198,7±57,8 µg/kg) i espiramicina (1539,8±469,4 µg/kg) que superaven àmpliament els LMR establits es van trobar en

la llet a les 24 hores després del tractament, fent impossible l'elaboració de formatge en la majoria dels casos. Després del període de set dies, només es va detectar espiramicina en la llet de cabra (79,6±19,2 µg/kg) encara que no es van trobar residus d'antibiòtics en els formatges. Els resultats en este estudi suggerixen que el període de supressió de set dies pareix adequat per a garantir la seguretat de la llet després de l'administració d'eritromicina i tilosina de forma extralabel, sense cap efecte negatiu sobre la llet ni sobre les propietats del formatge. Encara que per als tractaments amb espiramicina es recomana avaluar un període de supressió més prolongat.

Finalment, l'últim estudi va tindre com a objectiu avaluar la presència de residus de medicaments en la llet pasteuritzada i en els formatges frescos a partir de llet de cabra amb antibiòtics (amoxicilina, bencilpenicilina, cloxacilina, neomicina, eritromicina, ciprofloxacina, enrofloxacina i oxitetraciclina) a nivells de seguretat (UE-LMR). També es va avaluar el marge de seguretat d'estos productes lactis per als consumidors. Els resultats van mostrar que altes quantitats d'antibiòtics, entre el 71 i el 100% de la concentració inicial en la llet crua, van romandre en la llet de cabra pasteuritzada i que van ser transferides al formatge en gran manera, amb percentatges de retenció que van oscil·lar entre 37,5 i 75%. Respecte als marges de seguretat d'estos productes lactis, es van calcular tenint en compte diferent grups d'edats (xiquets, adolescents i adults), i els efectes negatius d'estos antibiòtics sobre la salut, els resultats van indicar que el mínim marge de seguretat en la llet pasteuritzada ho van presentar la ciprofloxacina, la enrofloxacina, i l'eritromicina per al grup de xiquets. En relació al formatge fresc, es va obtindre un elevat marge de seguretat per a tots els antibiòtics i grups d'edat considerats, la qual cosa suggereix que este producte probablement no tinga efectes negatius en la salut del consumidor.

En resum, dels estudis realitzats es pot concloure que el procés d'elaboració i les propietats de qualitat dels formatges Tronchón curats 60 dies només es van veure lleugerament afectats per la presència d'antibiòtics en la llet de cabra a una concentració equivalent d'UE-LMR. No obstant això, elevades quantitats d'antibiòtics altament estables, com les quinolones, romanen en el producte final. De manera semblant, és important emfatitzar que concentracions relativament altes d'antibiòtics podrien romandre en la llet de cabra pasteuritzada i productes relacionats, com els formatges frescos i de curta maduració. La presència d'estos antibiòtics podria contribuir al desenrotllament i propagació de la resistència als antimicrobians que actualment constitueix un greu problema a nivell mundial.

Els resultats d'este treball podrien servir les autoritats de salut pública per a avaluar si els sistemes actuals de control d'antibiòtics en la llet i els productes lactis són adequats o haurien de ser revisats. Considerant les diferències en la composició de la llet de les diferents espècies i la gran varietat de formatges existents, seria aconsellable continuar l'estudi de la transferència dels antibiòtics durant els processos d'elaboració per a augmentar el marge de seguretat dels productes lactis i garantir la seguretat alimentària.

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Chapter 1

Introduction

1. GOAT MILK PRODUCTION AND QUALITY

1.1. Goat milk and dairy products

Although goats produce only approximately 2% of the world's total annual milk supply, goat milk plays an important role in nutrition and socioeconomic wellbeing of developing and underdeveloped countries, where it provides basic nutrition and subsistence to rural people (Clark and Mora-García, 2017). In recent years, consumers have shown a greater interest in goat milk and derivatives related to their nutritional and digestive properties and providing gourmet foods to connoisseur consumers (Haenlein, 2004; Park et al., 2017).

Worldwide production of goat milk was 15.3 million tons in 2016, being distributed in a highly variable manner across the continents (Asia: 53%, Africa: 26%, Europe: 16% and America: 5%). It should be noted that countries in the Mediterranean region are relevant for dairy goats, including France (24%), Spain (16%) and Greece (15%) (FAOSTAT, 2018).

Goat milk is used to make fluid pasteurized milk and a wide range of dairy products, especially yogurt and different types of cheese (Park and Haenlein, 2010). In the last 20 years, goat cheese production in the world has significantly increased reaching 523,040 tons in 2014 (FAOSTAT, 2018). Figure 1 shows the distribution of goat cheese production in the world, and in the most relevant European countries.

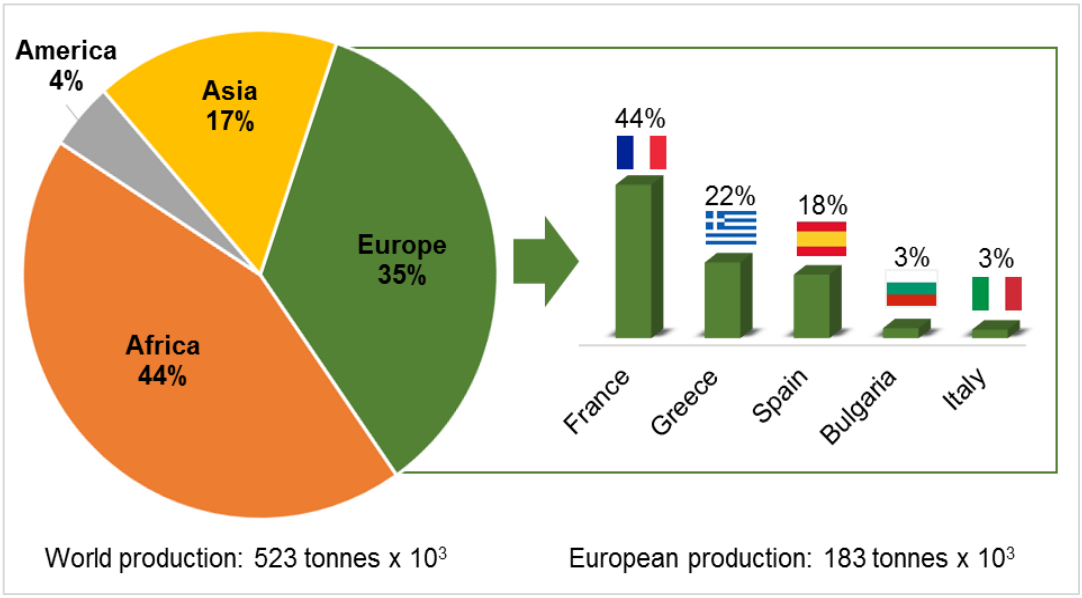


Figure 1. Goat cheese production in 2014.
Source: FAOSTAT (2018)

Europe (35%) represents an important part of the production of goat cheese, with France, Greece and Spain accounting for 84% of the total goat cheese production on the continent (FAOSTAT, 2018). These countries are characterized by a great tradition of goat milk production destined for the manufacture of pure goat or mixed milk cheeses, many of them under Protected Designation of Origin (PDO) and Protected Geographical Indications (PGI), among other brands of quality of international recognition.

Just as in other Mediterranean countries, in Spain, goat milk is traditionally destined for the cheese-making (approx. 98%), especially in Andalucía, Castilla-La Mancha; Murcia, Extremadura, Castilla-León, and Canarias (Figure 2), with the production of other dairy products such as fluid milk and yogurt being practically negligible in quantitative terms (Martínez et al., 2011; Medina and Núñez, 2017).

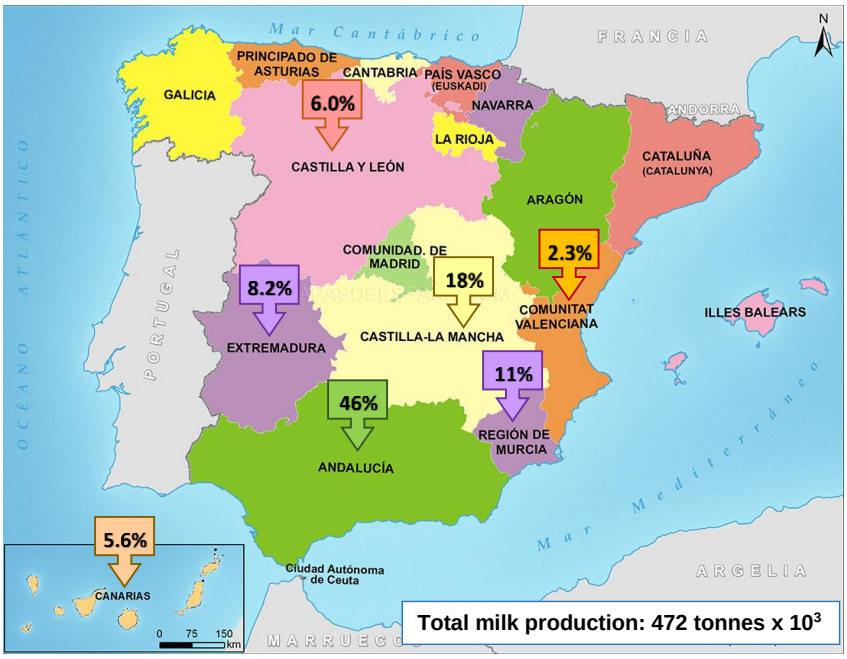


Figure 2. Production of goat milk destined to cheese-making in Spain by Autonomous Communities in 2017.
Source: MAPA (2018)

There are more than 150 varieties of cheese in Spain, including a considerable number of traditional goat cheeses. Six varieties of these (Camerano, Ibores, Murcia, Murcia al vino, Palmero, and Majorero) are made from pure goat milk and another five varieties (Guía, Picón, Gamonedo, Cabrales and Liébana) are from mixed cow, sheep and goat milk. All of them are protected by the Protected Designation of Origin (PDO), regime.

In Comunitat Valenciana region (Spain) different types of cheese are made, from goat and sheep milk (Figure 3). There is a long tradition of fresh cheeses (Cassoleta,

La Nucia, and Servilleta) and some cured cheeses such as Tronchón cheese stand out (Martínez et al., 2011). Tronchón is a very prestigious cheese, made from sheep, goat or mixed milk, coagulated by animal rennet, slightly ripened or ripened (>60 days), having a characteristic shape of a flat cylinder punched on the top.

Figure 3. Types of cheeses made in from Comunitat Valenciana.
Source: Modified from Soriano (2009)

With respect to cheese consumption, goat cheese (280 g/habitant/year); represents a small part of the total cheese consumption (8 kg/habitant/year), including all types of cheese from different species (MAPA, 2019). Despite the low volume compared to other cheeses, the consumer interest in goat milk products has grown owing to the availability of a wide range of high-quality dairy products.

1.2. Goat milk quality

The evaluation of goat milk quality is of fundamental economic importance, especially in those countries where the caprine sector is well developed, and the system of quality payment is mainly based on protein and fat contents as well as on the hygienic quality of milk (i.e., bacterial and somatic cell counts) (Pirisi et al., 2007).

Moreover, information on the physicochemical characteristics and composition of milk is essential for the successful development of the dairy goat industry as well as for the marketing of the products, since these characteristics are among the major factors determining the yield and quality of cheese. Some physicochemical parameters are of special interest (Table 1), acidity allows evaluating indirectly the hygienic quality of raw milk, while density or freezing point make it possible to identify fraud due to the addition of water in milk (Raynal-Ljutovac et al. 2005).

Table 1. Physical parameters of goat milk

Specific gravity (g/cm^3)	Freezing point (-ºC)	Acidity (% lactic acid)	pH	References
1.029-1.039	0.540-0.573	0.14-0.23	6.50-6.80	Park et al. (2007)
-	0.560	-	6.86	Salvador et al. (2006)
1.030	0.554	0.15	6.70	Romero et al. (2013)
1.028-1.030	-	0.17	-	Almeida et al. (2014)
1.029-1.032	0.540-0.550	0.15-0.17	6.4-6.60	Rawya and Ahmed (2014)
1.028-1.039	**0.540-0.573**	**0.14-0.23**	**6.4-6.86**	**Range**

Goat milk differs from cow milk in having a better digestibility, alkalinity, buffering capacity, and certain therapeutic values in medicine and human nutrition (Turkmen, 2017). Compared to cow and sheep milk, goat milk has its unique differences in several important constituents and physical parameters, such as proteins, lipids, minerals, vitamins, carnitine, glycerol ethers, orotic acid, enzymes, fat globule size, casein polymorphisms (Park and Haenlein, 2007).

The information currently available on the composition of goat milk has been published in the form of reviews (Jenness, 1980; Park et al., 2007; Park and Haenlein, 2010; Clark and Mora-García, 2017). The composition of the milk produced by a given species depends on the breed, individuals, lactation state, parity, season, feeding, management and the environment. Table 2 presents data on gross composition of goat milk for different goat breeds and countries.

As can be appreciated in Table 2, total solids cover a very broad range (9.00-14.80%). It should be noted that the fat (2.70-7.48%) and protein (2.40-5.03%) content in the milk can vary widely depending on the previously mentioned factors. However, the milk of more specialized dairy breeds generally presents a composition with lower amounts of fat and protein, mainly due to the differences in production levels between breeds.

Table 2. Chemical composition (%) of milk from different dairy goat breeds.

Goat breed	Country	Total solids	Fat	Protein	Lactose	References
Alpine	Slovenia	11.06	3.36	2.95	4.02	Žan et al. (2006)
Alpine	USA	9.00-10.90	2.60-3.10	2.40-2.80	-	Soryal et al. (2005)
Bischofshofen	Austria	11.70	3.74	3.15	4.32	Mayer and Fiechter (2012)
British Saanen	United Kingdom	11.60	3.48	2.61	4.30	Raynal-Ljutovac et al. (2008)
Boer	South Africa	-	6.13-6.39	4.97-5.03	4.48-4.97	Mmbengwa et al. (2000)
Capra Prisca	Greece	12.57-13.13	4.12-4.30	3.44-3.71	4.26-4.53	Kondyli et al. (2012)
Crossbreed Canarias	Venezuela	13.64	3.87	4.82	-	Salvador et al. (2006)
Damascus	Cyprus	11.31-13.70	2.36-4.43	3.85-4.31	3.85-4.15	Güney et al. (2006)
Local breed Greece	Greece	14.80	5.63	3.77	4.76	Raynal-Ljutovac et al. (2008)
Local breed India	India	12.33-13.67	3.55-4.55	3.22-4.10	4.19-4.89	Bhosale et al. (2009)
Nubian	USA	12.70-14.60	4.00-4.80	3.50-4.50	-	Soryal et al. (2005)
Nubian	United Kingdom	-	4.94	3.60	4.51	Raynal-Ljutovac et al. (2008)
Nguni	South Africa	-	6.04-7.48	4.54-4.95	4.27-4.50	Mmbengwa et al. (2000)
Murciano-Granadina	Spain	12.58	4.70	3.19	-	León et al. (2012)
Murciano-Granadina	Spain	14.5-14.7	5.0-5.1	3.9-4.3	4.6-4.8	Fernández et al. (2018)
Saanen	Slovenia	12.26	3.77	3.40	4.36	Žan et al. (2006)
Saanen	Brazil	11.61	3.55	3.15	4.85	Almeida et al. (2014)
Sardinian	Italy	-	5.10	3.90	-	Raynal-Ljutovac et al. (2008)
Range		**9.00-14.80**	**2.36-7.48**	**2.40-5.03**	**3.85-4.97**	

Lipids composition is of great importance for the technological and nutritional quality of goat milk. Lipids are involved in cheese yield and firmness, as well as in the color and flavor of goat dairy products (Chilliard et al., 2003). The characteristic flavor is a major criterion in the selection and consumption of goat milk cheeses by the consumer. Flavor is influenced largely by the profile and concentration of Free Fatty Acids (FFAs) present in the cheese.

A number of works have reported the Fatty Acid (FA) composition of goat milk fat (Raynal-Ljutovac et al., 2008; Núñez-Sánchez et al., 2016). The lipid composition of goat milk includes simple and complex lipids (mono-, di-, tri-glycerides, cholesterols, phospholipids and sterols) (Park et al., 2007). Goat milk is distinguished by having short and medium chain FA contents (C6-C12) higher than other types of milk, which are responsible for aromas and flavors. Generally, goat milk fat contains 53-72% saturated FA, 26-42% mono-unsaturated FA and 2-6% polyunsaturated FA. Among the poly-unsaturated FAs, conjugated linoleic acid (C18:2) should be highlighted, which is characterized by a high biological activity and potentially beneficial health effects (Zhang et al., 2006). Fatty acid concentration varies among animal species as well as with the season mainly related to the availability of feed (Chilliard et al., 2003).

Moreover, a high proportion of small fat globules in goat milk when compared to cow milk (3.5 μm *vs* 4.6 μm), is associated with a better digestibility (Park and Haenlein, 2010).

Regarding the proteins of goat milk, non-protein nitrogen (urea, nucleotides and nucleosides, creatinine, etc.) varies between 3% and 7% of the total nitrogen (Park et al., 2007; Prosser et al., 2008) and this level is higher than in sheep and cow milk, affecting cheese yield, cheese consistency and texture. Caseins (CN) represent approximately 80% of the total protein, being α_{s1}-CN (5.6% of the total casein), α_{s2}-CN (19.2%), κ-CN (20.4%), and β-CN (54.8%). The low level of α_{s1}-casein in goat milk compared to cow milk (38% of total casein) accounts for the hypoallergenicity of goat milk (Park, 2017). The lack of α_{s1}-casein in goat milk is caused by the high degree of genetic polymorphism among individual goats (Ballabio et al., 2011). Regarding whey protein, mainly containing α-lactalbumin and β-lactoglobulin (24 and 54% of total whey protein, respectively). Also, milk comprises other proteins, such as serum albumin, immunoglobulins, lactoferrin, transferrin, prolactin, and proteose-peptone among others (Park et al., 2007; Selvaggi et al., 2014).

Goat milk has been recommended as a substitute for cow milk for infants and patients who suffer from cow milk allergy (CMA), since cow milk proteins cannot be

tolerated (Getaneh et al., 2016). Moreover, casein micelles in goat milk differ markedly from those of cow milk, having a smaller size of micelles, more calcium and phosphorus, and low heat stability (Kalyankar et al., 2016).

Moreover, goat milk is a natural source of oligosaccharide derivatives from lactose, presenting a healthier lipid composition, a higher vitamin A and complex B contents as well as calcium content (Haenlein and Anke, 2011).

With respect to the hygienic quality of milk, the European Union published a legislative framework on the hygiene of foodstuffs for human consumption (Regulation EC Nº 852/2004) and more specifically those of animal origin (Regulations EC Nº 853 and 854/2004). In these regulations, the parameters to be controlled in milk are the bacterial count, the somatic cell count and the presence of antibiotics (Table 3).

Table 3. Hygienic quality parameters of raw milk according animal specie.

Parameters	Cow	Sheep and goat	
Bacterial count (cfu/mL)[1]	100,000	500,000[2]	1,500,000[3]
SCC (cell/mL)[4]	400,000	-	-
Presence of antibiotics	Absence of residues above MRL established in the EU		

[1] Geometric average in a two-month period, with at least two samples per month; [2] raw milk is intended for the manufacture of products made with raw milk by a process that does not involve any heat treatment; [3] raw milk is intended for the manufacture of products made with milk by a process that involve any heat treatment; [4] geometric average over a three-month period, with at least one sample per month. Source: Regulation (EC) Nº 853/2004.

As summarized in Table 3, regarding the bacterial count, Regulation (EC) Nº 853/2004 established for milk from species other than cow values of 1,500,000 and 500,000 cfu/ml, for the manufacture of products based on heat treatment or non-heat treatment, respectively. Somatic cell count (SCC) refers to a concentration of blood cells such as leukocytes, and epithelial cells in milk and is used as an indicator to estimate the health of the mammary gland and the prevalence of mastitis. Regulation (EC) Nº 853/2004 establishes a maximum threshold of 400,000 cell/ml for cow milk, while no limits have been established for sheep and goat milk. Both bacterial counts and SCC could be lowered by improved management conditions, which includes sanitation of the farm, animals, milking parlor, udder sealing, milking equipment maintenance, refrigerator conditions and timely transportation after milking.

Another parameter of great importance when evaluating hygienic quality is the possible presence of antibiotic substances in raw milk. Regulation (EC) No 853/2004 (Annex III, Section IX, Chapter III, 4) establishes:

"Without prejudice to Directive 96/23/EC, food business operators must initiate procedures to ensure that raw milk is not placed on the market if either:

(a) it contains antibiotic residues in a quantity that, in respect of any one of the substances referred to in Annexes I and III to Regulation (EEC) No 2377/90 (current Table 1 in the Annex to Commission Regulation (EU) Nº 37/2010), exceeds the levels authorized under that Regulation; or

(b) the combined total of residues of antibiotic substances exceeds any maximum permitted value."

The main aspects related to the control and the possible impact of the presence of antibiotic residues in milk will be treated in more detail in section 2 and 3 in the Introduction, since this thesis focuses on the effect of antibiotics in goat milk on cheese manufacture and quality.

1.3. Characteristics and quality parameters of goat cheese

Cheeses made from goat milk are greatly appreciated for their particular organoleptic characteristics and nutritive value. There is a considerable number of traditional goat cheeses worldwide as either pure goat milk cheese or blended milk cheese, together with cow and/or sheep milk. Some of them are produced at artisanal or semi-industrial level, being commercialized and consumed in very limited areas.

Most cheese properties are derived from the chemical composition of the milk used to produce it. However, the manufacturing process affects the nutritional and sensory characteristics of the finished product (Raynal-Ljutovac et al., 2011). Figure 4 shows the general cheese-making process for fresh and matured cheese.

The criteria for the classification of cheeses are multiple, since they can be based on numerous factors related: milk origin, raw or heat-treated milk, type of coagulation (rennet, lactic, mixed coagulation), ripening time, fat content of the cheese, internal and external aspect of the cheese, among others (IDF, 1981). Goat milk cheeses can be divided in different groups as fresh (unfermented) cheeses; soft cheeses that undergo lactic fermentation and mold surface flora, semi-hard and hard cheeses.

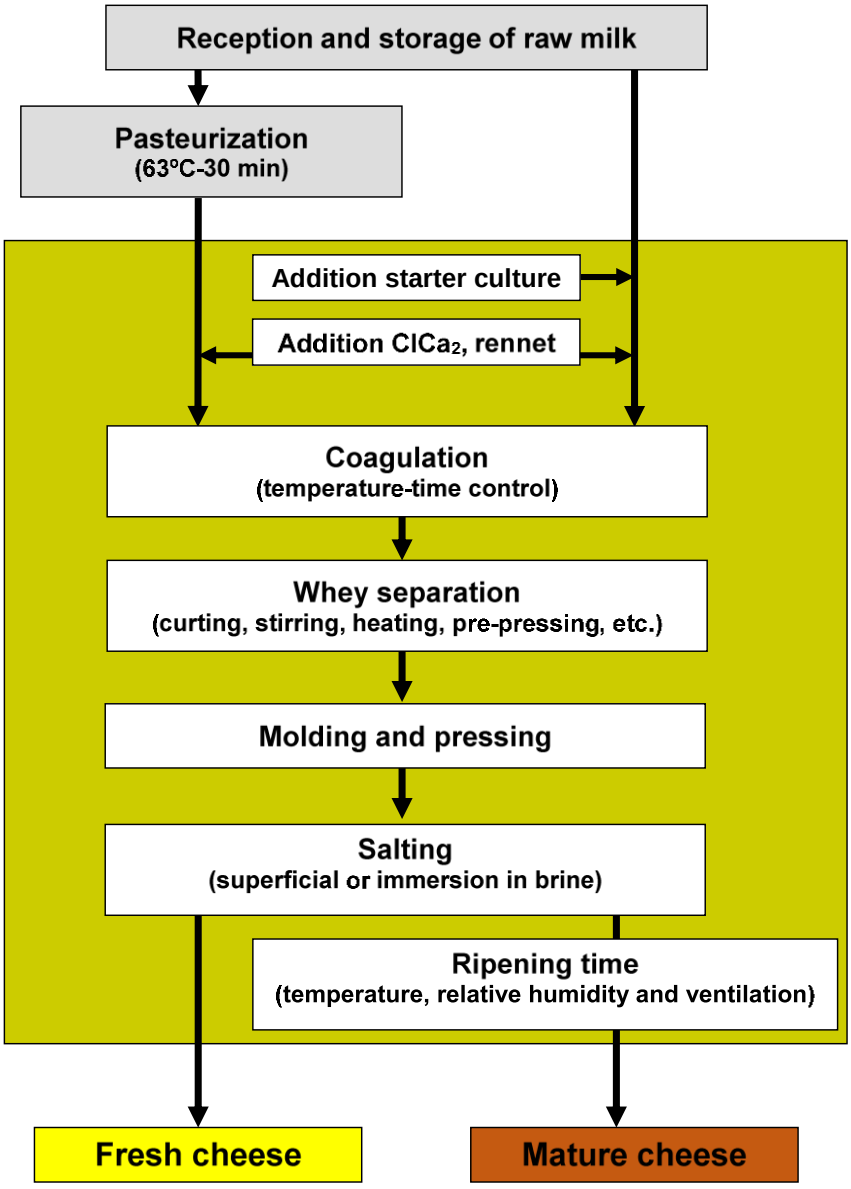


Figure 4. General cheese-making process for fresh and mature cheeses.

The composition of relevant types of goat cheese is reported in Table 4. The total solid content of fresh goat cheese ranges from 38 to 54%, while matured cheeses, with a ripening period of between 30 to 180 days, show a range of total solid content for 53 to 83%. This high total solids in mature cheese is due to the reduction of the water content during the ripening time. Fat (11 to 60%) and protein (12 to 31%) percentages have a wide variation among the different types of cheese, with the highest values of these components corresponding to mature goat cheese. Regarding the salt content, a wide range of variation (1.3 to 5.4%), depending on the type of cheese, can be appreciated.

Table 4. Chemical composition (%) of different goat milk cheeses.

Goat cheese	Type of cheese/ ripening time	Country	Total solids	Fat	Protein	Salt	References
Cacioricotta	Fresh, 7 days	Italy	43-51	11-23	15-19	1.3-4.5	Albenzio et al. (2006)
Cheddar type	Hard	USA	62	26	22	-	Fekadu et al. (2005)
Colby type	Semihard	USA	54	25	18	-	Fekadu et al. (2005)
Domiati	Fresh	USA	38	15	12	-	Soryal et al. (2004)
Feta type	21 days	S. Africa	41	17	15	4.1	Pitso and Bester (2000)
Fresh cheese	1 day	Spain	43-46	25-26	-	1.4-1.9	Trujillo et al. (1999)
Gokceada	~60 days	Turkey	50	24	19	5.4	Hayaloglú et al. (2013)
Ibores	90 days	Spain	63	36	23	-	Delgado et al. (2011)
Majorero	90 days	Spain	67	34	20	-	Fresno and Álvarez (2012)
Monterey Jack	Semihard	USA	58	23	27		Park et al. (2006)
Murcia al vino	60 days	Spain	65-66	40-44	22-24	1.9-2.1	Ferrandini et al. (2011)
Palmero type	Fresh	Spain	54	23	21	-	Guillén et al. (2004)
Plain soft	Fresh	USA	40	20	15	-	Park et al. (2006)
Travnicki	30 days	Bosnia	53	29	20	-	Saric et al. (2002)
Tronchón	60 days	Spain	66	37	25	2.2	Rivera et al. (2016)
Urfa	90 days	Turkey	51	22	-	5.0	Atasoy and Türkoglu (2009)
Xinotyri	180 days	Greece	83	60	31	2.6	Bontinis et al. (2008)
Range			**38-83**	**11-60**	**12-31**	**1.3-5.4**	

In general, goat milk cheeses have a very variable composition, related to the milk characteristics, the goat bread, the cheese-making region, as well as depending on the history and tradition of the manufacturing process, resulting in unique products according to the area of production.

In mature cheeses, especially from raw milk, the addition of starter cultures is common practice for the fermentation of lactose. The starter cultures are constituted by certain species of lactic acid bacteria (LAB) including *Lactococcus lactis, Leuconostoc species, Streptococcus thermophillus, Lactobacillus delbrueckii subsp. lactis and bulgaricus, and Lactobacillus helveticus,* but not all of them are used in every cheese variety (Hayaloglu et al., 2005; Parente et al., 2017). Moreover, the enzymes originating from starter cultures and non-starter LAB (i.e., proteinases, peptidases) together with indigenous milk and rennet enzymes (i.e. chymosin, pepsine, and esterase) play a major role in the biochemical changes during ripening (Fox et al., 2017). In general, complex commercial starter cultures and unpasteurized milk are used in the production of most artisan goat cheeses (Gámbaro et al., 2017).

Certain sensory characteristics, such as flavor, aroma and texture, are key to the identity, quality, and acceptability of goat cheese. Biochemical changes during ripening can be grouped into primary events that include the metabolism of residual lactose and lactate and citrate (glycolysis), and lipolysis and proteolysis pathways (McSweeney and Sousa, 2000; Garbowska et al., 2016).

The flavor of a cheese results from the presence of several compounds such as FFAs, esters, aldehydes, ketones, alcohols, amines and hydrogen sulphide that are formed by biochemical reactions (particularly proteolysis and lipolysis) that occur in the cheese during the ripening process (Delgado et al., 2010). Figure 5 summarizes the flavor formation compounds in cheese.

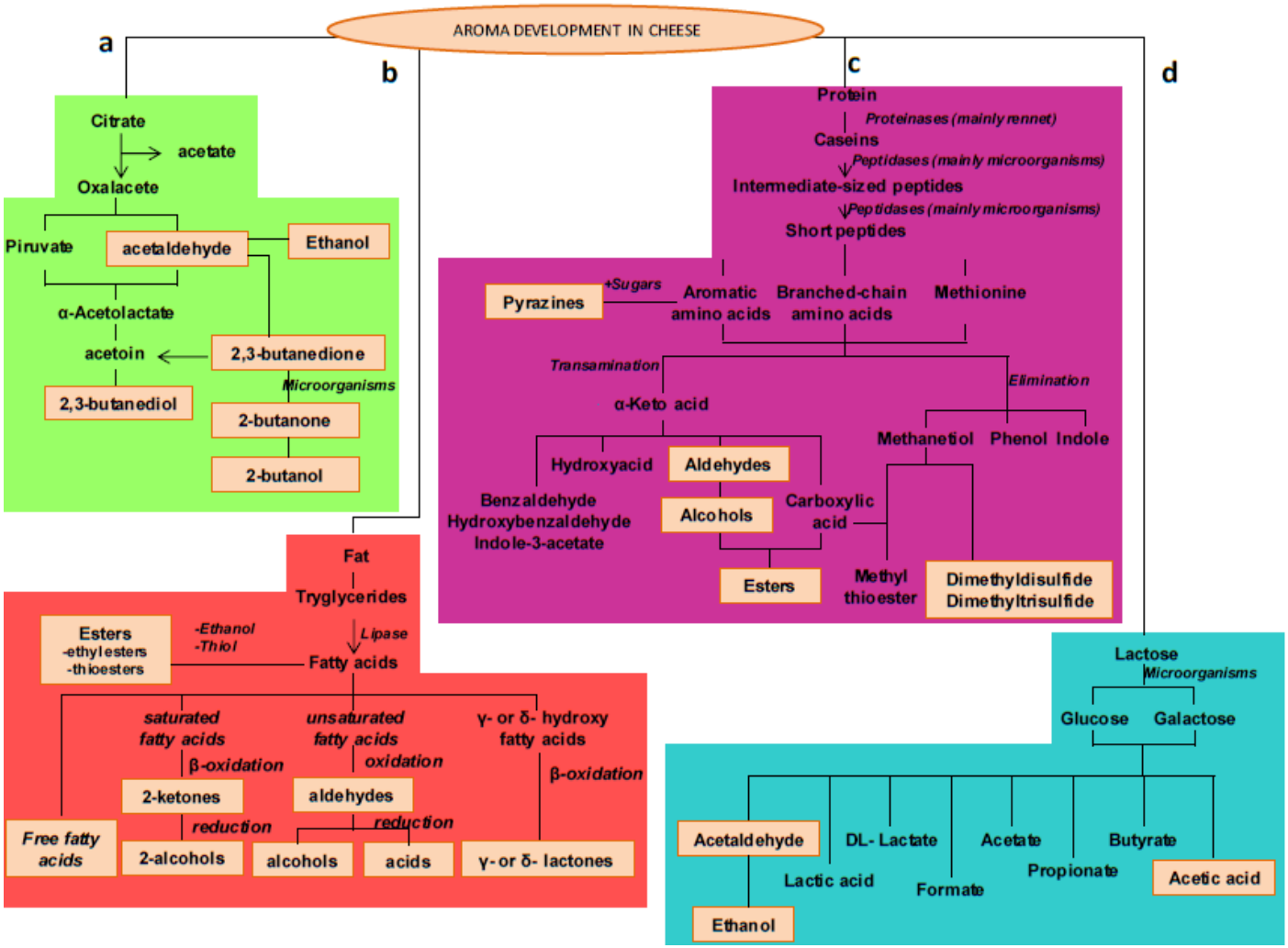


Figure 5. Main pathways of the metabolism of aroma compounds in cheese.
a) Citrate metabolism, b) lipolysis and free fatty acid metabolism, c) proteolysis and amino acid catabolism d) lactose metabolism
Source: Licón (2012)

Lipolysis is the hydrolysis of triglycerides releasing short- and medium-chain FFAs contributing to the flavor characteristics of goat cheese, or indirectly as precursors of aroma components (Curioni and Bosset, 2002; Park et al., 2017). The higher volatility and water solubility of these FFAs, compared with long-chain FAs, explain their considerable effect on the sensory properties of cheese despite being less abundant than long-chain FAs (Raynal-Ljutovac et al., 2011). Cheeses made with goat milk contain a high percentage of short- and medium-chain fatty acids (caproic, capric and caprylic). Additionally, certain branched-chain FFAs contribute, by themselves, to the goaty flavor of cheese (Collins et al., 2003; Clark and Mora-García, 2017).

Proteolysis is an important event which occurs during cheese ripening, influencing textural changes of the cheese matrix and, also, contributing directly to the flavor in most curd cheese varieties. Hydrolysis of milk proteins during cheese ripening occurs in different steps, starting when milk proteins (mainly caseins) are hydrolyzed by the rennet enzymes, releasing high molecular weight peptides, that are degraded to smaller peptides and further hydrolyzed to amino acids, amino-, carboxy-peptidases and di/tri peptidases, during cheese ripening by microbial peptidases. Finally, FAAs are metabolized producing volatile compounds (McSweeney and Sousa, 2000; Andiç et al., 2015; Fox and McSweeney, 2017).

Thus, proteolytic and lipolytic processes must occur in a coordinated way to give each cheese type its unique and appreciated sensory characteristics. The physicochemical and sensory characteristics of goat cheeses have been studied by different authors (Cabezas et al., 2005; Fresno and Álvarez, 2012; Gámbaro et al., 2017).

Sensory analysis is a valuable tool to measure consumer responses to different foods and to understand aspects, such as color, aroma, flavor, visual appearance, that differentiate cheeses (Foegeding and Drake, 2007; Clark et al., 2009).

The guidelines for sensory assessment are established by the International Standard Organization-ISO (ISO 4121, 2003; ISO 8587, 2007; ISO 5492, 2008). To carry out an adequate sensory analysis, an appropriate test (descriptive, discrimination, consumer acceptability), suitable conditions, panel types (consumers or trained panelists) and the statistical analysis have to be chosen in order to obtain reproducible, powerful, and relevant results (Drake, 2007).

2. USE OF ANTIBIOTICS IN DAIRY LIVESTOCK

2.1. Classification of antibiotics

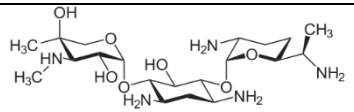
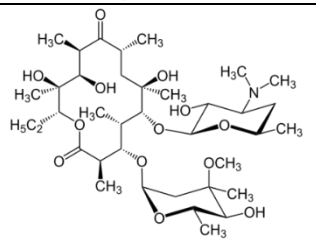
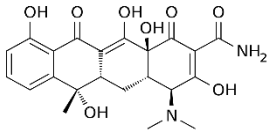
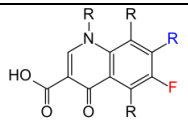
Since the discovery of penicillin, by Fleming in 1928, hundreds of antimicrobial agents have been developed for anti-infective therapy. The use of veterinary drugs, especially antibiotics, in the treatment and prophylaxis of mastitis and other infectious diseases in dairy livestock is a widespread practice nowadays.

The classes of antimicrobial agents, their physicochemical and pharmacological properties as well as the drug use in selected animal species have been presented by several authors in compendia on antimicrobial therapy in veterinary medicine (Botsoglou and Fletouris, 2001; Wang et al. 2012; Giguère et al., 2013; Papich, 2016; Daeseleire et al., 2017). The most relevant characteristics of antibiotic families used in goat livestock are summarized in Table 5 and are further described in this section.

β-lactam antibiotics are the most widely used antimicrobial drugs in veterinary practice. This group is comprised of a great variety of natural and semisynthetic penicillins (amoxicillin, ampicillin, benzylpenicillin, cloxacillin, etc.), including cephalosporin (cephalexin, ceftiofur, cefoperazone, cefquinome, etc.). All β-lactams have as basic structure a β-lactam ring responsible for the antibacterial activity and variable side chains account for the major differences in their chemical and pharmacological properties. β-lactams are polar organic acids and generally have a non-lipophilic nature; with a moderate to strongly acidic character (pK_a 2.7). β-lactam antibiotics are commonly used in goat livestock to treat mainly mastitis, infections reproductive disorders and urinary tract infections (De Briyne et al., 2014).

Aminoglycosides are elaborated naturally by bacteria of the genus *Streptomyces* and *Micromonospora*, and also semisynthetic derivates have been developed. The structure of aminoglycosides consists of one or more sugar units in the form of a glycosamine and/or a disaccharide, which are connected by a glycosidic linkage to a central aglycon moiety. In food-producing animals' streptomycin, gentamicin and neomycin are most commonly used. The pharmacokinetics of aminoglycosides are dictated by their highly polar and poorly lipid-soluble physicochemical properties and have limited capacity to enter cells. Aminoglycosides are active against mycoplasmas and are commonly used in dairy goats for treatment of different types of mastitis, pneumonia, and abortion diseases (Mavrogianni et al., 2011; Paterna, et al., 2013).

Table 5. Classification of the most commonly employed antibiotics in veterinary medicine.

Antimicrobial group	Structure	Bacterial effect/ Spectrum of action	Mechanism of action	Substances
β-lactams		Bactericide/ Broad-spectrum	Cell wall synthesis inhibitors	Penicillins: amoxicillin, ampicillin, benzylpenicillin, cloxacillin. Cephalosporins: cephalexin, ceftiofur, cefoperazone, cefquinome.
Aminoglycosides		Bactericide/ Narrow-spectrum	Protein synthesis inhibitors	Streptomycin, gentamicin, neomycin.
Macrolides		Bacteriostatic/ Narrow-spectrum	Protein synthesis inhibitors	Erythromycin, spiramycin, tylosin.
Tetracyclines		Bacteriostatic/ Broad spectrum	Protein synthesis inhibitors	Chlortetracycline, oxytetracycline, tetracycline.
Quinolones		Bactericide/ Broad- spectrum	Acid nucleic synthesis inhibitors	Enrofloxacin, marbofloxacin, norfloxacin.

Source: Beltrán (2014)

Most macrolide antibiotics are compounds isolated from cultures of *Streptomyces* strains. They have a common 14-, 16- or 17- membered macrocyclic lactone ring linked to one or more sugars, often amino sugars and lipophilic weak organic bases and possess moderate to high lipid solubility. Macrolides are highly effective against a wide range of gram-positive bacteria, representing the most effective medicines against diseases produced by *Mycoplasmas* and are, therefore, widely used in veterinary practice for prophylaxis and treatment of mycoplasmosis (Prats-Van der Ham et al., 2017).

In dairy goats, macrolides are usually employed in an off-label manner to treat mastitis, respiratory conditions as well as contagious agalactia in endemic areas (Atef et al., 2009; Young et al., 2011; Gómez-Martín et al., 2013).

Quinolones constitute an expanding group of synthetic antibiotics that are very effective in veterinary treatments. Fluoroquinolones, which are second-generation quinolones, include ciprofloxacin, enrofloxacin, marbofloxacin, among others. These antibacterial compounds are amphoteric, as they contain both carboxylic acidic and basic amino groups, with pK_a values between 5.5-6.5 and 7.5-9.3. Lipophilicity varies between drugs, always being moderate (ciprofloxacin, marbofloxacin) or high (enrofloxacin). In dairy goats, fluoroquinolones are usually administered by veterinarians in the treatment of gastrointestinal, respiratory and mammary diseases (Menzies and Ramanoon, 2001).

Tetracyclines are one of the most commonly broad-spectrum antibiotics used in animal husbandry. Naturally occurring tetracyclines (chlortetracycline and oxytetracycline) have been isolated from fungi and several others (i.e. tetracycline) have been prepared semisynthetically. These substances contain a linear fused tetracyclic nucleus (rings of four atoms) to which a variety of functional groups are attached. The lipid solubility of tetracyclines varies according to the substance, being moderate for oxytetracycline and chlortetracycline, and high for doxycycline. This group of antibiotics is widely used in caprine livestock in practically all types of diseases, such as mastitis, urinary tract infections or related to the nervous system, among others (Obaidat et al., 2017).

Currently, the use of different antibiotics is a common veterinary practice in caprine livestock for the treatment of diseases and health maintenance of the animals. The improper use of antibiotics is the major source of drug residues in milk (Sawant et al 2005). The International Dairy Federation published “The Guide to Prudent Use

of Antimicrobial Agents in Dairy Production" (IDF, 2013), which provides a generic framework to back up the responsible use of antimicrobial agents on dairy farms.

2.2. Use of antibiotics in veterinary medicine

The European Medicines Agency (EMA, 2018) reports the sales of veterinary antimicrobial agents for food-producing animals expressed in mg/Population Correction Unit (PCU), which is a technical unit of measurement for the estimated weight in the treatment of livestock and of slaughter animals (1 PCU = 1 kg). Figure 6 displays the sales of antimicrobials in 30 European countries in 2016, with Cyprus, Spain and Italy being the highest consumers of antimicrobials for food-producing species.

In general, regarding the largest amounts of sales of antimicrobials for the treatment of animal food-processing species, EMA (2018) lists tetracyclines (32 %), penicillins (26 %) and sulphonamides (12 %). This trend in sales according to antibiotic groups is also observed in the particular case of Spain (Figure 6).

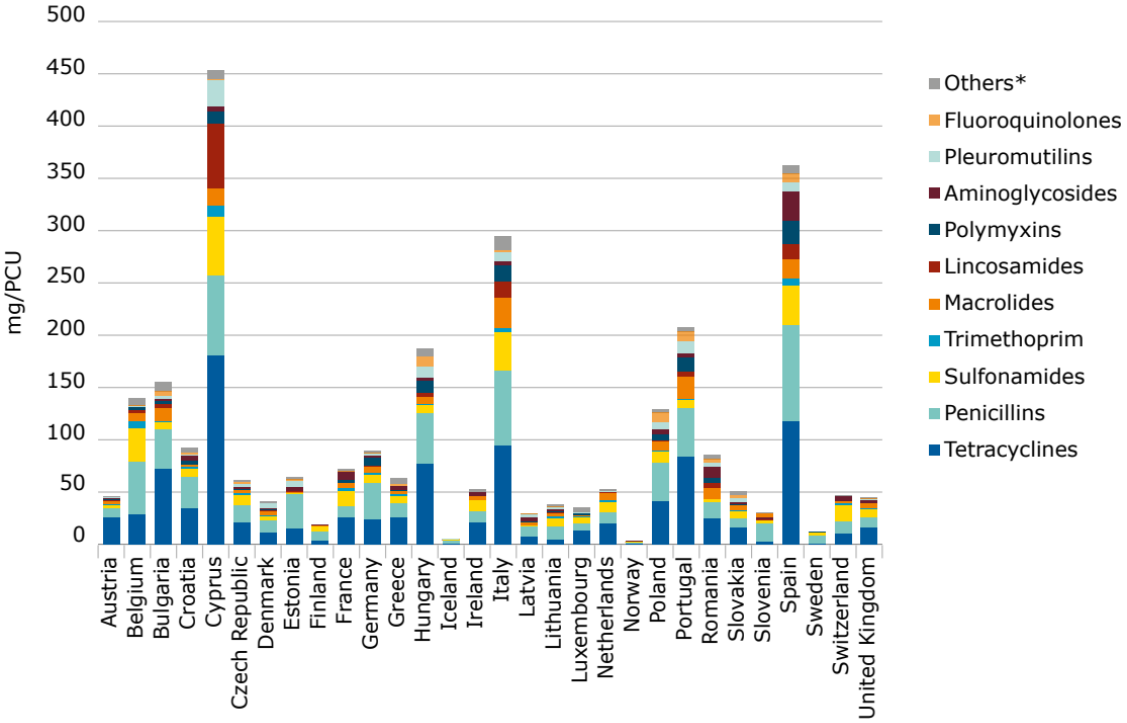


Figure 6. Sales of veterinary antimicrobials for food-producing species (mg/PCU) for European countries in 2016.
Source: EMA (2018)

On the other hand, it is important to note that the caprine sector has significantly evolved in recent years. The increase in production has, in turn, led to a further intensification of dairy goat farms, causing adverse effects on animal health issues,

such as a rising incidence of the prevalence of diseases (mastitis, pneumonia, etc.), which are usually treated with antimicrobial drugs.

In Spain, Berruga et al. (2008b) carried out a study on the treatments and drugs applied by veterinarians in dairy sheep and goats, commissioned by the Ministerio de Medio Ambiente, Rural y Marino-MARM (currently Ministerio de Agricultura, Pesca y Alimentación-MAPA). The cited study based on different surveys among veterinaries, laboratories and pharmaceutical companies showed that a high percentage of veterinarians in the caprine sector usually treat clinical mastitis during lactation (77%) and for the dry-off period (73%) choosing, mainly, β-lactam and macrolide drugs for treatments (Figure 7).

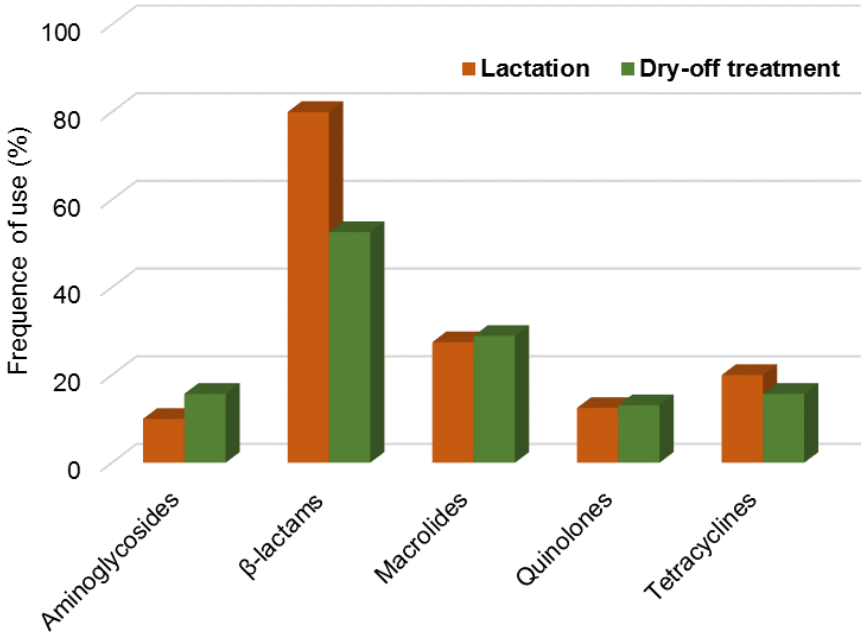


Figure 7. Frequency of use of antibiotics in mastitis treatments.
Source: Berruga et al. (2008b)

In addition to intra-mammary infections, there are respiratory, reproductive and digestive diseases, among others, that require antimicrobial therapy in dairy goats. In order to treat pathologies other than mastitis, Berruga et al (2008b) established that tetracyclines (43.6%) are the main antimicrobial group chosen by veterinarians, followed by β-lactams (26.3%).

An important aspect to be emphasized with respect to the use of antibiotics in small ruminants is, that due to the low volume of business which represents milk production, in comparison with cow milk, there is a limited availability of antibiotics registered for these species, especially for goats (Clark, 2013). In this sense, Berruga

et al. (2008b) indicated that 77% of veterinarians prescribe off-label antibiotic treatments for dairy goats employing drugs registered for other livestock species.

The off-label use of antimicrobials in dairy goats may cause residues in milk above the MRL since no correct depletion data are available. In this sense, studies carried out in dairy goats (Ferrini et al., 2010; Amer et al., 2012) showed that the minimum withdrawal period of seven days laid down in the legislation for off-label treatments is not always sufficient to ensure the absence of drug residues in milk.

To avoid the risk of the presence of antibiotic residues in goat milk, the treatments should be applied strictly following the veterinary prescription related to the antibiotic, dose, route of administration, and, particularly, the withdrawal period (Daeseleire et al., 2017). The application of Good Farming Practices (GFP) relative to the veterinary treatments in dairy goats is crucial to prevent the presence of antibiotic residues in milk and dairy products potentially posing a health hazard to consumers.

2.3. Legal aspects. Maximum Residue Limits

In order to warrant public health, regulatory agencies and international organizations, have established rules related to the presence of veterinary residues in milk and other foodstuffs of animal origin. Specifically, in the European Union, Regulation (EC) Nº 853/2004, lays down specific hygiene rules for foods, ensuring that raw milk is not placed on the market if antibiotic residues exceed the Maximum Residues Limit (MRL) permitted.

Regulation (EC) Nº 470/2009 defined the MRL as the *"maximum concentration of a residue of a pharmacologically active substance which may be permitted in food of animal origin"*. Thus, the MRL is calculated on the basis of the type and amount of residues considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI) using an additional safety factor to provide an adequate safety margin for the consumer.

MRLs for pharmacologically active substances in foodstuffs of animal origin are governed by Commission Regulation (EU) Nº 37/2010. Two separate tables have been established: one for allowed substances, listed in Annexes I, II and III of Regulation (EEC) No 2377/90, and other for prohibited substances, listed on Annex IV to that Regulation. Currently, the MRL has been fixed for raw milk but in not for all dairy products. The MRLs fixed for antibiotics in milk from different species are shown in Table 6.

Specific rules for official controls of products of animal origin are necessary. Thus, Regulation (EC) Nº 854/2004 lays down specific rules for the organization of official controls on products of animal origin intended for human consumption. Also, Regulation (EC) Nº 882/2004 regulates official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rule. In Spain, to comply with these regulations, Real Decreto 1728/2007 has been published, establishing the basic rules of control to be met by operators in the dairy sector.

Table 6. EU-Maximum Residue Limits (MRLs) fixed for antibiotics in raw milk.

Substance	MRL (µg/kg)	Milk	Substance	MRL (µg/kg)	Milk
β-lactams			***Macrolides***		
Amoxicillin	4	All species	Erythromycin	40	All species
Ampicillin	4	All species	Spiramycin	200	Bovine
Benzylpenicillin	4	All species	Tylosin	50	All species
Cefacetrile	125	Bovine	Tilmicosin	50	All species
Cephalexin	100	Bovine	***Lincosamides***		
Cefalonium	20	Bovine	Lincomycin	150	All species
Cefapirin	60	Bovine	Pirlimycin	100	Bovine
Cefquinome	20	Bovine	***Quinolones***		
Ceftiofur	100	All species	Danofloxacin	30	BOC
Cloxacillin	30	All species	Enrofloxacin	100	BOC
Dicloxacillin	30	All species	Flumequine	50	BOC
Nafcillin	30	All ruminants	Marbofloxacin	75	Bovine
Oxacillin	30	All species	***Tetracyclines***		
Penethamate	4	All species	Chlortetracycline	100	All species
Aminoglycosides			Oxytetracycline	100	All species
Abiocine	200	All ruminants	Tetracycline	100	All species
Gentamicin	100	Bovine	***Others***		
Kanamycin	150	All species	Bacitracin	100	Bovine
Neomycin	1,500	All species	Clavulanic acid	200	Bovine
Spectinomycin	200	All species	Colistin	50	All species
Streptomycin	200	All ruminants	Thiamphenicol	50	All species

Source: Regulation (EU) Nº 37/2010; BOC = Bovine, ovine and caprine

Regarding the control of the traceability of foodstuffs, the EU established Regulation (EC) Nº 178/2002, to verify that the relevant requirements of food laws are followed by food business operators at every stage of production, processing and distribution. To comply with this regulation Real Decreto 217/2004, specifies the tool (Letra Q database) to verify the traceability of raw milk in Spain. This database identifies agencies, establishments and entities involved in the dairy sector, tracking all movements of raw milk.

Additionally, Real Decreto 752/2011 fixes the mandatory minimum controls to be performed *in situ* (farms and dairy industries) concerning raw sheep and goat milk as well as the conditions required in quality control laboratories for the analysis of milk (Figure 8). Monitoring the presence of antimicrobials in raw sheep and goat milk has to be carried out in farms, if there is suspicion or certainty of the presence of drug residues in milk before loading, while, screening for antibiotic residues in dairy centers prior to the milk discharge is mandatory.

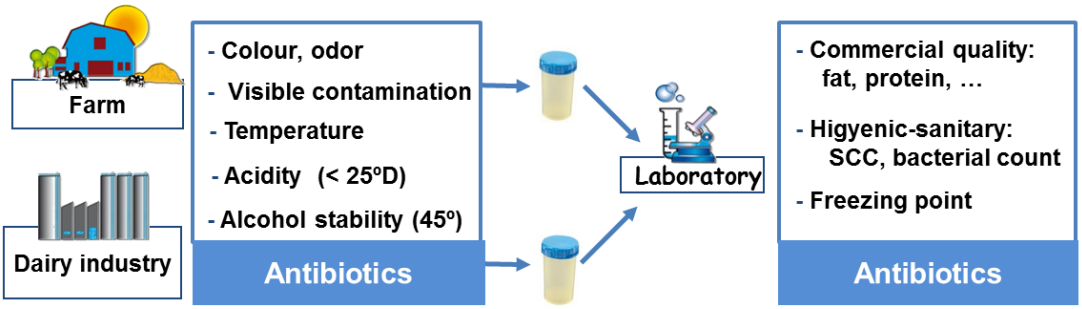


Figure 8. Quality checks and milk sample analysis in raw sheep and goat milk. Source: Real Decreto 752/2011

The control system for antibiotic residues in milk is usually performed in two steps, in which a primary screening to detect potentially non-compliant samples and a phase of confirmation to verify the presence of residues above the MRL (Figure 9). In general, microbial methods are most frequently used for screening antibiotics in dairy farms and industries. Microbial screening tests have been optimized for the use in cow milk but validation studies for sheep and goat milk are scarce. Recently, the detection capability for antibiotics of the microbial tests in goat milk was assessed by Beltrán et al. (2015a; 2015b), showing their high sensitivity to detect β-lactam antibiotics and other non-β-lactam drugs such as neomycin and tylosin. Nevertheless, in spite of the improvements made to these tests over the last years, they could be inefficient for other antibiotics belonging to aminoglycoside or quinolone groups.

A proper analytical strategy must be implemented to prevent antibiotic residues of veterinary treatments from reaching the food chain and, those guarantee the safety of milk and dairy products.

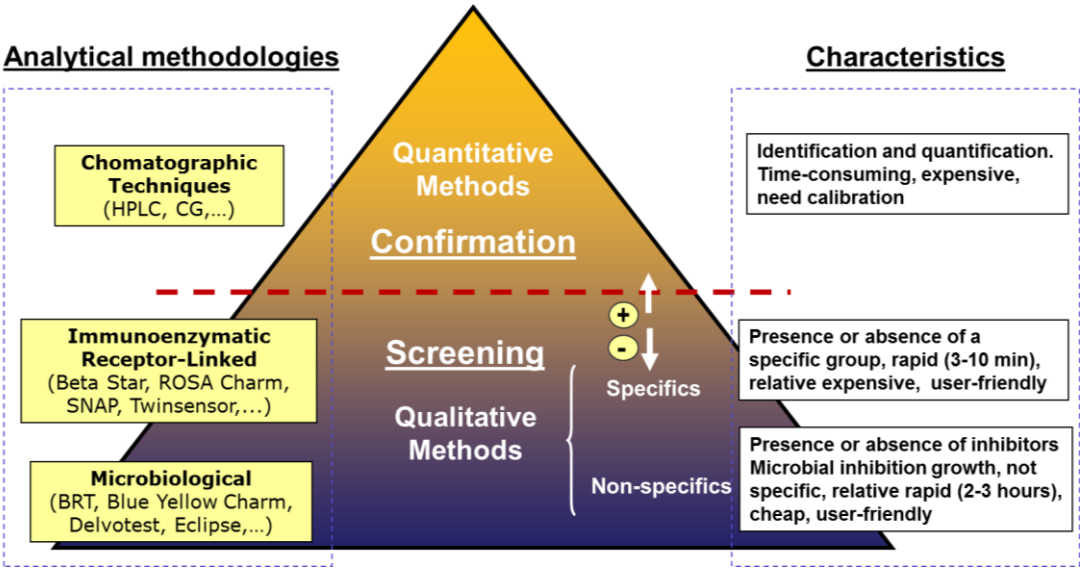


Figure 9. Control system for the detection of antibiotic residues in milk.
Source: Modified from Molina et al. (2010)

3. CONSEQUENCE OF THE PRESENCE OF ANTIBIOTICS IN MILK AND DAIRY PRODUCTS

3.1. General considerations

In the last decades, especially in industrialized countries, programs for the control of the presence of antibiotics in foodstuffs of animal origin have been implemented. The control measures for the detection of antibiotics have contributed enormously to the reduction of positive samples in raw milk. Thus, the US National Milk Drug Residue Data Base (NMDRD, 2018) shows that during 2017, of 3,888,093 raw milk from dairy farms samples analyzed, 356 (0.011%) were positive for drug residues. In the European Union, EFSA (2018) reported that the presence of antimicrobial residues in milk had been reduced with respect to previous survey, i.e. of the 11,929 samples analyzed, seven samples (0.06%) did not comply with the legal safety levels.

Specific information on the occurrence of antibiotic residues in goat milk produced in Spain is very scarce. Gonzalo et al. (2012) indicated a decrease, in the Castilla-León region, of positive samples for antibiotics in goat bulk milk from 0.31 % in 2007 to 0.001 % in 2011. That study evidenced that the implementation of Good Farming Practices in dairy livestock as well as control measures stipulated by Real Decreto 752/2011 has considerably improved the quality of raw goat milk.

As mentioned previously, some screening tests (i.e. microbial tests) used in the control systems present low sensitivity in the detection of antibiotics such as

quinolones, macrolides or aminoglycosides in goat raw milk (Beltrán et al., 2015a), these antibiotics would not be detected, and therefore can reach the food chain. Beltrán et al. (2015b) considered that it would be convenient to improve the current control strategy with the implementation of specific screening tests able to detect these substances at safety levels to guarantee consumer safety.

On the other hand, the economic impact of the possible presence of antibiotics in milk is of great concern for the farmer. If raw milk is declared "unfit for human consumption" (Regulation EC Nº 853/2004), the restriction of the commercialization of the contaminated milk together with the storage costs and subsequent elimination are the responsibility of the farmer and, therefore, represent significant economic losses.

Regarding less industrialized countries, programs for the control of the presence of antibiotic residues in foodstuffs of animal origin are practically inexistent. Several studies have reported antibiotic residues in milk at levels exceeding the safety limits. In Ethiopia, Abebew et al. (2014) in 400 bulk cow milk samples analyzed found positive samples for oxytetracycline (83%) and penicillin G (17%). A study by Shitandi and Sternesjö (2004) on the occurrence of antimicrobial drug residues in Kenya showed that of the total 1,600 milk samples, 13% contained penicillin G exceeding the established Codex maximum level (4 µg/kg). Research carried out in Algeria in 194 raw and fermented cow milk samples found high levels (65.5%) of non-compliant samples (Layada et al., 2016). All of the aforementioned results evidence the lack of controls as well as the negligent use of antibiotics in farms.

In general, the prevalence rate of veterinary drug residues in food of animal origin is below 1% in Europe, while it reaches 94% in some African countries (Mensah et al., 2014), it means that the presence of antibiotics in milk and dairy products in developing countries is a serious health hazard implying the necessity of establishing control systems.

Moreover, significant amounts of antibiotics administered to animals are not metabolized and eliminated by milk, urine and/or faces (Kemper, 2008), causing them to contaminate the top coat of the soil where they can accumulate or seep into the groundwater possibly affecting the microflora, fauna and groundwater quality (Martinez-Carballo et al., 2007).

Antibiotics enter the environment via multiple pathways that include effluents from the disposal of human waste, waste from agricultural and animal production as well

aquaculture (Qiao et al., 2018), having serious public health and environmental implications (Figure 10).

Therefore, the benefits of antibiotic therapy in dairy animals are counteracted by the presence of residues of these substances in milk and derivates with regard to possible repercussions for the dairy industry and public health.

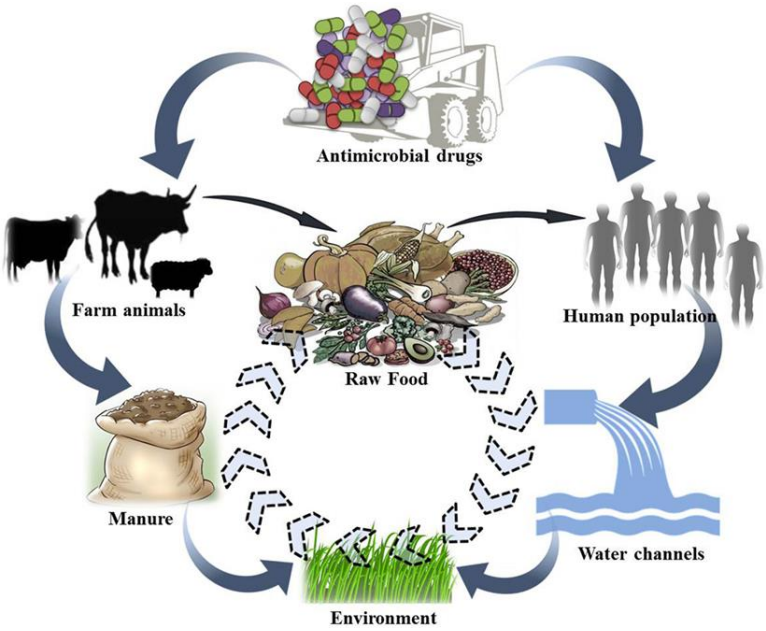


Figure 10. Cycle of the sources and transport mechanisms of antibiotics towards the environment.

Source: Sharma et al. (2018)

3.2. Effect of antibiotics in milk for the dairy industry

Milk and dairy products reach the consumer after different types of heat treatment, and processing, which in many cases does not prevent the presence of antibiotics in commercialized products. Zorraquino (2005) analyzed the effect of different dairy heat treatments on the antimicrobial activity of antibiotics (β-lactams, aminoglycosides, macrolides, quinolones and tetracyclines) LTLT in milk demonstrating that vat pasteurization (LTLT: low temperature-long time; 63ºC-30 min) caused a light reduction or did not alter the antimicrobial activity at all, only thermal treatment at 120ºC for 20 min produced a moderate reduction of the antimicrobial activity.

Other authors quantified by HPLC, the impact of the antibiotic concentration in milk conventional dairy heat treatments. Roca (2008) calculated the reduction of β-lactam and tetracycline antibiotics in milk, applying different temperatures and times

showing a degradation for vat pasteurization (63ºC-30 min) below 10% for penicillin and tetracyclines, while HTST pasteurization (HTST: high temperature-short time; 72ºC-15 sec) practically did not reduce the concentration of these antibiotics. In the case of quinolones, Roca et al. (2010) showed the high heat resistance of these substances at HTST pasteurization. Table 7 summarizes the reduction of the antibiotic concentrations in the previously mentioned studies.

Table 7. Degradation (%) of antibiotics in milk for different heat treatments used in dairy industry.

Antibiotic	63ºC 30 min	72ºC 15 s	120ºC 20 min	140ºC 4 s
Penicillins				
Amoxicillin	6.3	0.1	47.6	0.5
Ampicillin	3.3	0.1	84.0	2.1
Cloxacillin	6.9	0.1	53.1	0.6
Penicillin G	6.2	0.1	61.0	0.8
Quinolones				
Ciprofloxacin	-	0.01	12.71	0.11
Enrofloxacin	-	0.01	5.22	0.04
Norfloxacin	-	0.01	12.01	0.11
Tetracyclines				
Chlortetracycline	6.8	0.1	92.3	2.6
Doxycycline	1.3	0.0	37.4	0.5
Oxytetracycline	3.1	0.0	45.7	0.5
Tetracycline	1.5	0.0	62.1	1.2

Source: Roca (2008); Roca et al. (2010).

LTLT and HTST pasteurization commonly used in cheese-making processes do practically not reduce the concentration of antibiotics, remaining to a high extent in the milk destinated for cheese elaboration.

On the other hand, the presence of antibiotic residues in milk could have negative technological effects on products that require fermentative processes, such as yogurt and cheeses (Katla et al., 2001). In the manufacture of fermented products starter cultures containing different lactic acid bacteria (LAB) are frequently used for acidification, producing lactic acid from lactose (McSweeney and Sousa, 2000). LAB could be totally or partially inhibited by antibiotic residues even at or below safety levels. In this sense, a significant delay in the fermentation time has been reported in sheep milk yogurts spiked with penicillins (Berruga et al., 2007) and cephalosporins (Berruga et al., 2008c; Novés et al., 2015) at or below their respective MRLs. In sheep Manchego cheese, Berruga et al. (2008a) studied the influence of five β-lactams at 3

different concentrations on cheese manufacture, evidencing only a significant delay in the decrease of pH when ceftiofur was present.

Recently the presence of oxytetraciclyne at MRL (100 µg/kg) in raw and thermized sheep milk (heat 63 °C, immediately cooled) was evaluated (Cabizza et al., 2017; 2018) causing a delay in the cheese acidification process (50 and 78 min, respectively) when compared to cheese made from antibiotic-free milk due to the partial inhibition of the starter culture.

In addition, the physicochemical and organoleptic characteristics of dairy products could also be affected by some antibiotic residues in milk. Novés et al. (2012) observed that the presence of oxytetracycline at or below MRL has been related to lower firmness values in sheep milk yogurts. Nevertheless, other antibiotics, such as enrofloxacin (100 µg/kg) in goat milk did not affect the coagulation time nor most yogurt properties (Beltrán et al., 2017).

A few studies have been published on the effect of the presence of antibiotics on cheese characteristics. Cabizza et al. (2017, 2018) evaluated the differences in physicochemical parameters (pH, composition, soluble N levels, FFA) in cheese composition from sheep milk spiked with oxytetracycline when compared to antibiotic-free cheese, finding that no effect due to the antibiotic was observed.

Antibiotics in milk are not eliminated by technological processes (Adetunji, 2011). Hence, Beltrán et al. (2017) found that amounts of enrofloxacin, between 75 and 99% initially added to goat milk remained in the yogurt throughout its entire shelf life.

Currently, there are few studies on the transfer of antibiotics from milk to cheese. It should be stressed that FAO/WHO (2004), in a technical report suggested among other recommendations, to establish MRLs for fat-soluble veterinary substances in high fat content dairy products (i.e., butter or cheese).

Antibiotics could be retained in milk curd to a greater or lesser extent, according to the physicochemical properties of these antimicrobial substances depending on their ability to interact with the fat and/or protein fraction of the matrix (Sniegocki et al., 2015). Besides, other factors such as the origin of milk and the specific technological process in the manufacture of cheese might also affect the retention of these substances.

During cheese manufacturing from milk containing antibiotics, if the antibiotic is not retained in the curd, it may be transferred to the whey. A study (Hakk et al., 2016) based on whole milk spiked with radioactively marked antibiotics (benzylpenicillin,

sulfadimethoxine, oxytetracycline, erythromycin) calculated the distribution between skimmed milk and the fat fraction, reporting that more than 90% of the radioactivity was distributed in the skimmed milk fraction. Also, another study based on microscale cheese-making with radiochemical analysis found that the distribution of antibiotics in the curd ranged from 14% for oxytetracycline to approximately 28% for sulfadimethoxine, indicating that the remainder of the antibiotic was eliminated in the whey fraction (Shappel et al., 2017).

Reciently Giraldo et al. (2017) assessed the antimicrobial activity variation (AAV%) in whey as an indicator of antibiotic transfer from goat milk to whey and its potential retention in the curd. As displayed in Figure 11, antibiotics such as quinolones, aminoglycosides and tetracyclines presented a higher AAV%, and therefore a higher retention in curd, while β-lactam drugs and erythromycin are released in the whey.

It is important to emphasize that whey can be used as a by-product for animal feed, foodstuffs for human consumption, land disposal and agricultural applications, among other uses (Carvalho et al., 2013; Risner et al., 2018). This use can be compromised by the presence of antibiotic residual concentrations.

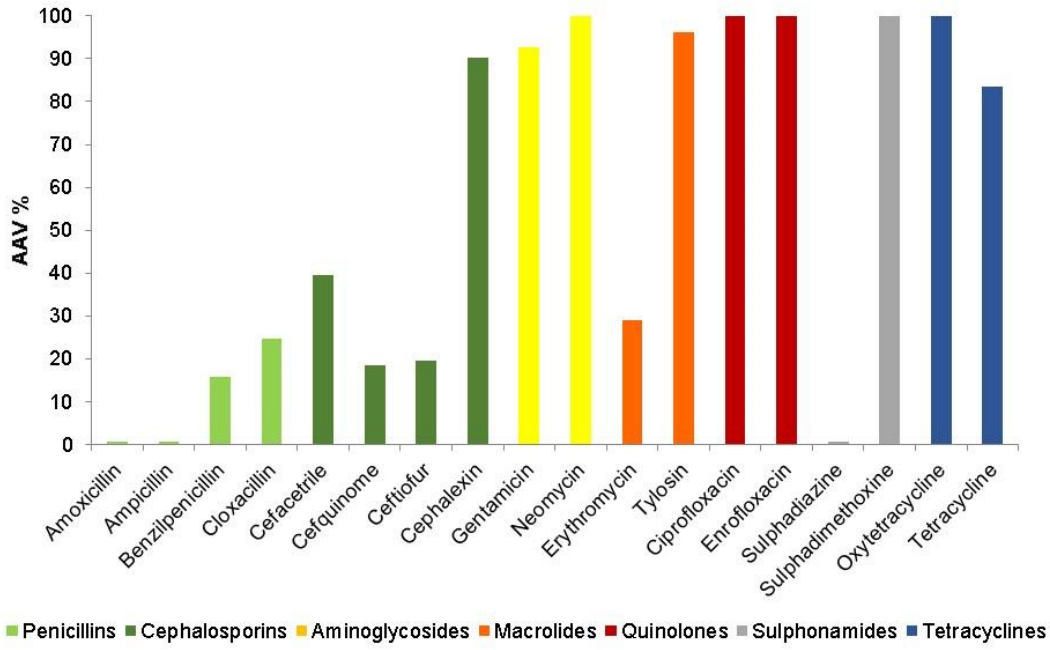


Figure 11. Antimicrobial activity variation (AAV; %) as indicator of the antibiotic drug transfer from goat milk to whey.

(AAV = 0% indicates total transfer; AAV =100% indicates no transfer)

Source: Giraldo et al. (2017)

Regarding to the retention of antibiotic in cheese, it depends largely on the type of product. In fact, Gajda et al. (2018) determined the antibiotic concentration in fresh cheese made from cow milk spiked at MRL (100 µg/kg), with different antibiotics of the tetracycline group, finding a retention from 280 µg/kg for doxycycline to 561 µg/kg for oxytetracycline. Also, in matured sheep cheese from milk spiked at MRL (100 µg/kg) with oxytetracycline, Cabizza et al. (2017) assessed the residual concentration of antibiotic at the beginning of ripening (1-day), which was equivalent to 3.8-fold higher than the initial per kg of milk (388 µg/kg) possibly leading to negative implications for consumer health.

Scientific literature data concerning the impact of the presence of antibiotics on the manufacturing process, physicochemical characteristics and the traceability of these substances from milk to cheese is scarce and insufficient. In order to know if it is necessary to establish MRLs in dairy products, it is of great interest to evaluate the impact of the manufacturing process on the retention of antibiotic residues in the final product.

3.3. Repercussion in public health

The presence of antibiotic residues in milk and/or dairy products may produce harmful effects on human health, causing disturbances in the intestinal flora (Jeong et al., 2009). Other possible effects are the allergic reactions that can, in extreme cases, lead to anaphylaxis (Dethlefsen et al., 2008). Allergies to β-lactam drugs are quite usual, with an incidence reported in about 8% of individuals in the USA (Sanders et al., 2011; Macy, 2014).

It is common knowledge that antibiotics have saved millions of lives and have contributed to an increase in life expectancy. However, in the last decades, the emergence and spread of antimicrobial resistance (AMR) to several microorganisms is complicating the management of many infectious diseases. AMR is a threat to global health, endangering the prevention and treatment of infections, animal health and welfare, as well as food production (Martens and Demain, 2017; WHO, 2018).

AMR is considered a global concern that must be managed with utmost urgency and is one of the most common priority areas identified by national and international agencies. It has been estimated that AMR causes around 700,000 human deaths each year, and AMR could lead to the deaths of ten million people a year by 2050 (O'Neill, 2014; WHO, 2016).

Concerning livestock, antibiotics have greatly served health and productivity, have also played an important role in the evolution of resistant strains, which can be

easily transferred to humans (Sharma et al., 2018). Besides, AMR can also spread along the food chain (IDF, 2017); antibiotic residues in milk may lead to problems of antimicrobial resistance of commercial starter cultures. In recent years, studies on the AMR of microorganisms from dairy products foods that can act as vehicles in the generation of resistance to certain antibiotics have increased (Devirgiliis et al., 2014; Flórez et al., 2017).

All the practices and actions that are being promoted must continue to be linked to the many practices of animal health, welfare and food safety. Recently the World Health Assembly has also called on the WHO, FAO, OIE and other relevant partners to develop a Global Action Plan for monitoring and evaluating AMR (WHO/FAO/OIE, 2018). The infographic of the OIE 6th strategic plan for 2016-2020 (Figure 12) included the concept "One Health", which is a global strategy to increase communication and interdisciplinary collaboration in the public health, animal health, plants and the environment, understanding that they are all linked together, to achieve better public health outcomes and provide guidance on how to reduce these risks.

Figure 12. The Office International des Épizooties (OIE)-World Organization for Animal Health 6th Strategic Plan 2016-2020.
Source: OIE (2018)

Also, in Figure 12 the concept of “Risk Management” as a tool to make decisions on food safety management policies is highlighted. The risk analysis able to provide a systematic means for assessing, in a qualitative or quantitative way, the probability of occurrence and the severity of known or potentially adverse health effects in a given

population based on hazard identification, hazard characterization, exposure assessment and risk characterization.

Different entities such as the European Food Safety Agency (EFSA, 2012) emphasized risk analysis as the starting point for setting priorities and allocating resources effectively based on risk. The Food Safety Margin (FSM) was introduced as a new risk characterization metric to verify compliance with Food Safety Objectives (FSO), addressing the effect of uncertainties. In this way, FSM is able to support the prediction of the exposure of consumers to risk management in a risk-informed decision-making framework (Doménech and Martorell, 2016, 2017).

Recognizing the repercussion of the presence of antibiotic residues in milk and dairy products is of great concern for farmers, the dairy industry and, mainly, public health. In this sense, studies on the transfer of antibiotics during food manufacturing processes, their possible impact on the quality of finished products, as well as risk assessment to protect public health are of utmost importance.

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Chapter 2

Objectives

The production of goat milk cheese has increased considerably in the last decades given the growing consumer interest in goat milk products related to their nutritional and digestive properties. However, the benefits of milk and dairy products can be jeopardized by the presence of veterinary drug residues.

Antibiotics in milk may pose a risk to public health in the form of allergic reactions, dysfunction of intestinal microbiota or due to the development of antimicrobial resistance. In the dairy industry, the presence of these substances in milk may have negative technological effects on the activity of starters employed in the manufacture of fermented products such as cheese.

Currently, hardly any information is available on the transfer of antibiotic residues from goat milk to cheese neither on the residual concentration that may be encountered in different kinds of cheese and their potential effect on consumer health. Moreover, research related to the effects of the presence of antibiotics in goat milk on the cheese-making process and the characteristics of cheese is rather limited.

Therefore, the aim of this thesis was to evaluate the effect of the most widely used antibiotics in dairy goats on the cheese-making process and the cheese characteristics as well as the transfer of these antibiotics from milk to fresh and mature cheese, assessing the potential risk for the consumer.

To reach this objective the following experimental studies were carried out:

Study 1. Evaluation of the characteristics of mature Tronchón cheese made from raw goat milk containing legally admissible amounts of antibiotics.

Study 2. Influence of oxytetracycline in raw goat milk on the characteristics of ripened cheese.

Study 3. Effect of antibiotic residues in milk and cheeses after the off-label use of macrolides in dairy goats.

Study 4. Food safety margin assessment of antibiotics in pasteurized goat milk and fresh cheese.

Various experiments, presented in the Results & General discussion sections, were performed in the framework of these studies.

Chapter 3

Results

Study 1. ***Evaluation of the characteristics of mature Tronchón cheese made from raw goat milk containing legally admissible amounts of antibiotics***

Evaluation of the characteristics of mature Tronchón cheese made from raw goat milk containing legally admissible amounts of antibiotics

Abstract

The aim of this study was to evaluate the transfer of the most widely antibiotics used in dairy goats, from milk to cheese as well as their effect on cheese-making process and the cheese characteristics during ripening. Antibiotic-free milk was spiked individually with seven veterinary drugs (amoxicillin, benzylpenicillin, cloxacillin, erythromycin, ciprofloxacin, enrofloxacin and oxytetracycline) at an equivalent concentration of the European Union Maximum Residue Limit (EU-MRL). Spiked goat milk was used to make mature Tronchón cheeses, which were analyzed at 0, 30, and 60 days of maturation to determine pH, chemical composition, proteolytic and lipolytic activities, color and textural properties. A sensory evaluation of 60 days ripened cheeses was carried out. Cheeses from raw antibiotic-free goat milk were made simultaneously to be used as reference. The cheese-making process was unaffected by the presence of most antibiotics evaluated. Only erythromycin and oxytetracycline significantly increased the time required for cheese production (122±29 and 108±25 min, respectively). However, variable amounts of antibiotics, ranging from 7.4 to 68%, were transferred from milk to cheese, with oxytetracycline and quinolones showing the highest retention rates. In general, antibiotic residues present in the cheeses at the beginning of maturation decrease significantly along time. Thus, β-lactams and erythromycin residues not being detectable after 30 days of ripening. However, relatively high concentrations of enrofloxacin (148±12 μg/kg) and ciprofloxacin (253±24 μg/kg) residues were found in the cheeses after 60 days of maturation. The quality characteristics of the Tronchón cheeses were only slightly affected by such substances, with few significant differences in the free fatty acid concentration, color and textural properties of the cheeses. Results herein indicate that the use of goat milk containing antibiotics, such as quinolones, at EU-MRL for cheese production could adversely affect the safety of the final products as relatively high concentrations of these substances could be retained in soft and semi-mature cheeses, making it necessary to assess the risk for consumer health. Studies on the partition of the antibiotic substances during cheese-making, using specific technologies, would be convenient in order to guarantee the safety of cheese and related products.

Key-words: Goat milk, antibiotics, cheese ripening, drug partition.

1. Introduction

The administration of veterinary drugs, especially antibiotics, in the treatment and prophylaxis of mastitis and other infectious diseases in dairy livestock is a widespread practice nowadays. However, the beneficial effects of antimicrobial therapy in lactating animals may counteract with the possible appearance of residues of these substances in milk. The consumption of milk or related products containing antibiotic residues can have harmful effects on human health, causing transient disturbances in the intestinal flora and allergic reactions (Stolker and Brinkman, 2005; Dethlefsen et al., 2008; Jeong et al., 2009). There is also the concern that the presence of antibiotics in foodstuff may be responsible for the development of bioresistance (Oliver et al., 2011; WHO/FAO/OIE, 2018).

To avoid potential risks related to drug residues in food, the control of the presence of antibiotics in milk and other products of animal origin is legally binding in many countries. The US Food and Drug Administration Center for Veterinary Medicine (FDA) established Safe Levels/Tolerance of antibiotic residues in milk to protect consumers (FDA, 2018). In the European Union, the regulatory levels or maximum residue limits (EU-MRL) are defined by Regulation (EC) 470/2009 (European Union, 2009), and established by Commission Regulation (EU) 37/2010 (European Commission, 2010).

Safety levels for milk minimize the potential risk of the consumption of dairy products as negative effects are not expected in most cases, if antibiotic residues do not exceed these thresholds. Thus, for example, pasteurized milk or yogurts made from contaminated milk generally show equal or lower concentration of antibiotics than raw milk used for their production (Grunwald and Petz, 2003; Adetunji, 2011), possibly related to the application of heat treatments, which tend to reduce the concentration of most antibiotics slightly (Roca et al., 2011; Gajda et al., 2018). However, in dairy products such as cheese, the residual amounts of antibiotics in the final products could be significantly affected by the elimination of most the aqueous fraction of the milk during the elaboration process, leading to the concentration of the main components such as fat and protein.

Antibiotics could be retained in milk curd to a greater or lesser extent, depending on the physicochemical properties of these substances and their ability to interact with the fat and protein fraction of the matrix (Giraldo et al., 2017; Shappell et al., 2017). The World Health Organization (WHO) suggested establishing MRLs of liposoluble antibiotics in milk products such as milkfat and cheese, being apprehensive that such

substances might reach levels far above the initial contents in milk and, thus, possibly posing a risk for consumers (FAO/WHO, 2004). However, EU-legislation has established only an MRL for raw milk, while safety limits for related products have not been fixed.

It should be noted that related studies currently available are scarce and focused on the transfer of tetracyclines from contaminated milk to different dairy products (Cabizza et al., 2017; Gajda et al., 2018). Information about the possible retention of antibiotics belonging to other families such as β-lactams, macrolides or quinolones widely used in dairy livestock is practically unavailable. Therefore, the impact of the presence of antibiotics in raw milk on the safety of dairy products such as cheeses is currently, unknown.

Besides the direct negative effects on consumer health, antibiotic residues in milk may lead to problems in the dairy industry by inhibiting the activity of starter cultures used in the production of fermented products such as mature cheese. Katla et al. (2001) evaluated antimicrobial resistance of commercial starter cultures, observing that antibiotic at concentrations below their respective MRLs reduce the activity of the microorganisms such as lactobacilli or streptococci. These starter cultures produce part of the enzymes responsible for the principal biochemical pathways involved during cheese ripening (McSweeney and Sousa, 2000) and, therefore, the presence of antibiotics could affect the typical cheese texture or flavor characteristics.

In the last decades, goat milk production has augmented considerably, reaching 15.3 million tons (FAOSTAT, 2018), as consumers have shown an increased interest in goat milk products related to their nutritional and digestive properties (Haenlein, 2004; Park, 2017). Goat milk is used to make fluid pasteurized milk and a wide range of dairy products, especially different types of cheese, often from raw milk, and under protected designation of origin (PDO) and other recognized quality brands. However, the presence of veterinary drug residues, especially antibiotics, can jeopardize the nutritional benefits and quality of milk and cheeses. Beltrán et al. (2015) indicate that the antibiotics most commonly employed in dairy goats are, in order to use, tetracycline (oxytetracycline and tetracycline), β-lactams (penicillin, amoxicillin, and cloxacillin), quinolones (enrofloxacine), and macrolides (tylosin and erythromycin), and therefore control strategies in goat milk should focus on these substances.

Studies on the retention of the antibiotic during dairy manufacturing processes are crucial to prevent the negative implications related to the presence of such substances in milk products. Therefore, the aim of the present work was to evaluate

the transfer of the most widely used antibiotics in dairy goats from milk to cheese, as well as their effect on the cheese-making process and the cheese characteristics during ripening.

2. Materials and Methods

2.1. Milk samples and antibiotics

Antibiotic-free milk was obtained from the experimental herd of Murciano-Granadina goats of Universitat Politècnica de València (UPV, Valencia, Spain). Animals did not receive any antimicrobial substances neither before nor along the experimental period. The milk chemical composition was analyzed by MilkoScan FT6000 (Foss, Hillerød, Denmark), somatic cell counts by Fossomatic 5000 (Foss), and total bacterial count by Bactoscan FC (Foss). The milk pH value was measured by a conventional pH-meter (model Basic 20, Crison, Barcelona, Spain). Screening test Eclipse 100 (Zeulab, Zaragoza, Spain) was used to detect inhibitors in milk.

The goat milk composition (g/100 g) presented an average (mean±standard deviation: SD) of total solids content of 14.4±0.7, fat content 5.3±0.5, and protein 3.7±0.8; with a pH of 6.80±0.05. The somatic cell count and total bacterial count presented a value of 1,227±314x10^3 cells/mL (6.08±0.11 log) and 106±137x10^3 cfu/mL (4.76±0.47 log), respectively.

The antibiotics (commercial reference) used in this study were: amoxicillin (A8523), benzylpenicillin (PENNA), cloxacillin (C9393), erythromycin (E6376), ciprofloxacin (17850), enrofloxacin (17849) and oxytetracycline (O4636), all supplied by Sigma-Aldrich Química, S.A. (Madrid, Spain). A stock solution (100 mg/100 mL) was prepared for each antibiotic trial using distilled water. For some antibiotics, the addition of 3 mL of a suitable solvent was necessary to dissolve the drug before adding water. These solvents purchased from Fluka (Barcelona, Spain), were ethanol for erythromycin; acetic acid (5%) for enrofloxacin and ciprofloxacin, and hydrochloric acid (0.1N) for oxytetracycline. Spiked milk samples were prepared to reach an antibiotic concentration equivalent to the EU-MRL (amoxicillin and benzylpenicillin: 4 µg/kg, cloxacillin: 30 µg/kg, erythromycin: 40 µg/kg, ciprofloxacin, enrofloxacin, and oxytetracycline: 100 µg/kg) according to the recommendations of the International Dairy Federation (ISO/IDF, 2003).

2.2. Cheese-making process

Cheese-making trials were carried out in duplicate per each antibiotic studied at the UPV pilot plant, following the artisanal process for mature Tronchón cheese, a traditional pressed cheese elaborated in the Maestrazgo area (Eastern Spain) from raw or pasteurized sheep, goats or mixed milk, enzymatic coagulation and different ripening times. For each replicate, 100 kg of raw goat milk were divided into two 50 kg vats. One vat was destined to make antibiotic-free (AF) cheese used as control, while the other vat was used to elaborate cheese from spiked milk (SM) with antibiotics prior to cheese manufacture at EU-MRL concentration.

Raw milk was inoculated with the commercial starter culture containing *Lactococus lactis ssp. lactis, Lactococcus lactis ssp cremoris, Lactococcus lactis ssp lactis biovar diacetylactis* and *Streptococcus thermophilus* (CHOOZIT MA4001, Danisco, Sassenage, France) at 5 Danisco culture units (DCU)/100 L. Milk was heated at 32ºC, and calcium chloride (Proquiga, A Coruña, Spain) at 0.013% (v/v) was added. Liquid calf rennet (chymosin: pepsin 70:30, 150 International Milk-Clotting Units-IMCU, Laboratorios Arroyo, Santander, Spain) at 0.07% (v/v) was used for coagulation. After coagulation (approx. 30-40 min), the curd was cut into grains (1 cm cubes). Subsequently, it was heated (35ºC) and stirred for approx. 90 min until reaching a pH value of 6.35±0.05. Then whey was drained off and the curd was distributed in cylindrical molds (800 g) and pressed in a pneumatic press (1.5 bars/90 min, 2.0 bars/90 min, and 2.5 bars/20 min). Ten cheeses were obtained from each elaboration. The acidification of the cheese was measured in each manufacture step, and after pressing, the pH was checked every 15 min until reaching the final pH of 5.30±0.05. Afterwards, the cheeses were salted in brine (23% w/v) for 3 h. Then, the cheeses were kept in an airing chamber (6ºC, 75% RH) for 48 h, and next in a ripening chamber under controlled conditions (11-12ºC, 80-85% RH) for two months. The 60 days period is the most commonly applied maturation time in Tronchón cheese manufacture from raw goat milk. Cheese samples for analysis were taken before (0 days) and during maturation (30 and 60 days).

2.3. Analysis of antibiotic residues in cheese

The extraction and purification of antibiotics in the cheeses was carried out according to the protocols established and validated at the Instituto Lactológico de Lekunberri (Lekunberri, Pamplona, Spain), according Commission Decision 2002/657/EC (European Union, 2002).

For the extraction procedure, 10 g of cheese samples were placed in a stomacher bag with 20 g of trisodium citrate (20% w/w) (Sigma-Aldrich) and homogenized twice for 3 min at 40ºC. The mixture was centrifuged at 9,000 g for 10 min at room temperature. Then 2 g of the supernatant were extracted by solid-phase extraction (SPE) using an Oasis HLB cartridge (60 mg, 3 mL, Waters Chromatography Division, Milford, MA), previously conditioned with 1 mL of methanol (LC gradient grade, Scharlau, Barcelona, Spain) and 1 mL of ultrapure water (generated in-house from a Milli-Q system, Millipore Corp., Billerica, MA). After the sample had passed through the cartridge, it was rinsed with 2 mL of water, eluted with 2 mL of methanol and dried under vacuum. After evaporation, 500 µL of 0.1% formic acid (LC gradient grade, Sigma-Aldrich) were added, and homogenized in an ultrasonic bath for 5 min. Finally, the redissolved extracts were filtered into a chromatographic vial, using a 0.45 µm polyvinylidene fluoride (PVDF) filter (Sigma-Aldrich), and 20 µL of this mixture were injected into the LC system.

Antibiotics were analyzed using a chromatography system consisting of an LC-MS/MS Alliance 2695 with a diode-array detector (Waters Chromatography Division) and a Micromass Quattro Micro TM triple quadrupole tandem mass spectrometer (Waters Chromatography Division). An XBridge^{TM} C_{18} column (100 x 34.6 x 2.1 mm, particle size of 3.5 µm) was used (Waters Chromatography Division). Chromatographic separation was carried out with a mobile phase A consisting of 0.1% (v/v) formic acid in water and mobile phase B consisting of 0.1% formic acid in acetonitrile (LC gradient grade, Scharlau). The solvent gradient conditions of the mobile phase for the antibiotics (except oxytetracycline) were as follows: time (t, min) t_0, 95% A and 5% B; t_8, A = 25%; t_{14}, A = 5%; t_{15}, A = 95%; t_{20}, A = 95%. In the case of oxytetracycline analyses, the mobile phase gradient profile was: t_0, 85% A and 15% B; t_6, A = 82%; t_8, A = 50%; t_{10}, A = 50%; t_{15}, A = 85%; t_{20}, A = 85%. The flow rate was 0.2 mL/min. The operating parameters for the mass spectrometer were needle voltage 3.0 kV, lens voltage 0.2 V; source block temperature 140°C; desolvation temperature 450°C. Desolvation and cone gas (nitrogen) was 750 and 50 L/h, respectively. Analytes were detected using electrospray ionization in the positive ion mode. The MassLynx 4.0 software (Waters Chromatography Division) was used to calculate the antibiotic concentrations in goat cheeses. The typical recoveries were approximately between 85-100% for the β-lactams and tetracyclines, 80-95% for the macrolides, and 90-110% for the quinolones.

The calibration curves had previously been established for each antibiotic considered. The limit of quantification (LOQ) being equal to 2 µg/kg for amoxicillin and

benzylpenicillin, 10 µg/kg for oxytetracycline and erythromycin, 15 µg/kg for cloxacillin and 50 µg/kg for ciprofloxacin and enrofloxacin.

2.4. Analysis of cheese samples

Tronchón cheese analysis was carried out with two cheeses of each batch being analyzed at the different ripening times (0, 30 and 60 days).

The pH of the cheese was measured in triplicate using a pH-meter (model Basic 20, Crison Instruments, Barcelona, Spain) with a penetration probe (model 5232, Crison Instruments).

The physicochemical characteristics were analyzed in duplicate. The chemical composition of the cheeses, i.e. total solids, fat, protein and salt contents, was determined using a FoodScan Analyzer (Foss). The calibration curve had previously been developed for matured goat cheeses.

The total contents of free amino acids (FAA) and free fatty acids (FFA) were used as indicators of proteolytic and lipolytic activities in the cheeses during maturation. FAA concentration (mg leucine/g of cheese) was analyzed using the Cd-ninhydrin reagent, as reported by Folkertsma and Fox (1992). FFA (meq/100 g of fat) was determined by titration using KOH ethanol solution according to Nuñez et al. (1986).

Color and textural properties were assessed in triplicate at room temperature (20±1ºC) using cylindrical samples taken 2 cm deep below the rind of the cheese (1 cm height x 2 cm diameter). The cheese color was assessed by CIELAB color space and determined using a spectrocolorimeter Minolta CM-3600D (Minolta, Tokyo, Japan). CIELAB color space expresses color as three numerical parameters: lightness (L*) has ranges between 0 and 100; redness (a*) represents red or green color (positive: red; negative: green) and yellowness (b*) stands for color ranging from yellow to blue (positive: yellow; negative: blue). Color coordinates CIE L*, a* and b* were obtained using observer 10º and illuminant D65. From these coordinates, color differences (ΔE_{ab}) of the cheeses made from milk spiked with antibiotics compared to their control cheeses were determined, applying the equation proposed by Bodart et al. (2008):

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

Where ΔE_{ab} is color differences using CIELab coordinates: ΔL*, Δa* and Δb* are the differences between the two samples in L*, a* and b*, respectively. The perception of the color difference ΔE varied according to the observed color and the sensitivity of the human eye. ΔE < 1 color differences could not be detected by the human eye;

$1 < \Delta E < 3$ minor color differences could be detected by the human eye, and $\Delta E > 3$ color differences could be detected by the human eye.

A Texture Profile Analysis (TPA) was carried out using a TA.XT Plus Texture Analyser (TA.XT Plus, Stable Micro Systems, Surrey, UK). A plunger with a diameter of 45 mm (P/45) was used. The cheese sample was compressed to 50% of its height at a constant deformation rate of 1 mm/s, leaving 5 s between the first and the second compression. The following texture parameters were measured from the force–deformation curve: hardness (N), adhesiveness (N*s), cohesiveness, springiness and chewiness (N).

2.5. Sensory evaluation

Sensory evaluation of the cheeses at 60 days of ripening was carried out by 100 un-trained consumers. Representative wedges (0.5 cm thick) of the AF and SM cheeses were prepared, at room temperature, coded with random three-digit numbers, and presented individually to the tasters. Consumer acceptance testing was carried out using a 9-point hedonic scale (1 = dislike very much to 9 = like very much) according to ISO 4121:2003 (ISO 4121, 2003). The attributes considered were: appearance, odor, color, texture, and overall preference. Since the cheeses with antibiotics could contain residues, the taste analysis was considered inopportune. The results are depicted as spider-web diagrams.

2.6. Statistical analysis

The data were analyzed using the Statgraphics Centurion XVI.II software (Statpoint Technologies, Inc. The Plains, Virginia, USA). When significant ($p < 0.05$) differences were found, means were separated by the Least Significance Difference test (LSD). A one-way analysis of variance (ANOVA) was applied to evaluate the relationship between the acidification time during cheese-making and the presence of the antibiotic in milk. Also, this analysis was used to evaluate the sensorial attributes of the cheeses. Furthermore, for each drug, the differences between cheeses from milk spiked with antibiotics and control cheeses were evaluated by means of a two-way analysis of variance applied to each of the parameters studied, considering as factors the antibiotic concentration (AF or SM cheeses) and the ripening time (0, 30 or 60 days) and their interaction was evaluated.

3. Results and Discussion

3.1. Antibiotic residues in goat milk cheeses

Table 1 displays the residual amounts of antibiotics found in the SM-cheeses at 0, 30 and 60 days of ripening. As shown in this table, variable concentrations of antibiotics were detected in all the cheeses from spiked goat milk before maturation (0-day) although such residues could not be quantified in the case of amoxicillin, whose residual concentration was below the LOQ of the LC-method (≤ 2 µg/kg).

Table 1. Antibiotic residues in Tronchón cheese made from goat milk spiked with antibiotics at European Union Maximum Residue Limit (EU-MRL) concentration during ripening (Mean±SD).

Antibiotic	EU-MRL[1] (µg/kg)	Antibiotic concentration in cheese (µg/kg)		
		Ripening time (days)		
		0	30	60
Amoxicillin	4	tr[2]	nd[3]	nd
Benzylpenicillin	4	4.8±1.3	nd	nd
Cloxacillin	30	28.8±1.7	nd	nd
Erythromycin	40	21.8±1.0	nd	nd
Ciprofloxacin	100[4]	362.5±36.5	309.4±19.6	252.9±23.7
Enrofloxacin	100[4]	268.7±55.7	153.8±0.6	147.5±11.5
Oxytetracycline	100	432.3±31.9	140.6±15.4	20.0±5.7

[1]EU-MRL: European Union Maximum Residue Limit in raw milk (European Commission, 2010); [2]tr = traces (LOD < result < LOQ); [3]nd = not detected (result < LOD); LOD = limit of detection; LOQ = limit of quantification; [4]Sum of enrofloxacin and ciprofloxacin.

The transfer of the different antibiotics from milk to cheese was calculated as a retention rate percentage (Figure 1) taking into account the residual amount of antibiotic retained in the cheeses before the maturation (0-day) and the cheese yield obtained in each cheese-making trial, which ranged from 13.2 to 17.7 kg of cheese/100 kg of milk.

The retention rates for β-lactams antibiotics and erythromycin were much lower than those obtained for quinolones and oxytetracycline, whose residues in the fresh cheeses (0-day) were 2.7-4.3 times higher than the initial drug concentration in raw goat milk, evidencing the elevated susceptibility of these substances to be retained in the cheese matrix.

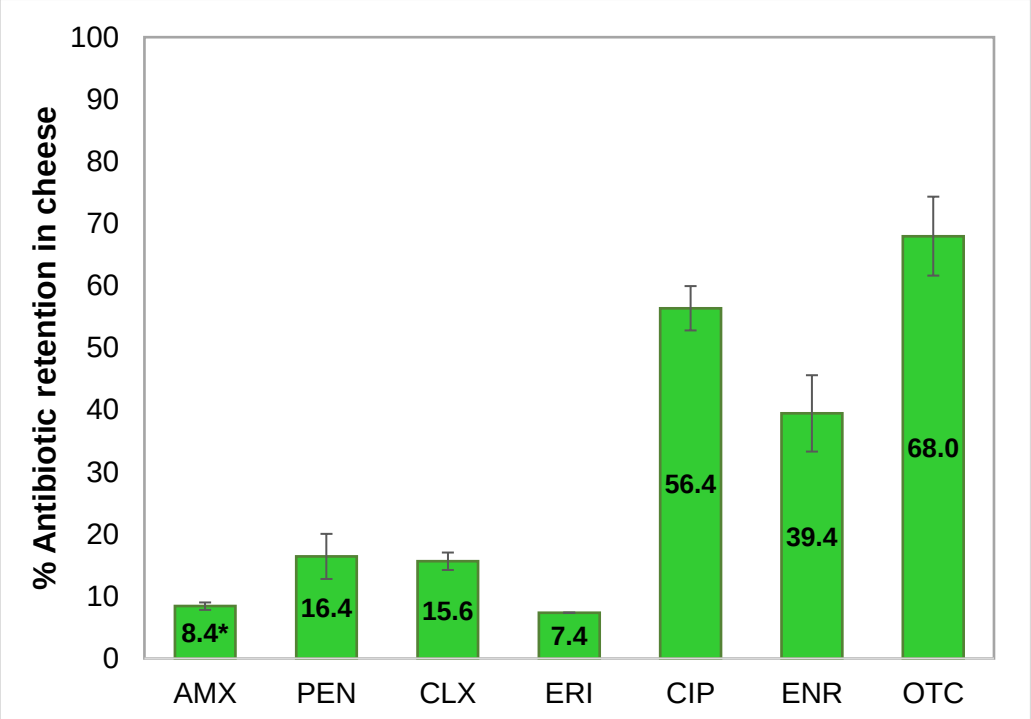

Figure 1. Retention rate percentage (mean±SD) of antibiotic Tronchón cheese before ripening made from goat milk spiked with antibiotics at EU-MRL concentration.

AMX = amoxicillin, CLX = cloxacillin, PEN = benzylpenicillin, ERY = erythromycin, CIP = ciprofloxacin, ENR = enrofloxacin, OTC = oxytetracycline *Retention calculated considering the equivalent value to the limit of quantification (LOQ amoxicillin: 2 µg/kg).

The higher or lower transfer of antibiotics from milk to cheese could be related to the solubility characteristics of these substances (Giraldo et al., 2017). Thus, the high water solubility of β-lactams and erythromycin with pK_a values ranging from 2.6 to 8.8 (Reeves, 2011; Giguère, 2013) could explain the lower retention rates obtained for those substances that are mostly transferred from the cheese to the whey during the draining-off. On the contrary, the high fat affinity of quinolones and oxytetracycline (Giguère, 2013) favors their trapping in the cheese matrix, containing high concentrations of fat and protein with which oxytetracycline can also interact to form stable chelates (Lees and Toutain, 2011), likely to explain the high retention rate (68%) calculated for this substance.

In all cases, the antibiotic concentration in the fresh cheeses decreases during maturation (Table 1). Thus, β-lactam drugs and erythromycin were not detected in cheeses having ripening for 30 days. However, quinolones and oxytetracycline were detected even after a period of 60 days although the residual concentrations of oxytetracycline in ripened cheeses was 95% lower than at the beginning of maturation possibly due to the lower stability of this substance under refrigerated conditions (Roca et al., 2008). Instead, quinolones were more stable showing a lower reduction

rate along maturation (30-45%) which also implies a higher concentration of these drugs in the final products making it necessary to assess their risk for consumer health. Also, the transfer of antibiotic residues from cheese to whey (Giraldo et al., 2017; Gajda et al., 2018), could have negative implications for humans, animals and environmental safety as this byproduct is used in the manufacture of foodstuffs for human consumption, animal feeding and agricultural applications, among other uses (Carvalho et al., 2013).

Our results are consistent with those reported by of other authors assessing the transfer of antibiotics from milk to cheese such as Giraldo et al. (2017), who evaluated the antimicrobial activity of the whey from contaminated milk or Cabizza et al. (2017) and Gajda et al. (2018), who investigated the transfer of oxytetracycline from sheep and cow's milk to cheese, respectively. The oxytetracycline retention in cheese at the beginning of the ripening process (0-day) is similar to that shown by Cabizza et al. (2017). However, the evolution of the content of this antibiotic along the maturation is different to that observed in the present study. The aforementioned authors observed a much lower degradation of the antibiotic at the end of the maturation. This greater denaturation of the antibiotic could be related to the type of milk (sheep vs goat) and the difference in production-related factors (acidification, ripening time and conditions, surface mold growth, etc.)

3.2. Effect of the antibiotics on cheese-making

The cheese-making process was unaffected by the presence of β-lactams and quinolones at safety levels in raw goat milk. However, the time required for cheese production using milk containing admissible amounts of erythromycin and oxytetracycline (40 and 100 µg/kg, respectively) increased ($p = 0.003$; $p = 0.013$, respectively). As shown in Figure 2, the kinetic of acidification of the cheeses during cheese production was considerably affected by the presence of these antibiotics, requiring additional time to reach the final pH (5.30±0.05) in the cheeses made from milk containing erythromycin (122±29 min) and oxytetracycline (108±25 min) with respect to the control cheeses. This suggests that the activity of the starter cultures was strongly inhibited by the presence of these antibiotics, leading to a lower acidification rate than in the control cheeses, especially for erythromycin which was also able to increase ($p = 0.006$) the heating and stirring time (25±5 min) prior to the draining off and molding of the curd (Figure 2.a). Similarly, Cabizza et al. (2017, 2018) also observed delays, ranging from 60 to 78 min, in the acidification process during ewe's cheese manufacture from milk with oxytetracycline at safety level (100 µg/kg). Moreover, a concentration below MRL for erythromycin (16 µg/L) reported by Katla et

al. (2001) was able to reduce by 50% the activity of *Streptococcus* spp., isolated from dairy products (yogurt, sour cream, fermented milk, whey and cheese), and commercial starter cultures.

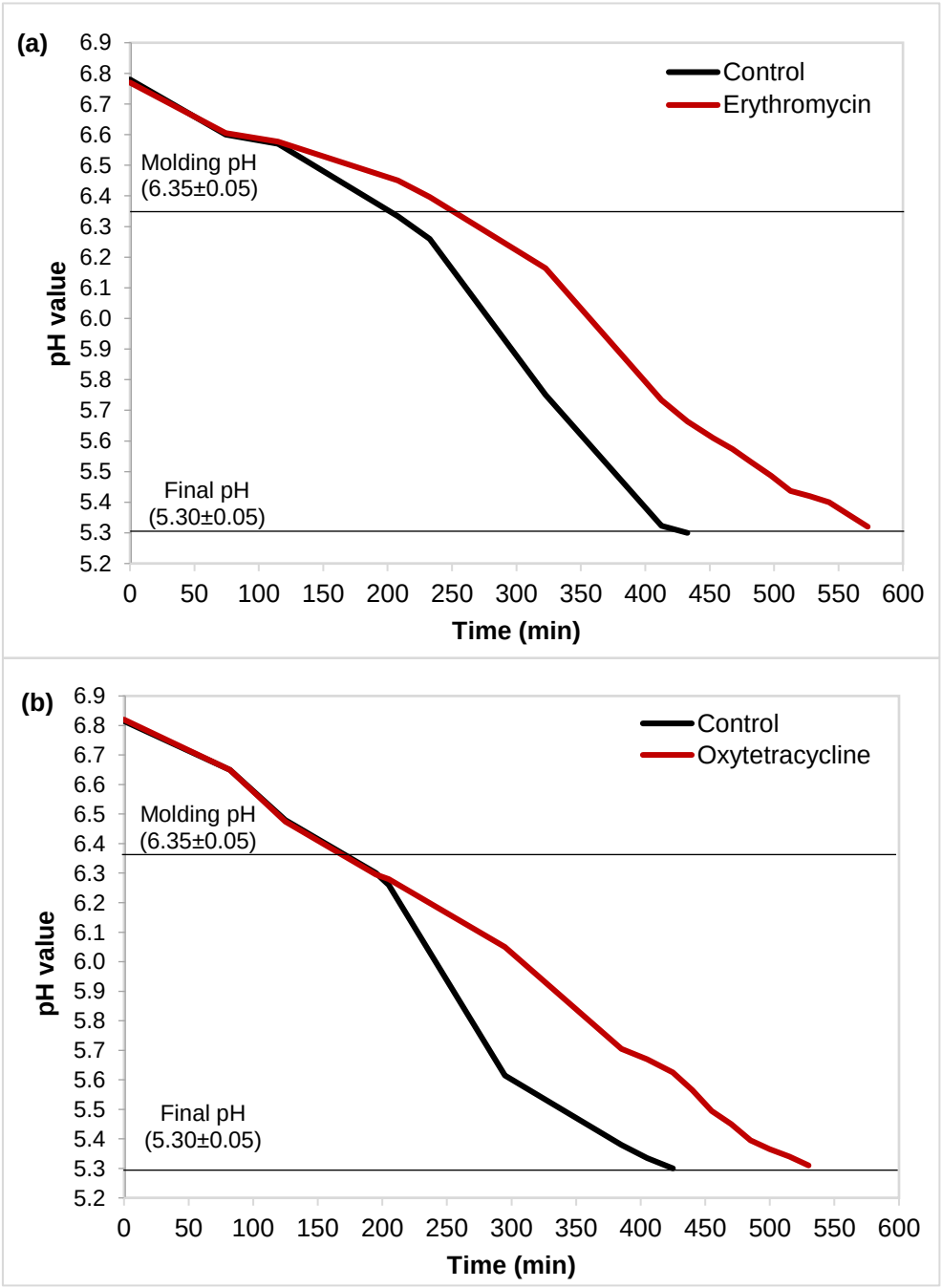

Figure 2. Kinetic acidification of the cheeses made from goat milk spiked with antibiotic during cheese-making. (a) Erythromycin; (b) Oxytetracycline.

As expected, the results herein suggest that admissible amounts of some antibiotic residues in milk could interfere in the metabolism of the microbiota present in the initial stages of the cheese production of ripened cheese, which could also affect its capacity to develop the complex biochemical processes necessary during

maturation, and the quality characteristics of the mature cheeses could, therefore, be affected. On the other hand, a low activity of the starter culture could result in more favorable environmental growth conditions for undesirable bacteria, like coliforms (Cabizza et al., 2018) during cheese-making, which could affect the microbiological safety of the cheeses (Choi et al., 2016).

3.3. Physicochemical parameters of the cheeses

Table 2 summarizes the quality characteristics of the experimental Tronchón cheeses according to the to the factors of variation "Antibiotic concentration" and "Ripening time" separately, given that the interaction between the two factors considered was not significant ($p > 0.05$). The use of raw goat milk spiked with antibiotics at EU-MRL concentration did not affect the physicochemical characteristics of the cheeses ($p > 0.05$) which presented similar values to their respective references. Nevertheless, the ripening time presented a significant effect on all the physicochemical parameters of the cheeses (Table 2) although both types of cheese (AFC and SMC) evolved in a similar way during maturation (non-significant interaction).

As shown in Table 2, all the variables evaluated in the experimental cheeses were affected by the ripening time ($p < 0.05$). In general, the pH of the cheeses diminished in the first 30 days of maturation, and remained invariable until the end of the ripening period, showing a similar trend as that reported by Salvador et al. (2014) being, however, higher than those obtained in other Spanish goat cheeses such as Ibores (pH 4.88; Delgado et al, 2011) and Majorero (pH 5.03; Fresno and Álvarez, 2012). On the whole, as ripening progressed the total solids, fat, protein and NaCl content of the cheeses increased, basically due to the loss of the water content along maturation. In any case, the concentration of the main cheese components was similar to that indicated by other authors in different goat milk cheeses (Freitas and Malcata, 2000; Ferrandini et al., 2011; Salvador et al., 2014) with slight differences mainly related to the fat content of cheeses, possibly being related to other factors such as animal breed, lactation period, feeding as well as the specific cheese-making process applied in each type of cheese (Chilliard et al., 2003; Lucas et al., 2006; Park, 2017).

Table 2. Effect of antibiotic concentration and ripening time on the physicochemical characteristics of Tronchón cheese made from goat milk.

Antibiotic	Parameters	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	pH	5.31	5.32	0.019	5.36^{b}	5.30^{a}	5.28^{a}	0.023
	Total solids (%)	59.9	59.3	0.28	57.4^{a}	59.6^{b}	61.9^{c}	0.34
	Fat (%)	32.6	32.6	0.23	31.9^{a}	32.4^{b}	33.5^{b}	0.29
	Protein (%)	22.2	22.0	0.10	20.7^{a}	21.9^{b}	23.7^{c}	0.13
	NaCl (%)	1.85	1.90	0.024	1.62^{a}	1.90^{b}	2.11^{c}	0.029
Benzylpenicillin	pH	5.18	5.21	0.012	5.32^{b}	5.14^{a}	5.13^{a}	0.014
	Total solids (%)	60.9	61.1	0.12	54.0^{a}	61.8^{b}	67.2^{c}	0.15
	Fat (%)	32.4	33.1	0.25	28.9^{a}	33.3^{b}	35.9^{c}	0.30
	Protein (%)	23.7	23.6	0.18	20.8^{a}	23.9^{b}	26.2^{c}	0.22
	NaCl (%)	2.03	2.08	0.019	1.65^{a}	2.19^{b}	2.32^{c}	0.022
Cloxacillin	pH	5.31	5.32	0.013	5.36^{b}	5.29^{a}	5.30^{a}	0.016
	Total solids (%)	59.8	59.2	0.25	57.5^{a}	59.2^{b}	61.7^{c}	0.30
	Fat (%)	32.3	32.0	0.18	31.7^{a}	32.1^{a}	32.8^{b}	0.22
	Protein (%)	22.3	22.0	0.13	21.0^{a}	21.9^{b}	23.6^{c}	0.16
	NaCl (%)	1.89	1.91	0.012	1.64^{a}	1.91^{b}	2.15^{c}	0.014
Erythromycin	pH	5.16	5.17	0.029	5.22^{b}	5.04^{a}	5.23^{b}	0.036
	Total solids (%)	62.3	62.7	0.25	55.8^{a}	64.1^{b}	67.7^{c}	0.30
	Fat (%)	35.0	35.5	0.33	31.2^{a}	36.4^{b}	38.0^{c}	0.41
	Protein (%)	22.7	22.9	0.23	20.0^{a}	23.4^{b}	25.2^{c}	0.29
	NaCl (%)	2.17	2.12	0.026	1.77^{a}	2.21^{b}	2.45^{c}	0.032
Ciprofloxacin	pH	5.19	5.21	0.022	5.26^{b}	5.15^{a}	5.19^{ab}	0.027
	Total solids (%)	62.1	62.6	0.18	54.6^{a}	65.0^{b}	67.4^{c}	0.22
	Fat (%)	34.8	34.4	0.28	30.1^{a}	36.0^{b}	37.7^{c}	0.34
	Protein (%)	23.1	23.6	0.21	19.7^{a}	24.4^{b}	25.9^{c}	0.26
	NaCl (%)	1.93	1.93	0.017	1.76^{a}	1.94^{b}	2.08^{c}	0.021
Enrofloxacin	pH	5.22	5.21	0.018	5.26^{b}	5.22^{ab}	5.17^{a}	0.022
	Total solids (%)	63.3	63.5	0.22	56.5^{a}	66.6^{b}	67.1^{b}	0.27
	Fat (%)	35.0	35.1	0.17	31.1^{a}	37.0^{b}	37.1^{b}	0.21
	Protein (%)	23.8	24.2	0.14	20.8^{a}	25.5^{b}	25.7^{b}	0.17
	NaCl (%)	1.86	1.80	0.023	1.71^{a}	1.77^{a}	2.01^{b}	0.027
Oxytetracycline	pH	5.23	5.19	0.037	5.33^{b}	5.11^{a}	5.19^{a}	0.046
	Total solids (%)	62.0	61.7	0.31	54.2^{a}	64.1^{b}	67.2^{c}	0.38
	Fat (%)	33.7	33.8	0.31	29.5^{a}	35.1^{b}	36.7^{c}	0.39
	Protein (%)	22.9	22.7	0.20	19.3^{a}	23.9^{b}	25.2^{c}	0.24
	NaCl (%)	2.17	2.14	0.031	2.00^{a}	2.19^{b}	2.28^{b}	0.038

[1]AFC: Antibiotic-free cheese, SMC: Spiked milk cheese; [2]SEM: standard error of the mean; $^{a, b, c:}$ Superscript letters in the same row for factor indicate significant differences ($p < 0.05$).

3.4. Proteolytic and lipolytic activities in the cheeses

Proteolysis and lipolysis play a major role in the development of texture and flavor in most cheese varieties during ripening, directly contributing to flavor via formation of peptides and FAA (Fenelon et al., 2000) as well as FFA from the lipolysis of triglycerides (Collins et al., 2003). The effect of the antibiotics on the proteolytic and lipolytic activities in the cheeses is presented in Table 3. Proteolytic activity in the SM-cheeses did not seem to be affected by the presence of antibiotics ($p > 0.05$), showing similar FAA concentrations than their respective references. However, a lower content of FFA in the SM-cheeses with amoxicillin ($p = 0.0001$) and cloxacillin ($p = 0.01$) were observed, suggesting a reduced biochemical activity in these cheeses, possibly due to inhibitory action of these β-lactams on the metabolism of the lipolytic bacteria (Berruga et al., 2016), which could adversely affect their typical textural and flavor properties (Collins et al., 2003; Thierry et al., 2017). The other drugs studied did not affect this metabolic pathway in the SM-cheeses, which presented similar FFA concentrations ($p > 0.05$) than their control counterpart.

Table 3. Effect of antibiotic concentration and ripening time on the proteolytic (FAA) and lipolytic (FFA) activities in Tronchón cheese made from goat milk.

Antibiotic	Parameters	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	FAA[3]	2.56	2.48	0.054	0.75^{a}	2.89^{b}	3.91^{c}	0.067
	FFA[4]	2.67^{b}	2.21^{a}	0.063	1.65^{a}	2.45^{b}	3.22^{c}	0.077
Benzylpenicillin	FAA	1.74	1.70	0.031	0.70^{a}	2.10^{b}	2.36^{c}	0.037
	FFA	2.45	2.48	0.071	1.90^{a}	2.27^{b}	3.22^{c}	0.089
Cloxacillin	FAA	2.56	2.45	0.054	0.79^{a}	2.83^{b}	3.88^{c}	0.066
	FFA	2.67^{b}	2.40^{a}	0.067	1.69^{a}	2.59^{b}	3.33^{c}	0.081
Erythromycin	FAA	2.28	2.12	0.060	0.68^{a}	2.26^{b}	3.65^{c}	0.073
	FFA	2.80	2.90	0.162	1.96^{a}	2.95^{b}	3.65^{c}	0.198
Ciprofloxacin	FAA	2.26	2.28	0.045	1.21^{a}	2.32^{b}	3.28^{c}	0.055
	FFA	3.12	2.96	0.053	2.44^{a}	2.89^{b}	3.80^{c}	0.065
Enrofloxacin	FAA	2.41	2.34	0.082	1.13^{a}	2.80^{b}	3.19^{c}	0.101
	FFA	3.01	3.03	0.069	2.53^{a}	2.85^{b}	3.66^{c}	0.084
Oxytetracycline	FAA	1.94	1.99	0.090	1.20^{a}	2.07^{b}	2.62^{c}	0.110
	FFA	3.45	3.55	0.154	2.82^{a}	3.29^{a}	4.39^{b}	0.188

[1]AFC: Antibiotic-free cheese, SMC: Spiked milk cheese; [2]SEM: standard error of the mean; [3]FAA: Free Amino-Acids (mg leucine/g of cheese); [4]FFA: Free Fatty Acids (meq/100 g of fat); [a, b, c:] Superscript letters in the same row for factor indicate significant differences ($p < 0.05$).

On the other hand, FAA and FFA concentrations of the cheeses increased, as expected, throughout the ripening period (Table 3) and no significant interactions between the two factors considered were found in any case.

In general, the FAA content in the experimental cheeses was in the order of those reported by other authors for cheeses of 60 days ripened (Juan et al., 2016), and the FFA content showed a similar trend that the data presented by Buffa et al. (2001) in mature cheese made from raw goat milk.

3.5. Color evaluation of the cheeses

As shown in Table 4, the color parameters evaluated in the cheeses were affected by the presence of some antibiotics in goat milk. Thus, a lower brightness (L*) value ($p = 0.0001$) was obtained in the SM-cheeses containing ciprofloxacin. The redness (coordinate a*) presented low values in the cheeses from milk spiked with benzylpenicillin ($p = 0.03$), cloxacillin ($p = 0.01$), and erythromycin ($p = 0.01$) when compared to control cheese. Similarly, the yellowness (coordinate b^*) value was lower ($p = 0.007$) in the SM-cheeses with oxytetracycline. However, differences found instrumentally could not be detected by the consumers, as the calculated ΔE value (Bodart et al., 2008) was ranging from 0.88 to 2.02 for the different antibiotics considered.

Regarding the effect of ripening on the color properties of the cheeses (Table 4), a significant reduction in L* and in the a* coordinate was observed, while b* coordinate value increased along time, possibly related to proteolysis and browning reactions that occur during maturation (Carreira et al., 2002; Tejada et al., 2007). A significant interaction (Antibiotic concentration x Ripening time, $p < 0.001$) was found for the a* coordinate which was only significantly lower in SM-cheeses with cloxacillin at 60 days of ripening.

A similar trend in color parameters was reported by Buffa et al. (2001) and Salvador et al. (2014), who analyzed goat cheese under similar conditions. The results obtained also agreed with those reported by Fresno and Álvarez (2012) in Majorero goat cheese ripened for a 60 days period (L*: 84.83; a*: -2.28; b*: 11.89).

Table 4. Effect of antibiotic concentration and ripening time on the color coordinates (CIE L* a * b*) of Tronchón cheese made from goat milk.

Antibiotic	Coordinates	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	L*	87.5	88.0	0.26	90.4^{b}	86.9^{a}	86.0^{a}	0.31
	a*	-1.21	-1.30	0.058	-0.34^{c}	-1.55^{b}	-1.88^{a}	0.071
	b*	11.5	11.5	0.21	10.7^{a}	12.2^{b}	11.7^{b}	0.26
Benzylpenicillin	L*	89.6	89.5	0.16	91.0^{c}	89.3^{b}	88.3^{a}	0.19
	a*	-1.43^{b}	-1.54^{a}	0.030	-0.43^{c}	-1.74^{b}	-2.29^{a}	0.037
	b*	10.1	10.2	0.18	9.3^{a}	10.7^{b}	10.4^{b}	0.22
Cloxacillin	L*	87.5	88.5	0.34	90.6^{b}	87.1^{a}	86.4^{a}	0.41
	a*	-1.21^{b}	-1.34^{a}	0.033	-0.30^{c}	-1.59^{b}	-1.94^{a}	0.041
	b*	11.5	11.5	0.21	10.6^{a}	12.2^{b}	11.7^{b}	0.25
Erythromycin	L*	89.8	89.2	0.19	90.2^{b}	89.7^{b}	88.6^{a}	0.23
	a*	-1.16^{b}	-1.23^{a}	0.018	-0.29^{c}	-1.61^{b}	-1.69^{a}	0.022
	b*	10.60	10.27	0.117	9.74^{a}	10.52^{b}	11.1^{c}	0.14
Ciprofloxacin	L*	89.4^{b}	88.3^{a}	0.16	90.1^{c}	88.7^{b}	87.8^{a}	0.19
	a*	-1.51	-1.47	0.030	-0.43^{c}	-1.81^{b}	-2.23^{a}	0.037
	b*	12.4	12.7	0.15	10.9^{a}	13.1^{b}	13.7^{c}	0.18
Enrofloxacin	L*	88.3	87.7	0.25	90.0^{b}	87.3^{a}	86.5^{a}	0.30
	a*	-1.27	-1.26	0.028	-0.20^{c}	-1.61^{b}	-1.97^{a}	0.035
	b*	12.1	12.1	0.19	10.7^{a}	12.4^{b}	13.0^{c}	0.23
Oxytetracycline	L*	88.3	88.9	0.26	90.8^{b}	88.0^{a}	87.1^{a}	0.31
	a*	-1.85	-1.77	0.042	-0.43^{c}	-2.17^{b}	-2.81^{a}	0.052
	b*	12.5^{b}	12.0^{a}	0.13	10.0^{a}	12.9^{b}	13.9^{b}	0.16

[1]AFC: Antibiotic-free cheese, SMC: Spiked milk cheese; [2]SEM: standard error of the mean; [a, b, c:] Superscript letters in the same row for factor indicate significant differences ($p < 0.05$).

3.6. Textural properties of the cheeses

The effect of the antibiotics on the textural properties of the Tronchón cheeses is shown in Table 5. Most of the drugs used in this study did not affect the texture profile of the cheeses, showing similar values than those obtained for the AF-cheeses used as a reference. The lower hardness ($p = 0.0002$) and chewiness ($p = 0.0025$) values observed in the cheeses from milk containing oxytetracycline, which also presented a higher cohesiveness value ($p = 0.007$), should be highlighted. These differences could be related to the interaction between oxytetracycline and the Ca^{2+} ion (Arias et al., 2007) forming stable bonds which could affect the conformation of the casein network (Everet and Auty, 2008) leading, consequently, to changes in textural properties of cheeses (e.g. hardness).

Table 5. Effect of antibiotic concentration and ripening time on the texture profile of Tronchón cheese made from goat milk.

Antibiotic	Parameters	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	Hardness (N)	20.8	20.9	0.99	28.0[b]	18.6[a]	15.9[a]	1.22
	Adhesiveness (N*s)	-0.93	-0.96	0.047	-0.61[b]	-1.09[a]	-1.13[a]	0.057
	Springiness	0.63	0.61	0.010	0.81[c]	0.57[b]	0.47[a]	0.011
	Cohesiveness	0.43	0.42	0.005	0.67[c]	0.33[b]	0.28[a]	0.006
	Chewiness (N)	7.0	6.9	0.36	15.3[c]	3.5[b]	2.1[a]	0.45
Benzylpenicillin	Hardness (N)	26.5	24.7	0.98	24.2[a]	23.0[a]	29.7[b]	1.20
	Adhesiveness (N*s)	-1.25	-1.19	0.085	-0.47[b]	-1.53[a]	-1.66[a]	0.103
	Springiness	0.62	0.62	0.014	0.83[c]	0.48[a]	0.56[b]	0.017
	Cohesiveness	0.40	0.44	0.016	0.73[c]	0.30[b]	0.23[a]	0.019
	Chewiness (N)	7.1	7.2	0.58	14.6[b]	3.1[a]	3.8[a]	0.71
Cloxacillin	Hardness (N)	20.8	19.9	0.64	27.1[b]	17.5[a]	16.5[a]	0.79
	Adhesiveness (N*s)	-0.93	-1.15	0.077	-0.63[b]	-1.20[a]	-1.30[a]	0.094
	Springiness	0.63[b]	0.60[a]	0.006	0.82[c]	0.55[b]	0.46[a]	0.007
	Cohesiveness	0.43	0.43	0.005	0.69[c]	0.33[b]	0.26[a]	0.007
	Chewiness (N)	7.0	6.7	0.28	15.4[c]	3.2[b]	2.0[a]	0.34
Erythromycin	Hardness (N)	31.9	30.1	0.82	24.4[a]	24.5[a]	42.1[b]	1.00
	Adhesiveness (N*s)	-1.95	-2.02	0.081	-0.79[c]	-2.16[b]	-3.00[a]	0.099
	Springiness	0.58	0.56	0.008	0.82[c]	0.48[b]	0.42[a]	0.010
	Cohesiveness	0.37[a]	0.39[b]	0.003	0.69[c]	0.23[b]	0.21[a]	0.004
	Chewiness (N)	6.8	6.8	0.39	13.8[b]	3.0[a]	3.7[a]	0.48
Ciprofloxacin	Hardness (N)	37.4	36.2	0.74	27.9[a]	37.3[b]	45.2[c]	0.91
	Adhesiveness (N*s)	-1.75	-1.95	0.099	-1.08[b]	-2.28[a]	-2.19[a]	0.122
	Springiness	0.59	0.57	0.009	0.82[c]	0.51[b]	0.42[a]	0.011
	Cohesiveness	0.38	0.38	0.007	0.65[c]	0.26[b]	0.23[a]	0.008
	Chewiness (N)	8.0	8.1	0.27	14.8[b]	4.9[a]	4.4[a]	0.33
Enrofloxacin	Hardness (N)	29.2	25.5	1.63	22.1[a]	27.5[ab]	32.4[b]	1.99
	Adhesiveness (N*s)	-1.99	-1.66	0.153	-0.77[b]	-2.35[a]	-2.36[a]	0.187
	Springiness	0.60	0.60	0.010	0.84[c]	0.52[b]	0.43[a]	0.012
	Cohesiveness	0.41	0.42	0.007	0.71[b]	0.29[a]	0.25[a]	0.008
	Chewiness (N)	7.2	6.6	0.28	13.0[b]	4.2[a]	3.5[a]	0.34
Oxytetracycline	Hardness (N)	35.0[b]	29.8[a]	0.79	23.0[a]	31.1[b]	43.0[c]	0.96
	Adhesiveness (N*s)	-1.55	-1.48	0.061	-0.47[b]	-2.07[a]	-2.01[a]	0.075
	Springiness	0.60	0.64	0.012	0.83[b]	0.54[a]	0.49[a]	0.015
	Cohesiveness	0.37[a]	0.38[b]	0.003	0.67[c]	0.24[b]	0.22[a]	0.004
	Chewiness (N)	7.5[b]	6.7[a]	0.18	12.7[c]	3.9[a]	4.7[b]	0.22

[1]AFC: Antibiotic-free cheese, SMC: Spiked milk cheese; [2]SEM: standard error of the mean; [a, b, c:] Superscript letters in the same row for factor indicate significant differences ($p < 0.05$).

During ripening, the cheese samples became significantly harder and more adhesive, while the springiness, cohesiveness and chewiness decreased significantly in the presence of most of the antibiotics evaluated (Table 5). In general, these changes are consistent with previous results under similar conditions (Delgado et al., 2011; Salvador et al., 2014) although values vary according to the type of cheese.

In cheeses made from milk spiked with amoxicillin and cloxacillin, however, hardness evolved in an inverse way during maturation, showing a similar trend to that indicated by Chen et al. (2010) in cheese from goat milk. These results could be related to the higher somatic cell count of goat milk used for cheese production (1.6 x 10^6 cell/mL) detected in a more advanced lactation stage than for the other antibiotics assessed. In addition to lower hardness values, the SM-cheeses with amoxicillin and cloxacillin, also presented a higher moisture and FAA contents (Tables 2 and 3), characteristics found by other authors (Revilla et al., 2007; Merin et al., 2008; Chen et al., 2010) in cheeses from milk having high somatic cell counts.

3.7. Sensorial analysis of the cheeses

The sensory analysis panel did not detect any sensorial differences between the Tronchón SM-cheeses containing antibiotics and their respective reference AF-cheeses ($p > 0.05$) at 60 days of ripening. Sensorial differences were only detected in the cheeses from milk containing amoxicillin and erythromycin at MRL concentration, whose score values are graphically represented as spider-web diagrams (Figure 3).

As shown in Figure 3.a, the SM-cheeses with amoxicillin had lower scores for the odor attribute than the reference cheeses ($p = 0.032$). This could be related to the lower concentration of FFA in the cheeses with the antibiotic (Table 3). The FFA content is closely related to the characteristic aroma to goat milk cheeses and precursors of other high-flavored compounds, such as methyl ketones and lactones (McSweeney and Sousa, 2000; Collins et al., 2003). Regarding mature SM-cheeses from goat milk containing erythromycin at safety level (Figure 3.b), the sensory analysis results indicated significant differences for the attributes odor ($p = 0.001$) and overall preference ($p = 0.002$), which were better valued than the control cheeses by the untrained consumers. Despite these differences, the SM-cheeses with these antibiotics were evaluated with high scores (overall preference: 7.3 and 7.1 for amoxicillin and erythromycin, respectively) suggesting a high degree of acceptance by the panelists.

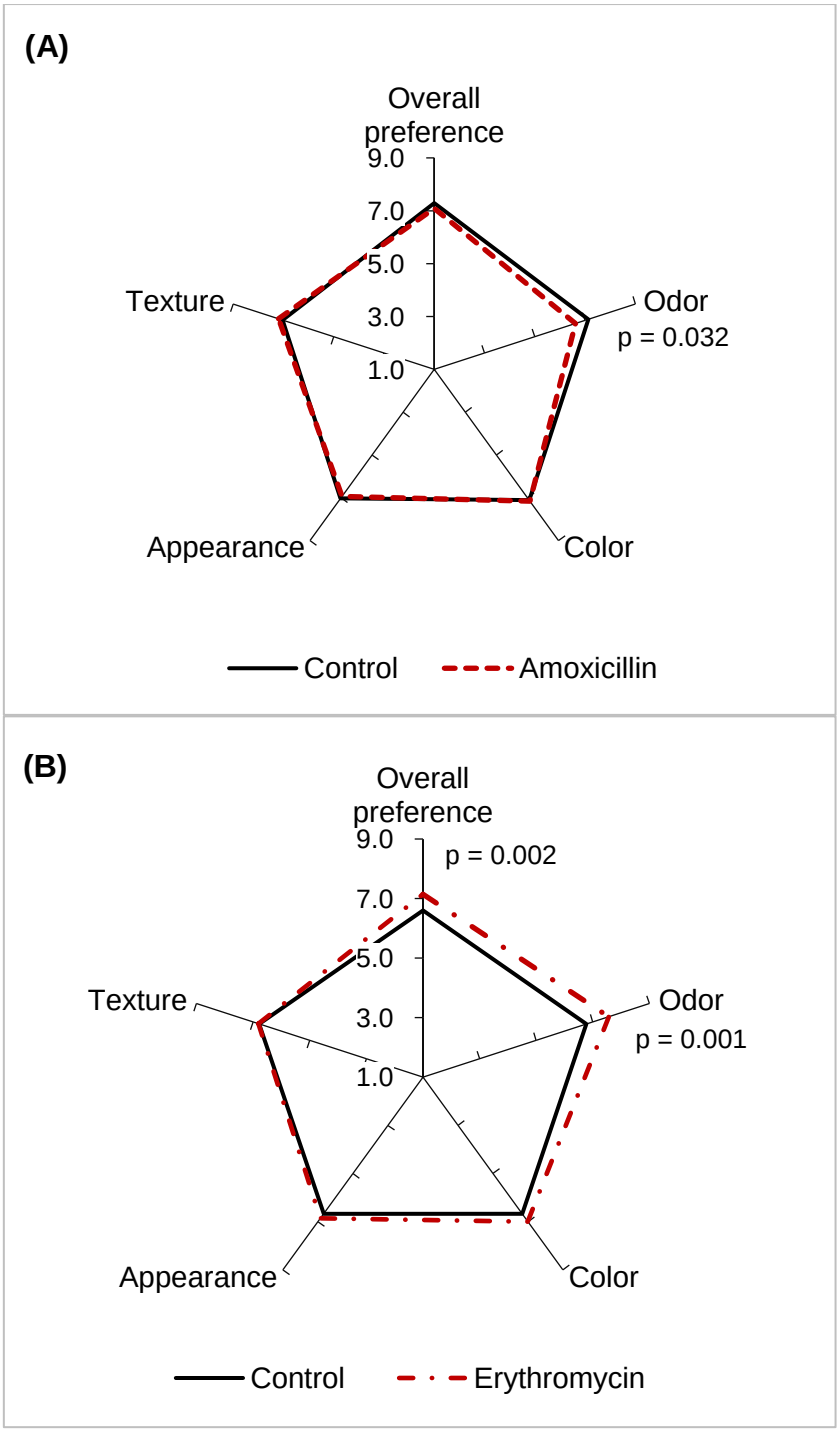

Figure 3. Sensory analysis of Tronchón cheese maturated for 60 days made from goat milk spiked with antibiotic at EU-MRL concentration. (A) Spider-web diagram for Amoxicillin; (B) Spider-web diagram for Erythromycin

Results herein suggest that the presence of some drug residues in ripened cheeses is undetectable for consumers as they reach high scores for several sensory attributes. Thus, antibiotics such as enrofloxacin, ciprofloxacin and oxytetracycline which remain in the cheeses after 60 days of maturation, did not affect the organoleptic characteristics of the final product in a negative way.

4. Conclusions

The cheese-making process and the quality properties of the ripened goat Tronchón cheeses were only slightly affected by the presence of antibiotics in milk at equivalent EU-MRL concentration. Moreover, the few differences that are related to the free fatty acid concentration, color and textural properties of the cheeses, remained mostly undetected by the sensory analysis panel.

However, it is important to emphasize that, depending on the physicochemical properties of antibiotic, drug residues are transferred from milk to cheese to a greater or lesser extent. In general, antibiotic residues in cheese decrease during ripening. However, large amounts of highly stable substances such as quinolones could remain in the final products, posing a potential risk for public health. Therefore, it is necessary to continue the study of the retention of antibiotics in cheese made from milk of different species and using specific cheese-making technologies in order to establish the corresponding regulations to guarantee the safety of dairy products.

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Study 2. ***Influence of oxytetracycline in raw goat milk on the physicochemical characteristics of ripened cheese***

Study 2.1. Impact of the presence of oxytetracycline residues in milk destined for the elaboration of dairy products: the specific case of cured goat cheese

Abstract

This study evaluates the instrumental parameters (chemical composition, color, texture and microstructure) and sensory characteristics in goat cheese (cured 60 days) made with milk spiked with oxytetracycline at different levels: 0, 50, 100 (Maximum Residue Limit “MRL”) and 200 µg/kg. Additionally, the influence of this antibiotic on the cheese making process was evaluated, proving that the presence of oxytetracycline in milk significantly slowed down the curd fermentation in proportion to its concentration. Oxytetracycline residues in cured cheeses remained at levels of <10, 20.0 and 79.0 µg/kg, with respect to the three spiked concentration. However, the residues of this antibiotic in ripened cheeses are not sensory detectable and almost undetectable in the case of instrumental analysis, even though the starting milk contains double the MRL allowed. Due to a lack of regulation and its undetectability this could raise serious health issues especially due to the development of antimicrobial resistance.

Key-words: goat milk, oxytetracycline, antibiotic residues, ripened cheese.

1. Introduction

The use of antimicrobial agents in dairy livestock is a necessary and widespread practice for the treatment of infectious pathologies. Tetracyclines are bacteriostatic antibiotics with a broad-spectrum of activity against gram-positive, gram-negative bacteria, intracellular and protozoan organisms (Papich, 2016), with oxytetracycline being the most commonly used for food-producing species. In dairy goats, oxytetracycline is used in the treatment of diseases, such as mastitis, urinary tract infections, skin and gastrointestinal infections (Clark, 2013). However, the improper use of oxytetracycline may result in the presence of residues of this drug in the milk supply.

The antibiotic residues in foodstuff is currently considered a serious public health problem, since it has been demonstrated that these residues can cause allergies, alterations in the intestinal microbiota and even the development of multibacteria resistance (EFSA, 2016; WHO, 2018). The European Union, to ensure food safety, has established Maximum Residue Limits (MRLs) for different pharmacologically active substances in foods of animal origin (European Commission, 2010). This regulation establishes for all tetracyclines a MRL of 100 µg/kg, in milk from any species. However, referring to dairy products, at present, no limits have been set for antibiotics; even though, the WHO Technical Report (FAO/WHO, 2004) recommended including MRL especially for fat-soluble veterinary substances in dairy products with a high fat content (butter or cheese) since a greater concentration of these substances could be retained.

In addition to the adverse effects on human health, the presence of antibiotic residues in milk, intended for the production of milk derivates, can also cause negative technological effects, even though they are below the established levels of MRL.

Residues of cephalosporin in sheep´s milk used in Manchego cheese (Berruga et al., 2008a) and in yogurt production (Berruga et al., 2008b; Novés et al., 2015) delay the decrease of pH during manufacturing. The same was found by Cabizza et al. (2017; 2018), during the manufacture of matured cheese made from sheep´s milk spiked with oxytetracycline at MRL.

It is worth mentioning that the thermal treatments, to which the dairy industry subjects the milk, practically do not alter the stability of the antibiotic molecule or its antimicrobial activity (Roca, 2008). Not even certain extreme and uncommon conditions of treatment (120ºC-20 min) in the dairy industry, meaningfully inhibited the activity of antibiotics (Zorraquino et al., 2010).

In commercial soft cheeses, different antibiotic residues such as benzylpenicillin, streptomycin and tetracycline were found (Adetunji, 2011) whereas in raw milk, yogurt, and cheese from Ghana, chloramphenicol, sulfathiazole, and oxytetracycline were detected (Darko et al., 2017), there are few studies focus on the transfer of antibiotics from milk to dairy products. Along this line, is highlighted the work of, Gajda et al. (2018) who determined the antibiotic concentration in dairy products, in the case of fresh cheese made from cow´s milk spiked at MRL (100 µg/kg), with different antibiotics of the tetracycline group. They found a retention between 280 µg/kg for doxycycline and 561 µg/kg for oxytetracycline. Cabizza et al. (2017) in matured sheep´s cheese from milk spiked at MRL with oxytetracycline showed a residual concentration of this antibiotic of 388 µg/kg at the beginning of the maturing stage (1-day). This value was equivalent to 3.8-fold higher than the initial concentration present in the original milk. In the same way, Darko et al. (2017) observed that quinolones, aminoglycosides and tetracycline presented an important tendency to be retained in cheese.

In general, the detection of antibiotic residues in dairy products reveals that the processing of milk in their manufacturing do not eliminate these veterinary drugs. Despite the fact that positives detected refer to samples that do not comply with the established MRL (EFSA, 2018), it should be kept in mind that there could be a presence of residues below this limit that are undetected or expressed as negatives in the controls. Hence, milk could arrive with residues to the consumer or the industry as a raw material to manufacture dairy products.

Contributing to the knowledge referring to the impact of the presence of antibiotics on the manufacturing process, physicochemical characteristics and their transfer from milk to cheese is of great importance for the industry, the consumer and the administration. Therefore, the present study is focused on matured cheese made from goat´s milk spiked at different levels (MRL and/or above and below) with oxytetracycline, evaluating its impact on the cheese-making process, as well as its chemical composition, color, texture, microstructure and sensory characteristics.

2. Materials and Methods

2.1. Experimental procedure

Antibiotic-free milk from the experimental flocks of Murciano-Granadina goats supplied by the Universitat Politècnica de València (UPV, Valencia, Spain) was used to produce the cheeses of this study. Animals had good health status and did not receive veterinary treatment before or during the experimental period.

Cheese making trials were carried out at the pilot plant of the UPV. The experiment was replicated over a consecutive three-week period. For each replicate 200 kg of raw goat milk was divided into four vats (50 kg each one): one for “control cheese” (not spiked) and to the other three (spiked) at different concentration of oxytetracycline [50 µg/kg (0.5 MRL), 100 µg/kg (1 MRL) y 200 µg /kg (2 MRL)] (Sigma-Aldrich Química, S.A., Madrid, Spain).

2.2. Cheese manufacture

Each vat of raw milk was inoculated with commercial starter culture MA4001 5 DCU (Choozit Cheese Cultures, Danisco, France). Then, milk was heated up to 32 °C, and 0.013% (v/v) of $CaCl_2$ (Proquiga, A Coruña, Spain) and 0.07% (v/v), of calf rennet (Laboratorios Arroyo, Santander, Spain) were added. After the coagulation (40-50 min), the curd was cut and heating (35-36ºC) whilst being stirred for 90-100 min. When the curd reached the optimum molding pH (6.35-6.4), the whey was removed, and the curd was molded into cylindrical pieces of about 800 g and pressed for 3.5 h, between 1.5-2.5 bars. The pH of the cheeses was monitored using a conventional pH-meter (Crison, Barcelona, Spain) between each stage, and every 15 minutes from the end of the pressing until a pH of 5.30-5.35 was reached (pH established for the immersion of cheeses to the brine). The time required to complete the acidification process, was recorded as additional acidification time. Afterwards, the cheeses were salted by immersion in brine (23% w/v). Finally, the cheeses were placed in a ripening chamber (10-12ºC, 80-85% RH) for a maturation of 60 days.

2.3. Antibiotic residue quantification

The liquid chromatography tandem-mass spectrometry (LC-MS/MS) method was used for the quantification of oxytetracycline in the cheese was carried out, according to the method validated at the *Instituto Lactológico de Lekunberri* (Lekunberri, Pamplona, Spain) and the conditions described by Quintanilla et al. (2019). For chromatographic analysis, an Alliance 2695 high-performance liquid chromatograph with a diode-array detector from Waters (Waters Chromatography Division, Milford, MA, USA) was used. Separation of compounds was accomplished using an XBridge^{TM} C_{18} column (Waters Chromatography Division). Mass spectral analyses were performed on a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters Chromatography Division). For quantification, calibration curves have previously been established. The MassLynx 4.0 software (Waters) was used to calculate the oxytetracycline concentrations in goat milk cheese. The repeatability and reproducibility were below 20% in agreement with this Commission

Decision. Limit of detection (LOD) and quantification (LOQ) was 2 and 10 µg/kg, respectively.

2.4. Chemical composition, pH and water activity

Cheese composition (moisture, fat, protein and salt content) was determined using a FoodScan infrared device (Foss, Foss Iberia, Barcelona, Spain). The pH value of the cheese ripened was measured using pH-meter with a penetration electrode (Crison Instruments, S.A., Basic 20, Barcelona, Spain) was employed, making 6 measurements per sample. The water activity of samples (a_w) was measured at 25 °C (±0.2 °C) by using a dew point hygrometer (Aqualab 4TE, Decagon Devices Inc., USA). All determinations were carried out in triplicate.

2.5. Assessment of proteolytic and lipolytic activities

The free amino acids (FAA) concentration (mg Leucine/g of cheese) were determined according to Cd-ninhydrin method described by Folkertsma and Fox (1992). The Free fatty acids (FFA) content (meq/100 g of fat) were determined in triplicate according to the methodologies described by Nuñez et al. (1986).

2.6. Color and texture profile analysis (TPA)

For color and texture measures cheese cylindrical samples (10 mm in height and 20 mm in diameter) were obtained from two cm below the rind of the cheese. Respect to color analysis, a Minolta spectrocolorimeter, model CM-3600D (Minolta, Japan), was used. The CIE color space L* a* b*, which was obtained by reflectance, using observer 10º and illuminant D65. Chromatic parameters Chroma (C*) and hue angle (H*) were obtained from the coordinates (L* a* b*) using the SpectraMagic v. 3.60 G software. This procedure was carried out three times for each cheese simple.

Total color differences (∆E) were calculated based on L*, a* and b* coordinates differences between each spiked condition (50, 100 and 200 µg/kg) with respect to the values of the control sample (0 µg/kg), applying the equation:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where ∆E < 1 indicates color differences could not be detected by the human eye; 1 < ∆E < 3 minor color differences could be detected by the human eye, and ∆E > 3 color differences could be detected by the human eye (Bodart et al., 2008).

With respect to textural determination of the cheese cylindrical samples, a texture profile analysis (TPA) was performed using a TA.XT Plus texturometer (Stable Micro

Systems, UK) as described by Salvador et al. (2014). Three replicates were carried out per cheese.

2.7. Cheese microstructure

2.7.1. Cryo scanning electron microscopy (Cryo-SEM)

A Cryostage CT-1500C (Oxford Instruments Ltd., Witney, UK) was used, coupled to a JSM-5410 scanning electron microscope (Jeol, Tokyo, Japan). The sample was immersed in slush N2 (−210 °C) and then quickly transferred to the Cryostage at 1 kPa where fracture of the sample took place. Sublimation (etching) was carried out at −90 °C, for 17 min. The sample was coated with gold at 0.2 Pa, with an ionization current of 2 mA. Observation in the scanning electron microscope was carried out at 15 kV at a working distance of 15 mm and temperature ≤−130 °C. Samples were taken from the centre of the cheese

2.7.2. Field Emission Scanning Electron Microscopy (FESEM)

Samples (0.5 cm each side) were frozen at –80 °C and lyophilized (Telstar, Lyoquest 55, Terrassa, Spain). The samples were coated with platinum in a vacuum, then observed in a field emission scanning electron microscope (FESEM) (Zeiss, Ultra 55, Oberkochen, Germany).

2.8. Sensory analysis

An acceptance test using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) was used to evaluate the following attributes: odor, color, appearance, texture and global preference (ISO 4121, 2003; ISO 5492, 2008). Attributes related to the tasting of the product were not included, due to the presence of the antibiotic in the samples. A trained panel composed of 16 members performed sensory analysis (8 women and 8 males aged from 30 to 60), all belonging to the Institute for Animal Science and Technology from UPV staff members and with vaste experience in sensory analysis of dairy products. The panel was trained for sensory analysis according to ISO 8586 (2012). The panelists evaluated “control cheese” (not spiked) and those elaborated with milk spiked at the different levels (0.5, 1 and 2 MRL). All samples were presented independently in closed containers. The sensory evaluations were conducted in individual booths in a homologated sensory room at the Institute of Food Engineering for Development at UPV, according to the international standards for test rooms (ISO 8589, 2007).

2.9. Statistical analysis

A one-way analysis of variance (ANOVA) was used to study the influence of “oxytetracycline concentration” (0, 50, 100 and 200 µg/kg) and “time” (minutes) on the pH evolution during the stages of cheese making process. In addition, other one-way analyses of variance (ANOVA) were carried out to evaluate the effect of “oxytetracycline concentration” on the parameters analyzed in ripened cheeses. In all cases, comparisons of mean values were made using the LSD’s test (Least Significant Difference) with a significance level of $\alpha = 0.05$, using Statgraphics Centurion XVI.II (version 16.2.04; Statpoint Technologies, Inc. Warrenton, Virginia, USA).

3. Results and Discussion

3.1. Cheese making process

As a first step, the influence of the presence of antibiotic (“control cheese”, 0.5 MRL, 1 MRL and 2 MRL) on the acidification stage of the milk and curd was evaluated. Figure 1 shows decrease of pH during cheese making, from the beginning (addition of starter culture) to the subsequent stages (coagulation (32ºC); heating & stirring (35ºC); molding & starting pressing; pressing after 90 min; pressing after 180 min; end of the pressing and final pH), until the curd reached the optimum pH of 5.30 (0.05). It is necessary that the curd reach this pH value (before immersion in the brine) since this type of cheese is made from raw milk, to accomplish with the technological requirements (Codex Alimentarius, 2004). At each stage, to eliminate the effect of time, the pH measurements at the four concentrations of oxytetracycline were carried out simultaneously.

At the previous stages to "Molding & starting pressing", the pH is not affected by the concentration of antibiotic, contrary to what happens in the following stages.

In the "Pressing after 90 min" stage, there were no significant differences in the pH between antibiotic concentrations, but the difference between the samples with oxytetracycline and the control samples was significant ($p < 0.05$). On the contrary, in the later stages, a delay in the pH decrease was observed as the antibiotic concentration increased, being the pH values at "End of pressing" of 5.36 (0.07), 5.41 (0.04), 5.50 (0.09) and 5.66 (0.04) for “control cheese”, 0.5, 1 and 2 MRL, respectively.

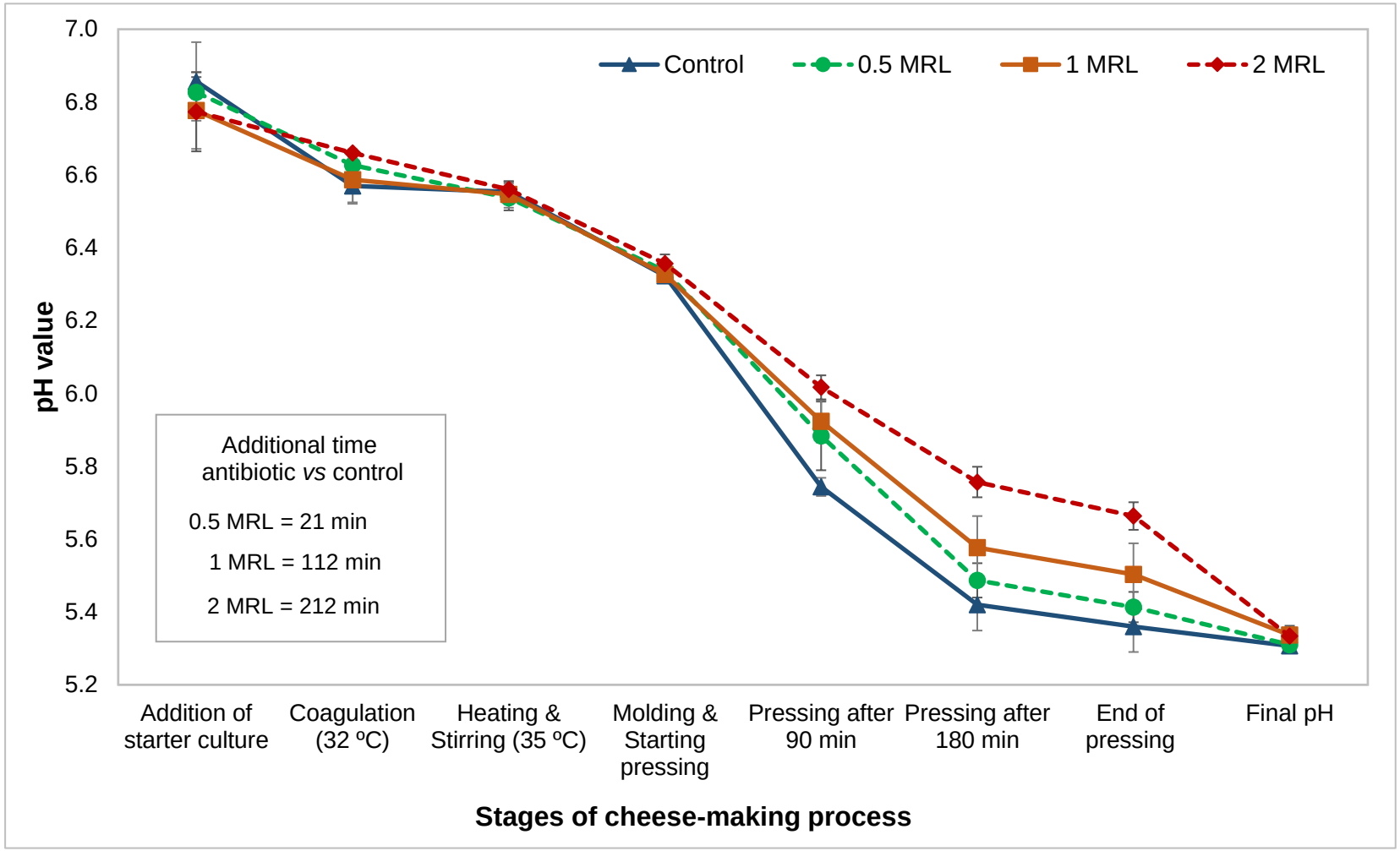

Figure 1. Kinetic acidification of the goat milk/curd during cheese manufacturing stages at different concentrations of oxytetracycline (Mean±SD). Additional time by different concentration necessary to reach final pH before immersion in the brine.

Figure 1 also shows (on "End of pressing" stage) the additional times, which from that stage and for each oxytetracycline condition were required to reach the pH of 5.3. Specifically, the average additional times to reach this pH were: 21 min, 112 min and 212 min for 0.5, 1 and 2 MRL, respect to the control cheese. Cabizza et al. (2018) reported in sheep's milk cheese, made from milk spiked with concentrations of 50 and 100 µg/kg of oxytetracycline, a delay in the pH decrease (35 and 78 min, respectively), similar trend to the delay in this study. In the above mentioned research, they considered that delay in acidification of milk spiked with oxytetracycline, provoked a dose-dependent influence of low levels of oxytetracycline presence in milk on the starter culture development and metabolism, which affected the cheese fermentation.

The acidification delay is the consequence of the behavior of the antibiotic on lactic acid bacteria (LAB), which are responsible for the conversion of lactose into lactic acid (Delorme, 2008). According to Tosi et al. (2007), S. thermophiles is susceptible to tetracycline at concentrations somewhat higher (125 µg/L) than the MRL (100 µg/L) in milk. It should be noted that, if growth of the starter is slow, due to the presence of antibiotic in the milk, considerable growth of pathogens can occur. The growth of pathogens will eventually cease due to the acidification (decrease pH) as significant amounts of lactic acid are produced (Fox et al., 2017).

3.2. Influence of oxytetracycline on the characteristics of cured cheese

3.2.1. Antibiotic concentration, chemical composition, water activity and pH

Table 1 shows the actual residual concentrations of oxytetracycline in the cheese at the end of ripening stage as well as the gross composition (moisture, fat, protein, salt) (g/100 g cheese, on wet basis), a_w and pH in ripened cheese manufactured with milk spiked with different concentrations of oxytetracycline (0, 50, 100 and 200 µg/kg milk). Taking into account these initial concentrations in the starting milk and that 8 kg of milk is required to obtain 1 kg of this type of cheese (Quintanilla et al., 2018), the oxytetracycline concentration at the end of the ripening (60 days), if there are no losses of antibiotic during the process, a concentration of 400, 800 and 1600 µg/kg cheese, respectively is expected. However, with the exception of “control cheese”, oxytetracycline residues were found, with values of < 10 (7 µg/kg approx. being an extrapolated value) 20.0 and 79.0 µg/kg cheese, respectively (which would correspond to 1.7%, 2.5%, and 4.9% of the initial spiked concentration). These losses are the consequence of the transfer of a large proportion of the initial antibiotic present in milk to the whey (Cabizza et al., 2018; Giraldo et al., 2017) and its continuous degradation during ripening at 10-12ºC (Roca, 2008). Although the amounts found in

the present study are well below MRL admitted by the regulations (considering the MRL of milk, since dairy products have not MRL fixed) the consumer would be exposed to a certain residual quantity of antibiotic. These findings should be considered a serious problem due to the health implications, especially for antibiotic resistance. Langford et al. (2003) observed that calves fed milk at different concentrations of penicillin showed greater resistance of gut bacteria to antibiotics, with increasing concentrations of this antibiotic in the milk fed to calves. Besides, many investigators have supposed that dairy bacteria including LAB may act as reservoirs of antibiotic resistance genes and can transfer resistance genes to pathogenic bacteria (Mathur and Singh, 2005).

Table 1 also shows the results of the ANOVA performed for a_w, pH and gross composition parameters. In the present work, for none of the cheeses made at different concentrations of oxytetracycline in milk, the presence of antibiotic had a significant effect on the parameters evaluated, except in the pH value. For the concentrations of 100 and 200 µg/kg the pH was slightly higher (5.23 and 5.24) than for the "control cheese" samples and those spiked with 50 µg/kg (5.13 in both cases). It should be noted that the pH values for all oxytetracycline concentrations are within other registered pH values for this type of goat cheese with two months of ripening: 5.25-5.32 (Quintanilla et al., 2018). The values of the gross composition are within what is considered usual for matured goat cheese, similar to the reported in other types of goat cheese (66.3% total solids, and 23.8% protein) by Ferrandini et al. (2011).

Table 1. Antibiotic concentration (µg/kg of cheese), composition (g/100 g of cheese), water activity (a_w) and pH (Mean±SD) in ripened cheeses manufactured with goat milk spiked with different concentration of oxytetracycline (0, 50, 100 and 200 µg/kg).

Parameters	Oxytetracycline concentration in milk (µg/kg)			
	0	50	100	200
Antibiotic concentration	-	7*±1.2^{a}	20.0±5.7^{a}	79.0±17.0^{b}
a_w	0.954±0.001	0.954±0.003	0.952± 0.003	0.953±0.002
pH	5.13±0.02^{a}	5.13±0.01^{a}	5.23±0.05^{b}	5.24±0.01^{b}
Total solids	65.42±0.74	65.66±0.30	66.45±0.58	65.82±0.82
Fat	35.96±1.59	35.95±1.62	35.69±1.69	35.32±1.35
Protein	24.35±1.56	24.70±1.62	25.97±1.79	25.47±1.52
Salt	2.34±0.07	2.25±0.16	2.19±0.13	2.16±0.09

* Concentration calculated with extrapolation outside the calibrated range; $^{a, b}$ Mean values with different letter in superscript within rows indicates significant differences ($p < 0.05$) due to oxytetracycline concentration in milk.

3.2.2. Assessment of proteolytic and lipolytic activities

Table 2 shows the concentration (average and standard deviation) of free amino acids (FAA) and free fatty acids (FFA) found in the ripened cheeses as indicators of the degree of proteolysis and lipolysis in cheese throughout ripening stage. FAA concentration showed no significant differences for any of the study conditions considered, with concentrations similar to those reported in goat cheese by Juan et al. (2016) (3.28 mg Leu/g of cheese). On the other hand, the concentration of FFA showed a significant and progressive decrease ($p < 0.05$) with the increase of the concentration of oxytetracycline. In this way, the concentration of FFA was higher in the "control cheese" (average of 4.02 meq/100 g of fat) and lower at the maximum concentration of antibiotic (average of 3.10 meq/100 g of fat). This seems to indicate a lower activity of the lipases due to the presence of the antibiotic. It should be noted that, although there are differences in the concentration of FFA among cheese, values are similar to those reported for the same type of cheese and ripening time with a range of 2.75 to 4.74 meq/100 g of fat (Quintanilla et al., 2018).

Table 2. Free amino acids (FAA) and free fatty acids (FFA) concentration (Mean±SD) in ripened cheeses manufactured with goat milk spiked with different concentration of oxytetracycline (0, 50, 100 and 200 µg/kg).

Parameters	Oxytetracycline concentration in milk (µg/kg)			
	0	50	100	200
FAA (mg Leu/g cheese)	3.01±0.45	2.93±0.53	2.72±0.44	2.80±0.37
FFA (meq/100 g fat)	4.02±0.48^{b}	3.69±0.68^{ab}	3.32±0.55^{a}	3.10±0.45^{a}

$^{a, b}$ Mean values with different letter in superscript within rows indicates significant differences ($p < 0.05$) due to oxytetracycline concentration in milk.

3.2.3. Color and texture profile analysis (TPA)

Color and texture parameters (average and standard deviation) in the ripened cheeses are shown in Table 3. Regarding color, the effect of the antibiotic has only been significant in terms of luminosity (L*) with higher values (approx. 89) for the "control cheese" and the lowest concentration of oxytetracycline, in comparison with 84-86 for concentrations of 100 and 200 µg/kg, respectively**.** On the contrary, coordinates a* (from -1.98 to -1.67) and b* (from 11.46 to 11.94) have not shown significant differences. Fresno and Álvarez (2012) reported similar results of L*: 84.83, a*: -2.28 and b*: 11.89. Concerning ΔE, the color differences were appreciable by the human eye ($\Delta E > 3$) for cheeses spiked with 200 µg/kg of oxytetracycline but not for those spiked with 50 or 100 µg/kg ($\Delta E < 3$).

Regarding the texture parameters, the springiness and cohesiveness were the only ones that showed significant differences related to the antibiotic concentration ($p < 0.05$). For springiness, the highest value was for "control cheese" observing a progressive decrease with increasing oxytetracycline concentration (average values from 0.54 to 0.44), on the contrary, an increase in cohesiveness was observed (average values from 0.23 to 0.29) when the level of antibiotic was equal to or above the MRL (100 and 200 µg/kg).

Table 3. Color and texture parameters (Mean±SD) in ripened cheeses manufactured with goat milk spiked with different concentration of oxytetracycline (0, 50, 100 and 200 µg/kg).

Parameters	Oxytetracycline concentration in milk (µg/kg)			
	0	50	100	200
Color				
L*	89.19±1.98^{b}	89.03±1.68^{b}	86.68±2.67^{ab}	86.00±2.28^{a}
a*	-1.74±0.28	-1.67±0.22	-1.89±0.14	-1.98±0.34
b*	11.52±1.29	11.46±1.09	11.94±0.91	11.50±1.08
ΔE_{ab}		0.18	2.55	3.20
Texture				
Hardness (N)	29.97±3.73	25.45±3.28	27.00±4.36	25.62±2.89
Adhesiveness (N.s)	-1.80±0.40	-1.57±0.54	-1.14±0.45	-1.28±0.45
Springiness	0.54±0.05^{b}	0.48±0.06^{a}	0.48±0.01^{a}	0.44±0.03^{a}
Cohesiveness	0.23±0.01^{a}	0.26±0.04^{ab}	0.28±0.05^{b}	0.29±0.03^{b}
Chewiness (N)	3.74±0.69	3.19±0.54	3.67±0.51	3.32±0.69

$^{a, b, c}$ Mean values with different letter in superscript within rows indicates significant differences ($p < 0.05$) due to oxytetracycline concentration in milk.

On the other hand, the hardness presented a similar tendency to springiness, but without significant differences between the cheeses. In the "control cheese", greater hardness and springiness were observed than the cheeses containing oxytetracycline. The differences found in the textural properties might have been due to the interaction between the oxytetracycline and the casein matrix (Cabizza et al., 2017). This is because the Ca^{2+} ions forming bonds with oxytetracycline (Arias et al., 2007) and being able to generate changes in the protein network of the cheese, therefore, affecting its texture characteristics. Calcium equilibria is known to impact considerably upon texture properties like hardness of cheese (Everett and Auty, 2008). According to the conformation of the protein network, the greater the number of intra and extra filament bonds, the protein matrix will be more elastic and more

difficult to deform, presenting a positive correlation between the volume of the protein fraction and the hardness of the cheese (Castro et al., 2014).

Despite the significant differences observed springiness and cohesiveness, the values of these parameters present similar results to those published by other authors such as Salvador et al. (2014), and Quintanilla et al. (2018) for goat cheese with the same ripening time: hardness (23; 33.95), adhesiveness (-1.1; -1.68), springiness (0.60; 0.49) and cohesiveness (0.38; 0.27) respectively.

3.3. Cheese microstructure

3.3.1. Cryo scanning electron microscopy (Cryo-SEM)

Figure 2 (a-d) shows the images of the “control cheese” and 2 MRL cheeses obtained using Cryo-SEM technique at different magnifications. In Figure 2.a and 2.b, a continuous three-dimensional network consisting of proteins (P) can be observed, leaving rounded holes (H) in its structure; these holes could originally have been occupied by fat as observed previously in fresh cheese (Hernando et al., 2000). Moreover, in the micrographs, fat is visualized as globules (G). No important differences are observed between the “control cheese” and the 2 MRL cheeses. However, when observing the microstructure at higher magnifications (Figure 2.c and 2.d), fat in some areas of the “control cheese” seems to have coalesced and it is observed as aggregates with an amorphous appearance (A), while fat in 2 MRL cheese has a better structure. Less coalescence of fat globules is observed in 2 MRL cheese if compared to “control cheese”. In the absence of oxytetracycline, the production of lipase by cheese microorganisms could cause the hydrolysis of some fat globules and thus, their coalescence. Rani and Jagtap (2019) observed by SEM that the fat globules were larger when adding lipase (to accelerate ripening of Swiss cheese) than in the absence of lipase.

3.3.2. Field Emission Scanning Electron Microscopy (FESEM)

In order to confirm the results obtained by Cryo-SEM technique, the observation of the “control cheese” and 2 MRL cheeses was also carried out by FESEM. This technique is used as a powerful tool to understand the relationships between structural properties and physical-chemical analysis, for example, observing the state of fat (Everett & Auty, 2008). Figure 2.e and 2.f show the structure of the cheeses, where the protein matrix (P), casein granules (C) and some fat globules (G) can be observed.

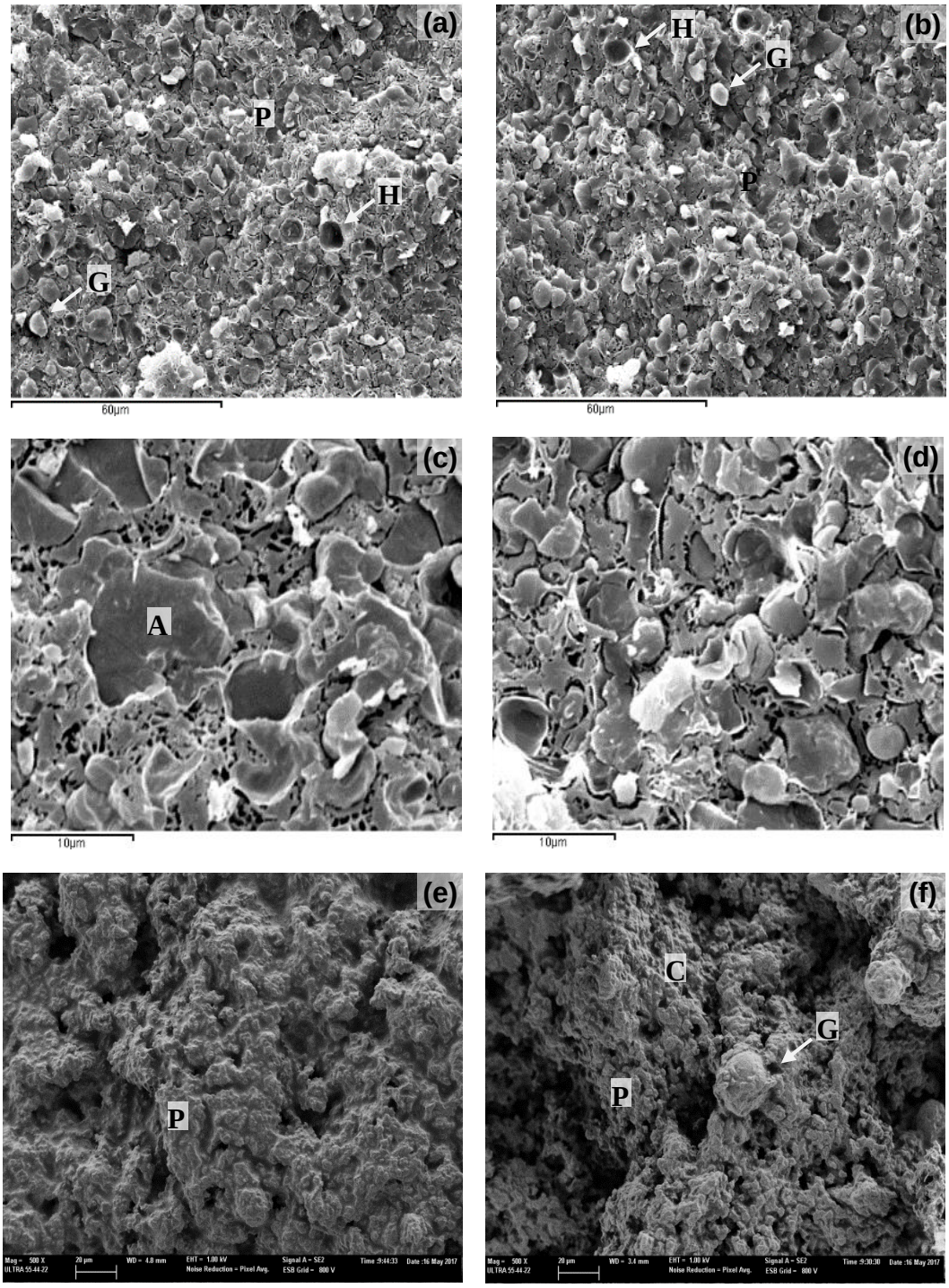

Figure 2. Cryo scanning electron microscopy (Cryo-SEM) images of **(a)** "control cheese" (x1,000), **(b)** 2-MRL cheese (x1,000) **(c)** "control cheese" (x3,500), **(d)** 2-MRL cheese (x3,500). Field Emission Scanning Electron Microscopy (FESEM) images of **(e)** "control cheese" (x500), **(f)** 2-MRL cheese (x500). The scale bars are 60 µm (a and b), 10 µm (c and d) and 20 µm (e and f) in length. P: Protein matrix, A: Aggregated fat, G: Fat globule, H: Hole. C: Casein.

In the Figure 2.e, corresponding to the "control cheese", fat globules cannot be clearly appreciated. On the other hand, in Figure 2.f corresponding to 2 MRL-cheese

the protein matrix can be better visualized, even some casein granules can be observed; some individualized globules of fat are observed as well. Likewise, smaller sizes of these globules produce micelles and tend to add and contract with greater intensity, generating a more rigid structure (Castro et al., 2014). This would correspond to the greater hardness observed in the “control cheese”, as commented before.

3.4. Sensory analysis

The result of the ANOVA values (using “concentration” as a factor), from the hedonic test carried out for the different attributes evaluated is shown in Table 4. This table shows the average score for each attribute evaluated by the panelists. The results indicate that presence of oxytetracycline residues in cheeses does not have an influence on the evaluated attributes ($p > 0.05$). This is because the judges scored similarly and satisfactorily the samples, without appreciating differences between the “control cheese” and the cheeses that contained different concentrations of oxytetracycline. Although, the measured color and texture characteristics are significantly different, the sensory analysis also indicates the presence of this antibiotic has not generated appreciable changes by the judges on these parameters in the cheeses of 60 days of ripening. As a result of these findings, it would lead to believe that people could be consuming cheeses with the presence of antibiotics without realizing it.

Table 4. Hedonic scores (Mean±SD) obtained for ripened cheese manufactured from raw goat milk (“control cheese”) and cheeses made to different concentrations of oxytetracycline in milk.

Parameters	**Oxytetracycline concentration in milk (µg/kg)**			
	0	**50**	**100**	**200**
Odor	5.80±1.63	6.00±1.47	6.27±1.30	6.24±1.42
Color	6.60±1.08	6.74±1.09	6.56±1.11	6.68±0.97
Appearance	6.59±1.02	6.61±1.14	6.57±1.15	6.76±1.00
Texture	6.71±1.01	6.71±1.01	6.59±1.25	6.73±1.12
Overall preference	6.44±1.11	6.43±1.18	6.50±1.11	6.58±1.16

Results of the hedonic test for each attribute, using a 9 cm linear scale (1: Dislike extremely, 9: Like extremely)

4. Conclusions

The presence of oxytetracycline residues in goat milk does not seem to have a significant impact on the cheese-making process nor on the properties of the ripened cheeses, even in amounts exceeding the legal limits established. The few differences were related to delays during cheese-making process and slight modifications in some parameters such as free fatty acid concentration, color and textural properties with respect to cheese free of the antibiotic. In addition, what could be even worse is the undetectability of the consequences of the antibiotic presence by a sensory analysis panel. Hence, cheese made from milk with oxytetracycline residues would go unnoticed in its sale, distribution and consumption.

Although the level of oxytetracycline residual in ripened cheeses was lower than MRL allowed in milk (since for cheese it is not regulated), different amounts of oxytetracycline remain in mature cheese reaching the food chain. This could have relevant health implications, especially due to the development and spreading of antimicrobial resistance, which is currently considered a major problem.

Future studies on the transfer of antibiotic from milk to related products including a wide range of antibiotics, different types of milk, and manufacturing techniques are therefore required in order to encourage policy makers to regulate the presence of antibiotics in cheese to guarantee the safety of this product

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Study 2.2. Volatile profile of matured Tronchón cheese affected by oxytetracycline in raw goat milk

Abstract

The presence of antibiotics in milk destined for cheese production may affect the biological processes responsible of the formation of volatile compounds, leading to alterations of the characteristic cheese flavor expected by consumers. The aim of this study was to evaluate the impact of the presence of oxytetracycline in goat milk on the volatile profile of ripened cheeses. Traditional Tronchón cheeses were manufactured from raw goat milk spiked with different concentrations of oxytetracycline (50, 100 and 200 µg/kg) and ripened for a 60-day period. Cheese from antibiotic-free goat milk was used as control. Residual amounts of the antibiotic and the volatile profile of the experimental cheeses were analyzed on a fortnightly basis during maturation by HPLC-MS and SPME-GC/MS methods, respectively. Results herein suggest that oxytetracycline was widely transferred from milk to cheese; the drug concentration in the cheeses being 3.5-4.3 times higher than the drug concentration in raw milk. Although the residual amounts of oxytetracycline significantly decreased during maturation (88.8-96.5%), variable amounts of residues remained in 60-days matured cheeses (<10-79 µg/kg). In general, the presence of oxytetracycline in goat milk did not affect, the volatile profile of Tronchón cheeses although some carboxylic acids, alcohols as well as some minority volatile compounds were slightly modified. However, the volatile profile of cheeses was significantly modified by the ripening time. In any case, the presence of oxytetracycline residues in the 60-day ripened cheeses could be of great concern for public health.

Key-words: Goat cheese, oxytetracycline, antibiotic residues, volatile profile.

1. Introduction

In the Mediterranean and eastern European countries, goat milk is mainly used for cheese-making, with a growing demand in the last decade due to its particular taste, nutritional value and the great variety of traditional cheeses. The cheese flavor is one of the most important organoleptic criteria for consumer acceptance, being the result of a complex balance between volatile and non-volatile chemical compounds. Biochemical processes such as glycolysis, lipolysis and proteolysis are the main pathways to produce impact-aromatic compounds like alcohols, aldehydes, carboxylic acids, esters, ketones, among others, during ripening (McSweeney and Sousa, 2000; Delgado et al., 2010).

The main enzymes involved in these primary degradations of the cheese components, and in the further catabolic reactions, originate from raw milk microbiota, starter cultures or rennet added during cheese-making. It is generally agreed that the presence of antibiotic residues in milk, besides the negative implications on consumer health, affects technological cheese-making processes as they could inhibit the activity of the raw milk microflora and/or of the starter cultures usually employed in the dairy industry (Katla et al., 2001), altering the liberation of enzymes and consequently, modifying the production of aromatic compounds in matured cheese.

One of the most commonly broad-spectrum antibiotics used in dairy goats is oxytetracycline currently used to treat mastitis, urinary tract as well as enteric infections among other diseases (Attaie et al., 2015). The improper use of this antibiotic can result in unwanted residues in milk and related products.

Studies available on the presence of oxytetracycline in matured cheese are very scarce and focus mainly on the transfer of tetracyclines from contaminated milk to cheese (Cabizza et al., 2017; Gajda et al., 2018); the effect of the antibiotic on the organoleptic properties of the ripened cheeses being unknown. Therefore, the objective of this work was to evaluate the impact of different concentrations of oxytetracycline in raw goat milk on the volatile profile of Tronchón cheese throughout ripening.

2. Materials and Methods

For each oxytetracycline concentration three elaboration of cheese were made at the pilot plant of Universitat Politècnica de València (UPV, Valencia, Spain) using Murciano-Granadina raw goat milk from the UPV experimental herd. For each cheese trial, 200 kg of milk were divided into four vats containing 50 kg each. Three of the vats were spiked with oxytetracycline (O4636, Sigma-Aldrich, Madrid, Spain) at different

antibiotic concentrations closely related to the maximum residue limit (MRL; European Commission, 2010) established for this substance: 50 µg/kg (0.5 MRL), 100 µg/kg (1 MRL) and 200 µg/kg (2 MRL), and the last one was not spiked to be used as reference.

The chemical composition of the three milk batches used for cheeses production were analysed using MilkoScan FT6000 (Foss, Hillerød, Denmark), the mean values (average±SD) being: 14.71±0.37% total solids, 5.37±0.19% fat, 3.98±0.22% protein and 4.63±0.07% lactose. Traditional semi-hard goat Tronchón cheese was made from raw milk following the cheese-making procedure reported by Quintanilla et al. (2019). Ten cheeses were obtained from each batch, which were sampled in duplicate at 0, 15, 30, 45 and 60 days of ripening for further analysis.

Oxytetracycline residues in the cheeses were quantified using the HPLC-MS/MS method validated at the Instituto Lactológico de Lekunberri (Lekunberri, Pamplona, Spain) previously described by Quintanilla et al. (2019). The volatile compounds analysis of the cheese samples was performed by the headspace SPME-GC/MS method at Laboratory of Food Quality and Design Group at Wageningen University & Research (Wageningen, Netherlands). For the solid phase micro-extraction (SPME) of volatile compounds, the method developed by Hettinga et al. (2008) was followed.

From each cheese, two samples were taken at a depth of 1 cm from the rind. For headspace extraction, one gram of a finely grated cheese sample was weighed in a 10 mL glass vial headspace GC vials (46 x 22.5 mm) and sealed with a 20 mm silicone/PTFE cap (Grace, Albany, OR, USA). Extraction of volatile compounds by SPME was carried out with a 75 µm Carboxen™–PDMS SPME fiber (Supelco, Bellefonte, PA, USA) at 45°C for 40 min using an auto-sampler. A vial filled with air was used as blank. For GC/MS analysis, the SPME fiber was desorbed for 10 minutes in the GC injection port at 225°C. GC/MS analysis was performed using a Trace GC Ultra connected to a DSQ II mass spectrometer (Thermo Scientific, Austin, TX, USA). The Stabilwax®-DA polyethylene-glycol column with 30 m length, 0.32 mm internal diameter, and 1 µm film thickness (Restek, Bellefonte, PA, USA) was used. The oven temperature was maintained at 40°C for 3 minutes, then increased to 220°C at a rate of 15°C/min. When the final temperature of 220°C was reached, it was maintained for 1 minute. Helium was used as carrier gas, which was fed at a constant flow rate of 1.5 mL/min. The MS ion source was maintained at 225°C. MS scans were collected in full scan mode, using a mass range of 33-250 m/z with electron impact mode at 70 eV. Each compound was identified with AMDIS software using the NIST/EPA/NIH database (NIST, Gaithersburg, MD, USA) and an in-house library (Hettinga, 2009).

Metalign and MetalignID software packages were used for noise reduction, peak selection, peak identification and peak integration (http://www.metalign.nl).

Statistical analysis was performed by Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. Warrenton, Virginia). A multifactor analysis of variance (ANOVA) was carried out to study the effects of the oxytetracycline concentration in raw milk (C: 0, 50, 100 and 200 µg/kg) and the ripening time (t: 0, 15, 30, 45 and 60 days), as well as their respective interaction (C x t) on the volatile profile of the cheeses. The Least Significant Difference (LSD) test was employed for multiple comparisons of the mean values. Furthermore, a Principal Component Analysis (PCA) data was applied by means of the software Unscrambler X.10.

3. Results and Discussion

Cheese-making from raw goat milk containing oxytetracycline close to MRL led to antibiotic residues in the cheeses (Figure 1). Results herein indicate that this antibiotic was widely transferred from milk to cheese, as the oxytetracycline concentration in the cheeses just before maturation (day 0) was about 4 times higher than the drug concentration in raw milk used for cheese production (0.5 MRL: 3.9±0.81; MRL: 4.3±0.32; 2 MRL: 3.5±0.57). High concentration factors (3.8-5.7) were reported also by Cabizza et al. (2017) and Gajda et al. (2018) when assessing the transfer of oxytetracycline from sheep and cow milk to cheese, respectively. The high fat affinity of this substance and its ability to form stable quelates with animal proteins (Giguère, 2013) could explain the high residual drug concentration in the experimental cheeses.

As shown in Figure 1, the residual amounts of oxytetracycline progressively decreased during maturation ($p < 0.001$) being on average 88.8-96.5% lower in 60-day ripened cheeses. However, it should be noted that variable amounts of oxytetracycline were present in the cheeses during the entire maturation period, which could negatively affect the growth of the cheese microbiota and consequently the release of the enzymes involved in the biochemical pathways responsible for the formation of the characteristic Tronchón cheese flavor.

Table 1 shows the areas measured for the volatile compounds identified in the experimental cheeses according to the two factors considered. Thirty-nine compounds including acids, alcohols, aldehydes, esters, ketones and others were identified; carboxylic acids and ketones being the most abundant compounds in the volatile fraction of the Tronchón cheeses, as similarly reported by Delgado et al. (2011) and Padilla et al. (2014) in other ripened goat milk cheeses. As shown in Table 1, the total amount of carboxylic acids was unaffected by the presence of oxytetracycline in the

cheeses ($p > 0.05$). However, significant differences were obtained when the most probable origin of these volatile compounds (Delgado et al., 2011) were considered.

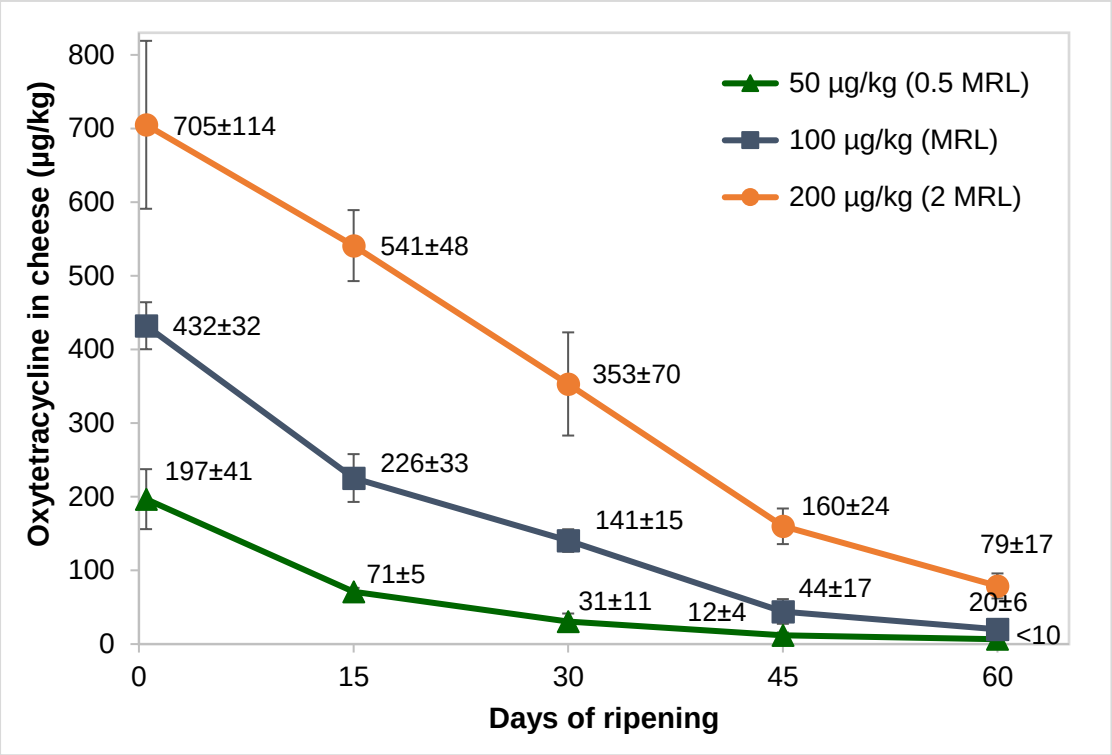

Figure 1. Antibiotic residues during ripening (Mean±SD) in Tronchón cheese made from goat milk spiked with different concentration of oxytetracycline.

Results herein suggest that the main biochemical pathways producing carboxylic acids were significantly affected by the presence of such residues in the cheeses. The most relevant differences were related to the main acids in the volatile fraction of the Tronchón cheeses, acetic and butanoic acids, flavor components typically isolated in ripened cheeses from raw milk (Beuvier and Buchin, 2004). Thus, a higher content of acetic acid ($p < 0.05$) and a lower content of butanoic acid ($p < 0.001$) were obtained in cheeses from milk containing oxytetracycline at or above MRL. These changes could have a noticeable effect on the flavor perception by consumers as acetic acid imparts sharp-vinegar notes to the cheese, whereas butanoic acid is related to a desirable cheesy sharp-piquant taste (Frank et al., 2004).

Table 1. Effect of oxytetracycline concentration in goat milk and ripening time on the volatile compounds (AU x 10^5) of Tronchón cheese.

Chemical group	Oxytetracycline concentration (µg/kg)					Ripening time (days)					
	0	50	100	200	SEM	0	15	30	45	60	SEM
Acids											
Acetic acid	3848.6^{a}	3958.3^{ab}	4189.7^{c}	4096.4^{bc}	76.4	3453.2^{a}	4285.6^{cd}	4075.1^{bc}	4366.2^{d}	3936.2^{b}	85.4
Propanoic acid	184.4	159.1	124.7	174.0	26.6	14.8^{a}	33.7^{a}	69.3^{a}	230.6^{b}	454.4^{c}	29.7
2-methyl-propanoic acid	51.7^{b}	41.3^{ab}	29.5^{a}	32.4^{a}	4.8	4.5^{a}	18.6^{a}	48.6^{b}	67.1^{c}	54.9^{bc}	4.6
Butanoic acid	4164.7^{b}	4011.2^{b}	3502.8^{a}	3581.2^{a}	128.1	1700.3^{a}	3008.0^{b}	3868.4^{c}	5140.8^{d}	5357.3^{d}	143.2
3-methyl-butanoic acid	95.9^{c}	73.1^{b}	53.2^{a}	65.1^{ab}	6.2	9.8^{a}	36.9^{b}	87.2^{c}	109.0^{d}	116.3^{d}	6.9
Pentanoic acid	69.7^{b}	65.1^{b}	54.3^{a}	56.2^{a}	3.2	23.1^{a}	45.3^{b}	67.1^{c}	86.4^{d}	84.9^{d}	3.6
Hexanoic acid	2179.1	2115.7	1954.1	1914.6	110.4	863.4^{a}	1474.1^{b}	2085.4^{c}	2820.7^{d}	2960.9^{d}	123.5
Heptanoic acid	33.3	32.2	32.6	32.7	1.9	15.4^{a}	22.5^{b}	32.3^{c}	42.8^{d}	50.5^{e}	2.1
Octanoic Acid	414.5	412.6	457.2	450.4	38.8	215.1^{a}	326.7^{ab}	442.3^{bc}	541.0^{cd}	643.3^{d}	43.4
Most probable origin:*											
Lactate metabolism	4033.0^{a}	4117.5^{ab}	4314.4^{b}	4270.4^{b}	77.4	3468.1^{a}	4319.3^{bc}	4144.5^{b}	4596.8^{d}	4390.6^{cd}	86.5
Lipolysis	6861.3^{b}	6635.5^{ab}	6001.0^{a}	6034.1^{a}	255.9	2817.3^{a}	4874.9^{b}	6494.3^{c}	8631.7^{d}	9096.7^{d}	286.1
Proteolysis	147.4^{c}	113.6^{b}	82.2^{a}	96.5^{ab}	9.8	11.2^{a}	55.4^{b}	135.8^{c}	176.2^{d}	171.2^{d}	11.0
Total Acids	**11042**	**10867**	**10398**	**10401**	**281**	**6297^{a}**	**9250^{b}**	**10775^{c}**	**13405^{d}**	**13659^{d}**	**315**
Percentage (%)	**52.1**	**52.0**	**51.7**	**52.9**		**36.3**	**40.6**	**52.1**	**66.0**	**66.0**	

Table 1 *(Cont.)*

Chemical group	Oxytetracycline concentration (µg/kg)					Ripening time (days)					
	0	50	100	200	SEM	0	15	30	45	60	SEM
Alcohols											
Ethanol	203.3	186.3	205.1	236.2	18.5	244.4^{b}	180.6^{a}	198.4^{ab}	n.d.	n.d.	15.0
1-butanol	88.8	108.8	89.7	108.2	11.8	n.d.	23.0^{a}	22.0^{a}	148.1^{b}	202.3^{c}	11.2
2-butanol	423.6	388.0	317.7	277.8	75.4	n.d.	n.d.	119.8^{a}	605.9^{b}	329.6^{a}	57.9
3-methyl-1-butanol	117.1	94.7	89.6	101.2	11.4	9.8^{a}	101.3^{bc}	191.4^{d}	123.9^{c}	76.9^{b}	12.7
1-Pentanol	24.3	22.6	25.8	20.1	1.6	11.9^{a}	31.2^{c}	34.3^{c}	23.3^{b}	15.3^{a}	1.8
2-Pentanol	318.8^{b}	264.4^{ab}	266.5^{ab}	208.7^{a}	25.2	24.4^{a}	22.6^{a}	223.1^{b}	609.9^{d}	442.9^{c}	27.6
1-Hexanol	45.7	44.3	30.0	37.9	14.0	19.8^{a}	95.9^{c}	53.5^{b}	18.6^{a}	9.6^{ab}	13.1
1-methoxy-2-propanol	17.8	20.3	23.2	17.0	3.8	15.9	24.8	23.5	19.2	14.5	4.1
Total Alcohols	**922.5^{b}**	**767.5^{a}**	**778.2^{a}**	**733.1^{a}**	**48.5**	**314.2^{a}**	**451.2^{b}**	**784.1^{b}**	**1534.7^{c}**	**917.5^{b}**	**54.3**
Percentage (%)	**4.4**	**3.6**	**4.1**	**3.8**		**1.9**	**1.9**	**3.7**	**7.8**	**4.4**	
Ketones											
Acetone	44.1	39.2	36.6	37.5	3.1	48.4^{c}	66.7^{d}	45.2^{c}	12.5^{a}	23.9^{b}	3.5
2-butanone	476.4	397.9	382.5	532.1	87.7	9.0^{a}	14.1^{a}	316.6^{b}	880.5^{c}	1016.0^{c}	102.8
2,3-butanedione	1651.1	1822.7	1696.9	1537.2	174.5	1445.3^{a}	2579.9^{b}	2117.4^{b}	1133.9^{a}	1108.3^{a}	195.1
2,3-pentanedione	21.4	18.8	20.4	19.1	2.2	15.5	18.2	24.2	21.8	n.d.	2.5
2-Pentanone	1868.2	2041.2	1945.3	1760.1	210.9	1407.1^{a}	2840.4^{b}	2555.5^{b}	1345.9^{a}	1369.4^{a}	235.8
2-Hexanone	41.7	48.0	39.4	35.6	6.1	11.7^{a}	54.7^{bc}	50.4^{b}	21.6^{a}	67.4^{c}	5.7
2-Heptanone	1052.0	1144.4	1409.7	1030.2	255.8	60.7^{a}	1982.2^{c}	1801.1^{c}	683.1^{ab}	1268.2^{bc}	286.0
3-hydroxy-2-butanone	3473.5	3693.7	3416.9	3322.5	166.1	7656.8^{d}	5925.1^{c}	2350.2^{b}	877.7^{a}	573.4^{a}	185.7
2-Nonanone	124.5	132.2	173.0	117.9	33.0	21.6^{a}	240.3^{c}	204.2^{c}	61.1^{ab}	157.1^{bc}	36.2
8-Nonen-2-one	14.5	14.6	15.9	18.7	2.1	n.d.	13.9^{ab}	23.2^{c}	9.4^{a}	17.3^{bc}	2.8
Total Ketones	**8732**	**9307**	**9097**	**8314**	**641**	**10641^{b}**	**13659^{c}**	**9390^{b}**	**5030^{a}**	**5592^{a}**	**716**
Percentage (%)	**41.3**	**42.1**	**42.0**	**41.2**		**61.4**	**55.9**	**41.7**	**23.5**	**25.8**	

Table 1 *(Cont.)*

Chemical group	Oxytetracycline concentration (µg/kg)					Time of ripening (days)					
	0	50	100	200	SEM	0	15	30	45	60	SEM
Aldehydes											
Hexanal	40.8	43.4	44.7	36.7	4.7	11.8^{a}	53.1^{b}	53.8^{b}	25.7^{a}	62.6^{b}	5.24
3-methyl-hexanal	163.0	144.1	125.1	148.8	22.4	12.6^{a}	189.5^{c}	276.9^{d}	131.0^{bc}	116.2^{b}	25.1
Nonanal	44.7	53.0	44.8	46.3	8.4	4.1^{a}	59.1^{b}	43.3^{b}	45.8^{b}	83.8^{c}	9.3
Benzaldehyde	10.2	9.2	8.3	12.8	1.9	5.1^{a}	16.8^{b}	11.1^{ab}	8.6^{a}	9.0^{a}	2.1
Total Aldehydes	**252.7**	**228.0**	**193.4**	**209.4**	**25.2**	**30.5^{a}**	**274.7^{bc}**	**330.1^{c}**	**197.5^{b}**	**271.5^{bc}**	**28.2**
Percentage (%)	**1.1**	**1.0**	**0.9**	**0.9**		**0.2**	**1.1**	**1.4**	**1.0**	**1.3**	
Esters											
Butanoic acid, ethyl ester	130.7	157.5	153.3	116.5	11.3	n.d.	50.9^{a}	94.5^{b}	195.6^{c}	217.0^{c}	11.3
Hexanoic acid, ethyl ester	117.4	124.3	134.3	93.6	13.0	n.d.	36.5^{a}	84.1^{b}	126.0^{c}	223.1^{d}	12.3
Propanoic acid, 2-methylpropyl ester	30.6	82.7	91.7	96.4	18.2	25.2^{a}	45.0^{b}	96.1^{b}	52.4^{ab}	158.2^{c}	20.0
Total Esters	**202.5**	**254.5**	**254.0**	**199.5**	**24.2**	**25.2^{a}**	**88.6^{a}**	**205.6^{b}**	**323.8^{c}**	**494.8^{d}**	**23.5**
Percentage (%)	**0.9**	**1.2**	**1.2**	**1.0**		**0.1**	**0.4**	**1.0**	**1.6**	**2.4**	
Others											
2-methyl-1,3-butadiene	14.9^{b}	14.9^{b}	14.6^{b}	12.4^{a}	0.5	10.2^{a}	17.1^{d}	15.9^{cd}	15.0^{c}	12.7^{b}	0.4
Dimethyl disulfide	4.1	4.0	3.6	3.6	0.4	4.5^{bc}	2.3^{a}	3.7^{b}	5.1^{c}	3.6^{ab}	0.4
Dimethyl sulfone	8.4^{b}	7.7^{ab}	7.1^{a}	7.9^{ab}	0.3	10.3^{c}	7.7^{b}	8.3^{b}	6.7^{a}	5.8^{a}	0.4
3-carene	5.6	6.6	6.4	7.1	1.1	2.9^{a}	15.8^{b}	3.3^{a}	3.7^{a}	n.d.	1.1
2, 4-dimethyl-heptane	10.3	12.0	13.1	9.0	2.0	n.d.	n.d.	7.6^{a}	12.9^{ab}	12.8^{b}	1.8
Total Others	**28.2**	**27.5**	**27.3**	**25.6**	**1.6**	**24.1^{a}**	**30.9^{b}**	**30.5^{b}**	**30.9^{b}**	**19.4^{a}**	**1.8**
Percentage (%)	**0.1**	**0.1**	**0.1**	**0.1**		**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	

Values are means of six determinations (two cheeses per ripening time x three batch replicate); SEM: standard error of mean; $^{a, b, c, d, e:}$:Superscript letters in the same row for factor indicate significant differences ($p < 0.05$). *Most probable origin of carboxylic acids (Delgado et al., 2011) from: lactate metabolism (sum of acetic acid and propanoic acid), lipolysis (sum of butanoic acid; pentanoic acid; hexanoic acid; heptanoic acid and octanoic acid) and proteolysis (sum of 2-methyl-propanoic acid and 3-methyl-butanoic acid).

Likewise, the presence of oxytetracycline affected the total concentration of alcohols in the cheeses ($p < 0.05$), leading to a significant reduction in the 2-pentanol content, the main and most abundant alcohol in the volatile profile of the cheeses, formed by the enzymatic reduction of the corresponding methyl ketone (Molimard and Spinnler, 1996). Lower amounts of minority volatile compounds like 2-methyl-1,3 butadiene ($p < 0.01$) and dimethyl sulfone ($p < 0.05$) were also obtained in the cheeses from milk containing oxytetracycline at or above MRL. On the contrary, aldehydes, esters, and ketones were unaffected by the presence of this antibiotic. The volatile compounds modifications could be related to the bacteriostatic activity of the oxytetracycline potentially able to produce an imbalance in the raw cheese microbiota (Cabizza et al., 2018) involved in the biochemical changes during maturation. Regarding the effect of the ripening time, statistical analysis showed that the volatile profile values were significantly modified throughout maturation (Table 1).

In general, the total amounts of organic acids, alcohols, and esters significantly increased during the first 45 days of maturation ($p < 0.001$), whereas ketones, the group with the highest concentrations in the two first weeks of ripening, progressively decreased ($p < 0.001$) in this period, possibly due to a reduction of these compounds into secondary alcohols (Andiç et al., 2015). Minor changes were observed in the last two weeks of maturation, which could be related to the reduced microbial activity in the cheeses, due to the lower viable microbial population at this ripening stage (Urbach, 1995; Delgado et al., 2011), and the lower water activity which also limits the enzymatic activity in the cheeses (Beresford et al., 2001).

In 60-day ripened Tronchón cheeses, acetic, butanoic, and hexanoic acids were the most abundant volatile compounds in the cheeses, more than 50 %, being typical flavor components perceived as a goat-like smell (Castillo et al., 2007). High amounts of methyl ketones were also detected in the mature Tronchón cheese, as occurred in other Spanish goat cheeses such as Majorero (Castillo et al., 2007) and Ibores (Delgado et al., 2011). The 2-pentanone linked to a smell described as orange peel and sweet, fruity (Curioni and Bosset, 2002) was the most important along the entire maturation period. Large amounts of 2,3 butanedione (diacetyl) with an intensive creamy, buttery flavor (Le Bars and Yvon, 2008) were also detected. No significant interactions were obtained between antibiotic concentration and ripening time factors ($C \times t$; $p > 0.05$), suggesting that all the cheeses evolved in a similar way over time, regardless of the concentration of oxytetracycline assessed. The only exception was 2-methyl-1,3-butadiene ($p < 0.01$) with a higher content in the last two weeks of ripening in cheeses made from milk spiked with oxytetracycline at or above MRL.

Once the individual behavior of each compound was studied, a PCA was conducted to assess the overall effect of the concentration of oxytetracycline and days of ripening on the volatile compounds. Figure 2 shows the score (A) and loading (B) plots of the PCA performed.

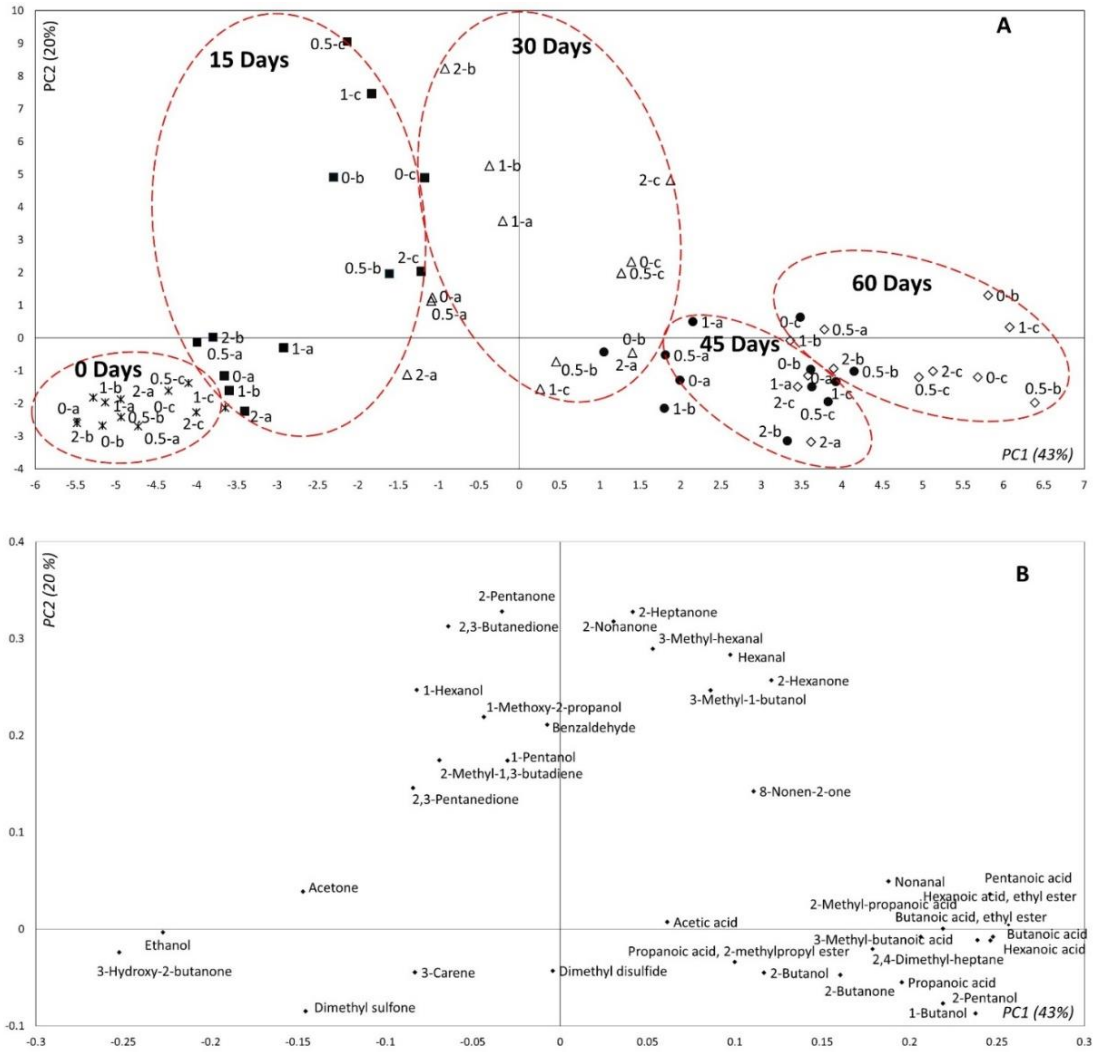

Figure 2. Score (A) and loading (B) plots of the two first principal components (PC1 and PC2) in volatile profile of Tronchón cheese during ripening time (0:*; 15: ■; 30:Δ , 45:● and 60 days: ◊). Codes in the score plots refer to oxytetracycline concentrations in goat milk (0, 0.5, 1 and 2 MRL) and triplicate of cheese manufacture (a, b, and c).

In the score plot the code for each point refers to: oxytetracycline concentration in goat milk (0, 0.5, 1 and 2 MRL) and the triplicate of cheese manufactured for each concentration (a, b, c). In this plot, proximity between samples means a certain similarity among them. Three principal components were found to explain 70% of the variations in the data set in which PC1 represents 43% of the variability and PC2 20%. This PCA showed that, in general, the presence of oxytetracycline did not have an

overall effect on the volatile profile. However, the different stages of maturation were very well separated along PC1, progressively from the left to the right quadrants. The circles represent the different stages of maturation (0, 15, 30, 45 and 60 days). The loading plot shows a wider distribution of the volatile compounds along PC1 according to the ripening times, which amounts of acid compounds at 45 and 60 days of ripening standing out.

4. Conclusions

The presence of oxytetracycline in goat milk close to the legal maximum concentration (0.5-2 MRL) modified the volatile profile of Tronchón cheeses only slightly, larger amounts of residues of this antibiotic could be present especially when cheese is matured for a short time (2-4 weeks). Although ripening conditions lead to the degradation of residual oxytetracycline in cheese, variable amounts of this antibiotic may persist in matured cheeses posing a risk to consumer health.

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Study 3. ***Effect of antibiotic residues in milk and cheeses after the off-label use of macrolides in dairy goats***

Effect of antibiotic residues in milk and cheeses after the off-label use of macrolides in dairy goats

Abstract

The limited availability of drugs registered for dairy goats makes veterinarians prescribe off-label treatments with a legally established minimum safety period of seven days. The aim of this work was to verify if the exceptional use of macrolide antibiotics in dairy goats generates residues in milk and cheeses within that period. Hence, three macrolide drugs (erythromycin, tylosin and spiramycin) were administered in an *in vivo* experiment in dairy goats. Ripened cheeses were made from bulk milk obtained before drug administration, 24 hours after treatment, and at the end of the recommended withdrawal period. Residual amounts of erythromycin (234.9±52.7 µg/kg), tylosin (198.7±57.8 µg/kg) and spiramycin (1,539.8±469.4 µg/kg), widely exceeding their legal maximum residue limits (MRLs) established, were detected in milk collected 24 hours after treatment, making the cheese production in most cases impossible. After the seven-day period, only spiramycin was detected in goat milk (79.6±19.2 µg/kg) although no antibiotic residues were found in the cheeses. A withdrawal time of seven days seems suitable to guarantee milk safety after the administration of erythromycin and tylosin without any negative effects neither on the milk nor on the and cheese properties. However, given the rapid elimination of these substances, a shorter withdrawal period might be considered. For spiramycin, persisting in milk for a longer period, further studies on its pharmacokinetics in dairy goats would be recommendable to avoid a potential risk to consumer health.

Key-words: antibiotics, macrolides, goat milk, goat cheese.

1. Introduction

Antibiotic therapy plays an important role in dairy livestock health and consequently in milk production. In dairy goats, antibiotics are usually applied to treat mastitis and other infectious diseases. However, it should be noted that due to the low volume of business which milk production from small ruminants represents, in comparison to cow milk, there is evidently a limited availability of drugs registered for these species leading veterinarians to employ unregistered drugs. Although the exceptional use of such drugs is legally considered (European Parliament and the Council of the European Union, 2001; European Parliament and the Council of the European Union, 2004), the risk of drug residues in milk and dairy products might increase as the required elimination period is not always known. In this sense, studies carried out in dairy goats (Ferrini et al., 2010; Amer et al., 2012) showed that the minimum withdrawal period of seven days laid down in legislation for off-label treatments is not always sufficient to ensure the absence of drug residues in milk. It should be noted that the presence of antibiotic residues in milk may have negative implications for consumer health, causing transient disturbances in the intestinal flora and allergic reactions which can, in extreme cases, lead to anaphylaxis (Graham et al., 2014). Also, there is concern that the development of bioresistance may be caused by such residues (EFSA, 2016). Finally, the bacterial processes required for the elaboration of fermented products such as cheeses and yogurt may be inhibited by such residues (Berruga et al., 2008; Cabizza et al., 2017), an important aspect when considering that goat milk is primarily intended for cheese-making.

Respect at the use of veterinary drug Spain is the second country after United Kingdom, which has used the most antimicrobial agents for goats and sheep species. In addition, among the sales of antimicrobial agents for food-producing species, the macrolides constitute the fourth most important group of antimicrobials applied, behind the tetracyclines, penicillins and sulfonamides (EMA, 2017). Macrolides are antibacterial compounds usually applied in veterinary medicine showing *in vitro* activity against a wide range of pathogenic microorganisms including mycoplasma, Gram positive bacteria, and some Gram-negative bacteria like *Pasteurella* spp. (Clothier et al., 2012). The antibacterial activity of such drugs is based on the inhibition of bacterial protein synthesis by binding to bacterial 50S ribosomal subunits (Papich and Riviere, 2001). Macrolides may also have an immune-modulating effect on cell-mediated immunity (Cao et al., 2006).

In dairy goats, macrolides are usually employed in an off-label manner to treat respiratory conditions, and mastitis (Atef et al., 2009; Young et al., 2011), as well as contagious agalactia in endemic areas (Gómez-Martín et al., 2013).

Systemically administered macrolides are distributed through the udder tissues and milk, reaching concentrations higher than those measured in plasma (Al-Wabel, 2008; Avci and Elmas, 2014). Xenobiotics cross the blood-milk barrier by passive diffusion, thus, the basic nature of macrolides (pK_a values ranging 6-9) and their low degree of ionization (18-30 %) favor their trapping in the udder, as milk has a lower pH than blood (Ambros et al., 2007).

Studies have been performed to evaluate the pharmacokinetics of macrolides in tissues and plasma of some animal species including goats (Taha et al., 1999; Cárceles et al., 2005). However, very little information is available on residual patterns of macrolides in goat milk with excretion times ranging from a few hours to several days (Ambros et al., 2007; Amer et al., 2012). The aim of this work was: 1) to verify if the exceptional use of macrolide antibiotics in dairy goats leads to residues in milk and cheese, thus posing a risk for consumer health, and 2) to evaluate the effect of these treatments on cheese manufacturing and the characteristics of matured cheeses.

2. Materials and Methods

2.1. Experimental procedure

The study was carried out with the experimental herd of Murciano-Granadina goats of Institute of Animal Science and Technology at Universitat Politècnica de València (UPV, Valencia, Spain). Animal management protocols were approved by the Ethics Committee of UPV.

For each antibiotic treatment, 24 healthy goats were used, each weighing 45-55 kg, randomly allocated in two groups (2x12), being in mid-lactation and not having received any veterinary drug prior to the experiment. Machine-milking was carried out once a day in the morning (08:00 a.m.).

Three macrolide antibiotics (erythromycin, tylosin and spiramycin) registered for the use in cattle and pigs, were selected for this study. All the treatments were administrated after morning milking by the intramuscular route. The veterinary drugs used were: Pantoyet® (Laboratorios Syva, S.A. León, Spain), 200 mg/mL of erythromycin, dose: 0.5 mL/10 kg body weight on three consecutive days; Trelacón® (Laboratorios Elanco Valquímica S.A., Madrid, Spain), 200 mg/mL of tylosin, dose:

0.5 mL/10 kg body weight on three consecutive days; and Mycogal® (Laboratorios Ovejero, S.A. León, Spain), 276.3 mg (1.05 MUI)/mL of spiramycin, dose: 1 mL/10 kg body weight in a single dose.

The withdrawal period considered was seven days after the last drug administration, as stipulated by European legislation for the exceptional use of antibiotics, except for Mycogal® (spiramycin) for which two withdrawal times (seven and 14 days) were considered as the manufacturer's specification sheet indicates a withdrawal period (11 days) for dairy cows. During the experimental period, bulk milk samples (50 mL) were taken on a daily basis to detect the presence of drug residues.

Different cheese making trials of ripened cheese were made for each experimental animal group: one day before the antibiotic treatment was applied (pre-treatment cheeses: PT-cheeses, which were then used as reference), 24 hours thereafter (after treatment cheeses: AT-cheeses), and after the safety period of seven days, in the case of spiramycin additional cheese-making after withdrawal of 14 days (after withdrawal period cheeses: AW-cheeses). Therefore, it supposes a total of six cheese-making for each one of the substances tested except for spiramycin, which were eight manufactures. In all cases, bulk milk samples (100 mL) were analysed prior to the cheese production.

Immediately after the milking had taken place, the cheese was made at the UPV pilot plant, following the artisanal making-process for mature Tronchón cheese. A vat of raw bulk milk was inoculated with mesophilic starter cultures (Choozit MA4001, Danisco, Paris, France), and heated to 32±1ºC. Then, calcium chloride (Proquical, Proquiga, A Coruña, Spain) and calf rennet (Suministros Arroyo, Santander, Spain) were added at 0.013 % (v/v) and 0.07 % (v/v), respectively. After the coagulation (30-40 min), the curd was cut and scalded (33-35ºC) whilst being stirred for 90-100 min. The curds were moulded, pressed for 3.5 h, and salted by immersion in brine (23% w/v). The cheeses ripened for a 60-day period and the cheese sample analysis was carried out at the beginning and the end of the ripening period, using one piece of cheese from each of the cheese-making and ripening times considered.

2.2. Milk Analysis

Milk samples were analysed by MilkoScan FT6000 (Foss, Hiller∅d, Denmark) to determine the chemical composition (fat, protein and total solids); somatic cell count (SCC) and total bacterial count (TBC) were obtained using Fossomatic 5000

(Foss) and Bactoscan FC (Foss), respectively. The milk pH value was measured by a conventional pH-meter (model Basic 20, Crison, Barcelona, Spain).

2.3. Cheese Analysis

The kinetic acidification of the milk curd was checked periodically during cheese-making using a pH-meter (model Basic 20, Crison, Barcelona, Spain) with a penetration probe (model 5232, Crison, Barcelona, Spain).

Cheese samples were characterized twice whilst ripening (0 and 60 days) by assessing quality variables such as pH, water activity (a_w), free fatty acids (FFA) and free amino acids (FAA) contents.

The pH value of the cheese samples was measured in duplicate using a pH-meter with a penetration probe (model 5232, Crison, Barcelona, Spain). A dew point hygrometer (Aqualab 4TE, Pullman, Decagon Devices Inc., Washington, USA) was employed to determine the water activity (a_w) making two replicate analysis.

The FFA concentration (meq/100 g of fat) and the FFA content (mg of leucine/g of cheese) were determined in duplicate according to the methodologies described by Nuñez et al. (1986) and Folkertsma and Fox (1992), respectively.

Color and textural properties were made in triplicate using circular samples (20 mm in diameter and 10 mm in height) obtained from an intermediate area between the rind and the center of the cheese. The color coordinates CIE L*, a* and b* were obtained employing a spectrocolorimeter (model CM-3600D, Minolta, Tokyo, Japan) using observer 10º and illuminant D65. A Texture Profile Analysis (TPA) was carried out using TA.XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a cylinder probe of 45 mm diameter (P/45). The cheese sample was compressed to 50 % of its height in two sequential compression events (constant deformation rate of 1 mm/s) separated by a rest phase of 5 s.

2.4. Antibiotic residues analysis

Antibiotic residues in goat milk and cheeses were analysed at the Instituto Lactológico de Lekunberri (Pamplona, Spain) using a liquid chromatography tandem mass spectrometry (LC-MS/MS) technique. For chromatographic analysis, an Alliance 2695 high-performance liquid chromatograph with a diode-array detector from Waters (Waters Chromatography Division, Milford, MA, USA) was employed. Separation of compounds was accomplished using an XBridge™ C_{18} column (Waters Chromatography Division). Mass spectral analyses were performed on a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters

Chromatography Division). The calibration curves had previously been established for each macrolide considered; the quantification limit (LOQ) being equal to 10 µg/kg for tylosin and erythromycin, and 30 µg/kg for spiramycin. MassLynx 4.0 software (Waters) was used to calculate the macrolide concentrations in goat milk and cheeses.

2.5. Statistical analysis

Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. The Plains, Virginia, USA) was used for the statistical analysis. The milk quality variables were analyzed using a multifactorial ANOVA including the effects of the Milk sampling (PT: pre-treatment, AT: 24 hours after treatment, and AW: after the withdrawal period) and the animal group (1 to 2).

In cheeses, multifactorial ANOVA was applied to study the influence of the different factors considered according to the model:

$$Y_{ijkl} = \mu + C_i + R_j + G_k + (C_i \times R_j) + (C_i \times G_k) + (R_j \times G_k) + e_{ijkl}$$

Where: Y_{ijkl} = dependent variable; μ = mean; C_i = cheese-making (PT: pre-treatment, AT: 24 hours after treatment, and AW: after the withdrawal period); R_j = ripening time (0 or 60 days); G_k = animal group (1 or 2). $C_i \times R_j$ = Effect of interaction cheese-making and ripening time; $C_i \times G_k$ = effect of interaction cheese-making and animal group; $R_j \times G_k$= effect of interaction ripening time and animal group; e_{ijkl}= residual error.

In both analyses, multiple comparisons of the mean values were made using the LSD test (least significant difference) with a significance level of $\alpha = 0.05$.

3. Results and Discussion

The off-label use of erythromycin, tylosin, and spiramycin in dairy goats under conditions described in this study had no significant effect ($p > 0.05$) on the milk quality parameters such as gross composition, pH, SCC, and TBC. In all cases, similar characteristics were observed in bulk milk obtained before drug administration as well as in the milk collected 24 hours after treatment, and at the end of the withdrawal period considered for each antibiotic. The mean values for gross composition (g/100 g) of raw milk used for cheese manufacture were as follows: 14.40±0.40 for total solids, 5.30±0.29 for fat, and 3.74±0.18 for protein. The pH-value was 6.72±0.05, SCC 707,800 cells/mL, and TBC 21,900 cfu/mL. However, residues of erythromycin (234.9±52.7 µg/kg), tylosin (198.7±57.8 µg/kg) and spiramycin (1,539.8±469.4 µg/kg) were found in goat milk 24 hours after the last

drug administration. In all cases, the residues decreased markedly along time becoming undetectable in milk 3-5 days after completing antibiotic therapy, except for spiramycin, whose residues were quantified in milk until the eighth day of the withdrawal period (Figure 1).

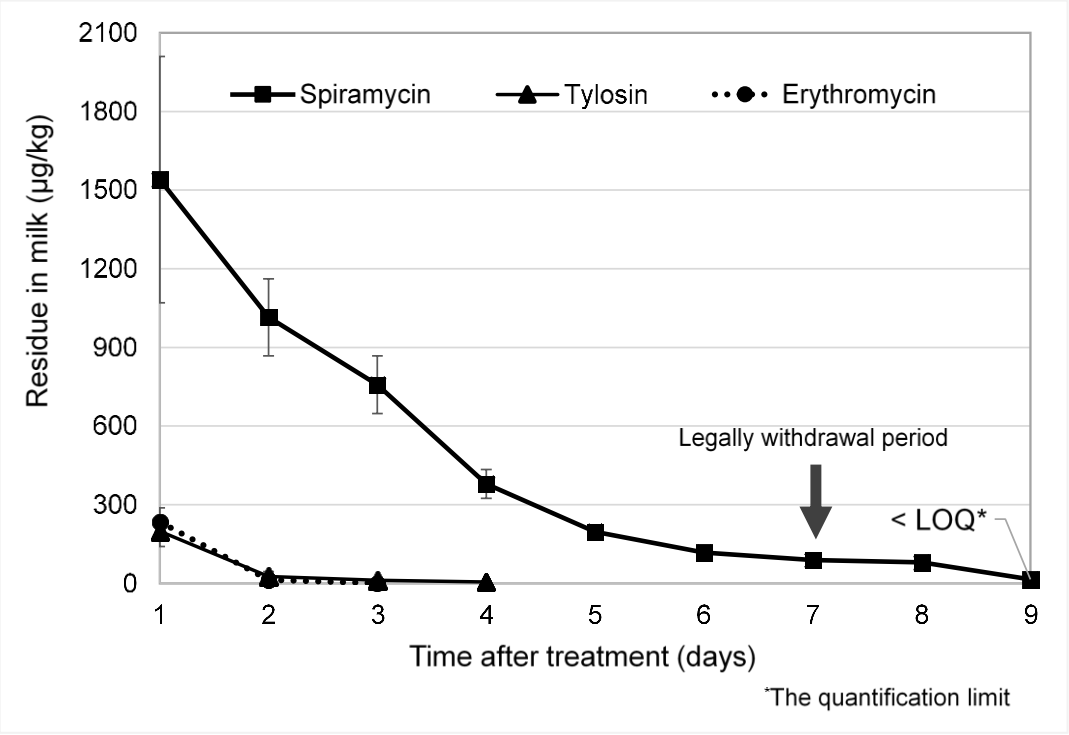

Figure 1. Concentration (µg/kg) of macrolides in goat milk at different time after antibiotics treatment.

Regarding cheeses, the residual amounts of erythromycin and spiramycin in bulk milk collected on the first day post-treatment inhibited the starter-culture activities, thus impeding the acidification process necessary for the cheeses to reach their final pH of 5.3 required for maturation. Therefore, the manufacturing of AT-cheeses due to these substances was not feasible.

However, cheese-making remained unaffected ($p > 0.05$) by the presence of tylosin above the safety limits in milk from treated goats (198.7±57.8 µg/kg), although residual amounts of this substance were detected in cheeses along the entire ripening period. Thus, the tylosin concentration in AT-cheeses at the beginning of maturation was 178.9±3.3 µg/kg which decreased significantly along time reaching a final concentration of 86.8±4.7 µg/kg at 60 days of ripening.

Table 1. Average values of parameters analysed in cheeses made at difference time after erythromycin treatment and ANOVA F-ratio for each factor: Cheese-making (C), ripening time (R) and animal group (G).

Parameters	Cheese-making (C)[1]			Ripening time-days (R)			Animal Group (G)			ANOVA (f-ratio)		
	PT-cheeses	AW-cheeses	SE	0	60	SE	1	2	SE	C	R	G
Physico-chemical												
pH	5.29	5.33	0.02	5.30	5.32	0.02	5.32	5.30	0.02	1.86^{ns}	0.67^{ns}	0.67^{ns}
a_w	0.962	0.964	0.00	0.971	0.955	0.00	0.962	0.964	0.00	37.41^{ns}	958.12*	11.69^{ns}
FFA	2.97	3.16	0.18	1.39	4.74	0.18	3.09	3.05	0.18	0.56^{ns}	167.76*	0.02^{ns}
FAA	2.74	2.55	0.05	0.70	4.59	0.05	2.74	2.56	0.05	8.47^{ns}	3705.53*	7.90^{ns}
Color												
L*	89.29	87.37	0.36	89.80	86.86	0.36	87.82	88.85	0.36	14.22^{ns}	33.05^{ns}	4.01^{ns}
a*	-1.11	-0.83	0.08	-0.25	-1.69	0.08	-0.96	-0.98	0.08	6.87^{ns}	174.44*	0.05^{ns}
b*	11.1	10.5	0.09	10.6	11.07	0.09	11.04	10.64	0.09	21.20^{ns}	12.56^{ns}	9.42^{ns}
Texture												
Hardness (N)	25.36	33.88	0.63	25.29	33.95	0.63	29.45	29.79	0.63	91.64^{ns}	94.48^{ns}	0.15^{ns}
Adhesiveness (N.s)	-1.16	-1.16	0.07	-0.64	-1.68	0.07	1.19	-1.13	0.07	0.00^{ns}	101.54^{ns}	0.30^{ns}
Springiness	0.66	0.65	0.02	0.82	0.49	0.02	0.66	0.65	0.02	0.07^{ns}	147.44^{ns}	0.02^{ns}
Cohesiveness	0.49	0.49	0.02	0.72	0.27	0.02	0.48	0.50	0.02	0.01^{ns}	445.27*	0.67^{ns}
Chewiness (N)	9.25	10.33	0.43	14.98	4.60	0.43	9.17	10.41	0.43	3.13^{ns}	285.07*	4.04^{ns}

[1] The manufacture of 24 h after treatment cheeses (AT-cheeses) was not possible; PT-cheeses: Pre-treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error; a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/g of cheese); *$p < 0.05$ indicate significant difference; ns: non-significant.

Table 2. Average values of parameters analysed in cheeses made at difference time after tylosin treatment and ANOVA F-ratio for each factor: Cheese-making (C), ripening time (R) and animal group (G).

Parameters	Cheese-making (C)				Ripening time-days (R)			Animal Group (G)			ANOVA (f-ratio)		
	PT-cheeses	AT-cheeses	AW-cheeses	SE	0	60	SE	1	2	SE	C	R	G
Physico-chemical													
pH	5.32	5.34	5.36	0.03	5.43	5.25	0.02	5.32	5.36	0.02	0.61^{ns}	30.19*	0.91^{ns}
a_w	0.962	0.963	0.963	0.00	0.969	0.957	0.00	0.962	0.964	0.00	0.61^{ns}	225.37**	7.13^{ns}
FFA	2.18	2.06	2.75	0.10	1.92	2.75	0.08	2.68	1.99	0.08	14.46^{ns}	54.85*	38.02*
FAA	2.14^{b}	1.89^{a}	2.21^{b}	0.01	0.59	3.57	0.01	2.11	2.04	0.01	179.02**	41509.12***	22.56*
Color													
L*	89.84	89.71	89.26	0.14	90.36	88.85	0.11	89.37	89.83	0.11	4.85^{ns}	92.28*	8.28^{ns}
a*	-0.88	-0.92	-0.87	0.04	-0.25	-1.53	0.03	-0.86	-0.92	0.03	0.57^{ns}	960.87**	2.11^{ns}
b*	10.41	10.49	10.79	0.06	10.00	11.12	0.05	10.67	10.47	0.05	12.57^{ns}	289.13**	11.86^{ns}
Texture													
Hardness (N)	26.33^{a}	38.65^{b}	31.44^{ab}	1.20	21.78	42.47	0.98	30.18	34.09	0.98	26.49*	222.00**	7.95^{ns}
Adhesiveness (N.s)	-1.40	-1.77	-1.73	0.08	-0.73	-2.53	0.07	-1.49	-1.77	0.07	5.86^{ns}	341.12**	8.35^{ns}
Springiness	0.62	0.62	0.62	0.02	0.83	0.40	0.01	0.62	0.61	0.01	0.04^{ns}	430.88**	0.18^{ns}
Cohesiveness	0.44	0.48	0.47	0.01	0.69	0.24	0.01	0.45	0.48	0.01	2.60^{ns}	1528.25***	5.96^{ns}
Chewiness (N)	6.35	10.91	7.85	0.89	12.57	4.17	0.73	7.98	8.76	0.73	6.74^{ns}	66.24*	0.56^{ns}

PT-cheeses: Pre-treatment cheeses; AT-cheeses: After treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error; a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/g of cheese); $^{a, b:}$ Different letters in the same row indicate significant differences ($p < 0.05$); ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns: non-significant.

Table 3. Average values of parameters analysed in cheeses made at difference time after spyramicin treatment and ANOVA F-ratio for each factor: Cheese-making (C), ripening time (R) and animal group (G).

Parameters	Cheese-making (C)[1]				Ripening time-days (R)			Animal Group (G)			ANOVA (f-ratio)		
	PT-cheeses	AW-cheeses (7 days)	AW-cheeses (14 days)	SE	0	60	SE	1	2	SE	C	R	G
Physico-chemical													
pH	5.40	5.38	5.43	0.03	5.48	5.32	0.02	5.42	5.39	0.02	0.96^{ns}	29.47*	1.41^{ns}
a_w	0.960	0.961	0.963	0.00	0.967	0.956	0.00	0.962	0.961	0.00	4.43^{ns}	134.62**	1.83^{ns}
FFA	2.54	2.27	2.31	0.08	1.40	3.35	0.06	2.28	2.47	0.06	3.58^{ns}	492.45**	4.78^{ns}
FAA	3.69	3.91	4.09	0.08	1.15	6.64	0.06	3.87	3.92	0.06	6.20^{ns}	3604.30***	0.34^{ns}
Color													
L*	88.79	89.47	87.22	0.55	89.92	87.07	0.45	88.22	88.77	0.45	4.37^{ns}	19.90*	0.74^{ns}
a*	-0.95	-0.88	-0.94	0.02	-0.19	-1.66	0.02	-0.95	-0.90	0.02	3.35^{ns}	3854.48***	3.37^{ns}
b*	10.87	10.34	11.82	0.19	10.44	11.58	0.15	10.98	11.04	0.15	15.66^{ns}	27.81*	0.06^{ns}
Texture													
Hardness (N)	33.20	27.69	28.23	2.95	22.49	36.92	2.41	30.77	28.64	2.41	1.06^{ns}	17.95^{ns}	0.39^{ns}
Adhesiveness (N.s)	-1.71	-2.01	-1.20	0.11	-0.97	-2.31	0.09	-1.79	-1.49	0.09	13.60^{ns}	108.45**	5.16^{ns}
Springiness	0.60	0.62	0.64	0.03	0.81	0.43	0.02	0.60	0.64	0.02	0.49^{ns}	138.68**	1.49^{ns}
Cohesiveness	0.47	0.46	0.49	0.01	0.70	0.25	0.01	0.47	0.48	0.01	1.28^{ns}	1013.63**	0.30^{ns}
Chewiness (N)	8.13	7.76	9.24	0.88	12.76	3.99	0.72	8.23	8.52	0.72	0.77^{ns}	74.63*	0.08^{ns}

[1] The manufacture of 24 h after treatment cheeses (AT-cheeses) was not possible; PT-cheeses: Pre-treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error; a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/g of cheese); Significant differences ($p < 0.05$); ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns: non-significant.

On the other hand, the cheese-making from goat milk obtained after the legally established minimum withdrawal period, seven days, remained unaffected by the antibiotic treatment applied ($p > 0.05$), even for goat milk containing residual amounts of spiramycin (79.6±19.2 µg/kg) which was not detected in cheeses, regardless of the ripening time considered.

The characteristics of ripened Tronchón cheeses produced in this study after the off-label use of erythromycin, tylosin and spiramycin are presented in Tables 1, 2, and 3, respectively.

As shown in Table 1, the cheeses made from bulk milk obtained seven days after the last administration of erythromycin showed similar characteristics to cheeses produced before the antibiotic treatment ($p > 0.05$). These results were observed in the two experimental goats group performed ($p > 0.05$) with the cheese properties being only affected by the maturation period ($p < 0.001$) leading to lower cohesiveness, chewiness and water activity values as well as higher concentrations of FFA and FAA at 60 days of maturation. None of the interactions considered in the statistical model affected the quality variables studied ($p > 0.05$).

Results for the antibiotic treatment with tylosin (Table 2) indicate that cheeses made from milk after the last drug administration (AT-cheeses) showed higher hardness values ($p < 0.05$) and a lower FAA concentration ($p < 0.01$) than the other two types of cheese. As shown in Figure 2, statistical differences in the FAA concentration were only detected at the end of the 60-day ripening period (interaction $C_i \times R_j$, $p < 0.05$) evidencing a lower proteolytic activity in the AT-cheeses during maturation. On the other hand, both lower lipolytic and proteolytic activities were also observed in cheeses made in the second experimental replicate ($p < 0.05$) although the related interactions ($C_i \times G_k$ and $R_j \times G_k$) were non-significant.

Regarding spiramycin, it should be noted that the characteristics of cheeses made prior to drug administration (PT-cheeses) did not differ significantly ($p > 0.05$) from those of the cheeses manufactured after the antibiotic treatment (Table 3). It has not had a significant effect on the characteristics of the cheese among the different cheese elaborations. Nevertheless, the ripening time was the only factor able to significantly affect most cheese properties evaluated, evolving similarly during this period as in cheeses previously described for the other macrolides studied. None of the interactions between factors analyzed were significant.

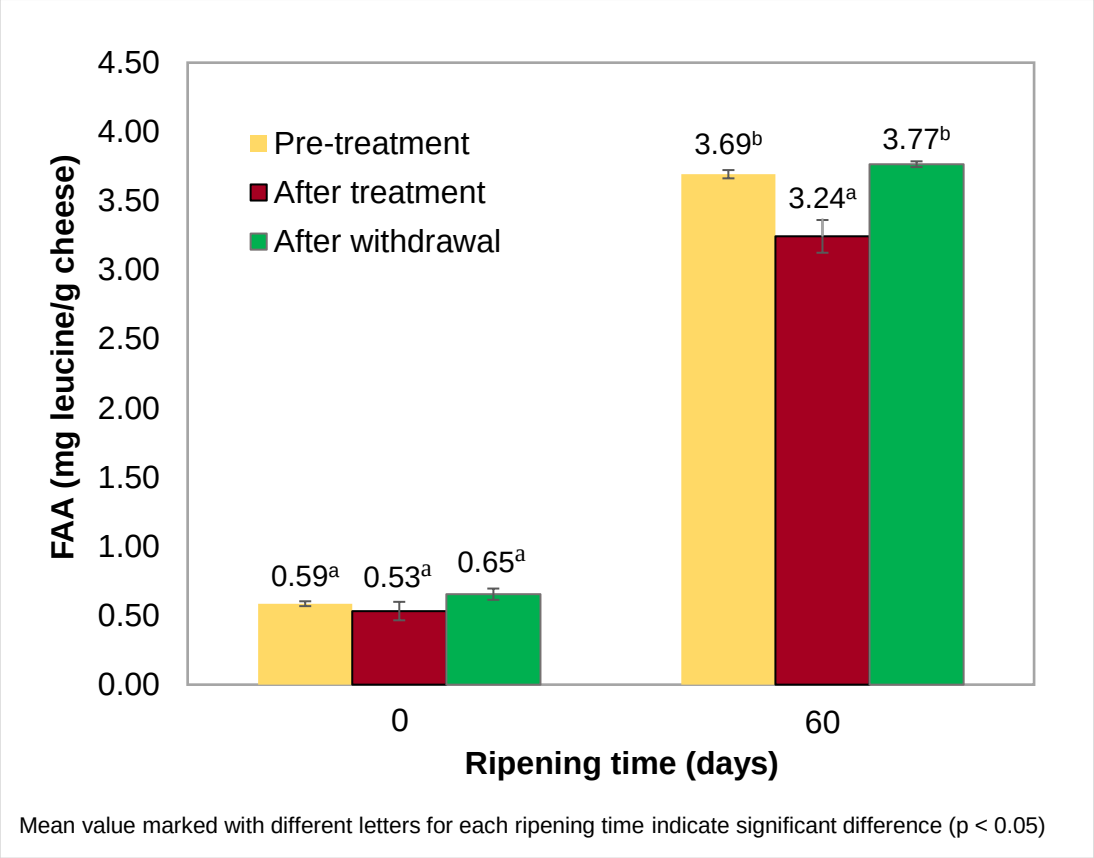

Figure 2. Free Amino-Acids (FAA) concentration in cheese made at different time after tylosin treatment during ripening.

Results herein suggest that the off-label use of macrolides in dairy goats did not significantly affect the bulk milk quality characteristics; the mean values being similar to those reported by other authors in milk from Murciano-Granadina bread goats (Blasco et al., 2016). However, this veterinary practice produces high concentrations of erythromycin, tylosin, and spiramycin, widely exceeding their respective safety levels (40, 50, and 200 µg/kg) in bulk milk obtained on the first day post-treatment making it unsuitable for human consumption, whether fresh or turned into dairy products such as cheese. In fact, the erythromycin (234.9±52.7 µg/kg), and spiramycin residues (1,539.8±469.4 µg/kg) present in goat milk 24 hours after treatment rendered the production of ripening cheese infeasible due to the complete inhibition of the starter cultures activity.

Cabizza et al. (2017) observed a delay of 60 minutes in the completion of the acidification process of ripened cheeses from sheep milk spiked with 100 µg/kg of oxytetracycline, in comparison to the cheese made from antibiotic-free sheep milk, due to the inhibitory effect of this substance. It should be noted that an increase in the acidification time to reach the final pH in the cheese poses a risk to consumer health as high pH values could facilitate the growth of pathogenic or undesirable

microorganisms (Fox and McSweeney, 2017). In our study, the inhibition of the starter bacteria by the erythromycin and the spiramycin residues was so pronounced that the pH values of the curd remained at 6.4-6.5 along the entire production process, impeding the maturation of the cheeses.

However, the presence of high concentrations of tylosin in goat milk collected 24 hours after drug administration (198.7±57.8 µg/kg) did not affect the cheese-making processes. Nevertheless, tylosin residues were detected in AT-cheeses along the entire ripening period which could be related to the lower proteolytic activity in such cheeses containing lower FAA at the end of this period. Results herein suggest that 48.5 % of the antibiotic retained in the soft cheeses remain in the final product, the rest being degraded during maturation. The lower stability of this substance in acidic conditions (Papich and Riviere, 2001) could be related to the antibiotic losses in cheeses which presented a pH final ranging from 5.1 to 5.3. In any case, macrolides show a low protein binding ability due to their low degree of ionization. Thus, considering a mean cheese yield value for mature cheese like Tronchón of 12.5 kg of cheese/100 kg of milk, the antibiotic retained in the cheese could represent 5-6 % of the drug initially present in the milk supply. It should be noted that the information related to the presence of macrolide residues in cheeses is practically non-existent.

After the legally established withdrawal period, seven days, erythromycin and tylosin residues were not detected in bulk milk from treated goats. Hence, considering that after 48 h the residues of these substances in bulk milk are lower than the MRLs (European Commission, 2010) prescribed, the shortening of the legal withdrawal period could be considered. However, spiramycin residues can be found in milk until the eighth day of this period although being below the MRL established for this substance (200 µg/kg). These results are in agreement with those observed by other authors when studying the pharmacokinetics of macrolide antibiotics in dairy goats. Thus, while erythromycin and tylosin, given their lipophilic nature, are rapidly eliminated from the animal's organism by excretion in milk during the first hours after their systemic administration (Ambros et al., 2007; Atef et al., 2009), spiramycin requires a longer elimination period.

The lower absorption rate of spiramycin could be related to its higher pK_a value, possibly a result of the high degree of ionization in acidic conditions making the excretion in milk slower (DrugBank, 2018), Therefore, in spite of the fact that even after seven days, the antibiotic is detected below the MRL, further pharmacokinetic studies on spiramycin are recommended to establish its adequate withdrawal period to avoid negative implications on the consumer health.

On the other hand, the absence or lower level of macrolide residues in bulk milk from goats used for cheese-making after the withdrawal period could explain the similarity of the mature Tronchón cheeses obtained with those made before initiation of the veterinary treatments. It could also explain the fact that the cheese-making processes did not differ significantly. No antibiotic residues were detected in cheeses made from milk contaminated with spyramicin obtained after a seven-day withdrawal period, evidencing the previously commented low retention capability of this substance in cheese.

4. Conclusions

The off-label use of macrolides in dairy goats can result in drug residues in the milk supply if appropriate measures are not taken. The legally established minimum withdrawal period of seven days seems suitable to guarantee milk safety after the intramuscular administration of erythromycin and tylosin, without negative effects neither on the raw goat milk properties nor on the quality of the ripened cheese obtained. However, given the rapid elimination of these substances a shorter withdrawal period would be recommendable. Spiramycin residues can be detected in goat milk after the minimum safety period, thus making further studies on the behavior of this substance in dairy goats necessary to establish a more convenient withdrawal period, which also guarantees the quality of the dairy products as well as consumer safety.

5. Acknowledgments

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Study 4. *Food safety margin assessment of antibiotics in pasteurized goat milk and fresh cheese*

Food safety margin assessment of antibiotics in pasteurized goat milk and fresh cheese

Abstract

Traces of antimicrobials in milk are of great concern for public health. The European Union established the Maximum Residue Limits (MRLs) for antibiotics in milk, which, however, by themselves do not guarantee the absence of drug residues in milk and related products. Currently, very little information is available on the transfer of antibiotic residues from milk to other dairy products and their potential effect on consumer health. The objective of this work was to evaluate the presence of antibiotic residues in pasteurized fluid milk and fresh cheeses from goat milk containing the veterinary drugs at legal safety levels (MRL) and, also to assess the safety margin of these dairy products for consumers. Eight antibiotics (amoxicillin, benzylpenicillin, cloxacillin, neomycin, erythromycin, ciprofloxacin, enrofloxacin and oxytetracycline) were selected and three batches of fresh cheese were made from pasteurized goat milk spiked with each of these drugs. Drug residues in milk and cheese samples were analyzed by LC-MS/MS. The safety margin of goat milk products was calculated taking into account different age groups (children, teenagers and adults) and the negative effects of such antibiotics on consumer health. Results showed that most antibiotics present in raw milk remained in pasteurized milk and were transferred to cheese to a high extent, with retention being above 50% in most cases. The minimum safety margin in pasteurized milk was obtained for enrofloxacin, ciprofloxacin and erythromycin in the group of children. Regarding fresh cheese, an elevated safety margin was obtained for all antibiotics and age groups considered, which seems to indicate that the presence of antibiotics in, this product is not likely to cause toxicological effects on consumer health. However, the large amounts of antibiotics retained in the cheese might contribute to the development and spread of antimicrobial resistance. Considering the differences in milk from different species and the great variety of cheeses, it would be advisable to continue the study of traceability of antibiotics in order to increase the safety margin of dairy products.

Key-words: antibiotics, goat milk, fresh cheese, safety margin.

1. Introduction

The consumption of milk and dairy products from different livestock species is recommended because of their positive contribution to dietary intake of protein, calcium, and other nutrients (USDA, 2010). However, the nutritional benefits of milk and dairy products can be jeopardized by the presence of veterinary drug residues in milk from treated animals. These substances may endanger public health as allergic reactions might ensue as well as the dysfunction of human intestinal microbiota or given the potential health risk due to the development of antimicrobial resistance (Jeong et al., 2009; WHO/FAO, 2016). In recent years, the use of antibiotics at sub-therapeutic dose as well as the presence of residues of antibiotics in foodstuff of animal origin and other agriculture products has gained much importance related to the selection of antibiotic resistant bacteria, which has become a great public health concern. Thus, WHO/FAO (2017) regularly publishes a list of Critically Important Antimicrobials for Human Medicine (WHO CIA list) intended to assist in managing antimicrobial resistance, ensuring that antimicrobials are used prudently both in human and veterinary medicine.

On the other hand, antibiotic residues in raw milk might be present in dairy products as they remain stable in spite of heat treatments and other processes applied by the dairy industry. Thus, some authors reported the presence of drug residues in milk after pasteurization and UHT treatments (Zhang et al., 2014) as well as in yogurts and soft cheeses made from contaminated milk (Adetunji, 2011).

In order to warrant public health, regulatory agencies and international organizations have limited the presence of veterinary residues in milk and other foodstuffs of animal origin. Thus, in the European Union, Maximum Residue Limits (MRLs) for such substances have been established by Regulation (EU) No 37/2010 (European Commission, 2010), based on the type and amount of residues considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI), that uses an additional safety factor to provide an adequate safety margin for the consumer. However, it should be noted that, despite the undeniable protection afforded by the establishment of these safety levels in raw milk for most dairy products, it could be insufficient in the case of related products such as cheese, whose process of elaboration may lead to a concentration of the main milk components (fat and protein) that could also affect the antibiotic residues potentially present in milk. However, very little information is available on the transfer of antibiotic residues from milk to other dairy products and their potential effect on consumer health. Recent studies have evaluated the traceability of antibiotics during

the cheese making process, indicating that substances such as aminoglycosides, quinolones and tetracyclines have a high susceptibility of being retained in the curd (Giraldo et al., 2017), capable of reaching higher concentrations in the cheese than in the milk used for cheese production (Cabizza et al., 2017; Gajda et al., 2018), posing a risk for consumer health. However, safety levels for drug residues in dairy products have not been established yet.

The last decades have seen the introduction of risk assessment as a discipline able to provide a systematic means for assessing, in a qualitative or quantitative way, the probability of occurrence and the severity of known or potentially adverse health effects in a given population based on hazard identification, hazard characterization, exposure assessment and risk characterization.

Concerning hazard characterization, in particular for non-genotoxic hazards, two metrics, i.e. hazard quotation (HQ) and the hazard index (HI) have been introduced to deal with predicting and managing the exposure of humans to multiple chemicals and their associated toxicological effects (Zheng, et al., 2007; Evans, et al. 2015; Yu et al., 2016). However, these metrics do not take into account the effect of variability and the uncertainties of the many factors affecting their quantification. To address this drawback, a new metric, the Food Safety Margin (FSM) was formulated based on the fundamentals behind HQ and HI definitions but also accounting for the effect of variability and uncertainty (Doménech and Martorell, 2016; 2017). Thus, FSM allows assessing whether the margin between exposure to an estimated daily intake (EDI) and the safety threshold as the acceptable daily intake (ADI) for the food chemical of concern is sufficient or not and to estimate the increase or decrease of the margin after any change in food chain conditions.

On the other hand, goat milk production has increased considerably in regions such as Europe, North and South America or Oceania reaching nowadays 18.3 million tones yearly (FAOSTAT, 2018). The consumers' growing interest in goat milk, mainly related to its nutritional and digestive properties, has led to the current availability of a wide range of quality dairy products such as pasteurized fluid milk, yogurts, and especially different types of cheese (Haenlein, 2004). However, in many cases, this increment in production goes together with the intensification of farming which implies an increased use of veterinary drugs, especially antibiotics which could cause milk contamination if not properly applied.

The objective of this work was to evaluate the presence of antibiotic residues in pasteurized fluid milk and fresh cheese made from goat milk containing these

veterinary drugs at safety levels (MRL). Likewise, the safety margin for consumers will be assessed in different age groups by making a balance between the exposure to the antibiotic present in both products and the ADI established for each of the antibiotics.

2. Materials and Methods

2.1. Experimental procedure

Antibiotic-free milk from goats was spiked with a veterinary drug at MRL equivalent to the antibiotic concentration. Immediately after spiking, raw milk was pasteurized and analyzed to determine the residual amounts of antibiotics after heat treatment. Pasteurized goat milk was used to produce fresh cheeses which were also analyzed to evaluate the transfer of antibiotics from milk to cheese.

The experiment was performed in triplicate on different days making a total of three batches of pasteurized milk and fresh cheeses per each antibiotic considered.

Antibiotic-free bulk milk was obtained from the experimental herd of Murciano-Granadina goats of Universitat Politècnica de València (UPV, Valencia, Spain). Animals had a good health status and had not received any veterinary drugs, neither before nor along the experimental period.

Eight antibiotics provided by Sigma-Aldrich Química, S.A. (Madrid, Spain) were used in this study (commercial reference): amoxicillin (A8523), benzylpenicillin (PENNA), cloxacillin (C9393), neomycin (N1876) erythromycin (E6376), ciprofloxacin (17850), enrofloxacin (17849), and oxytetracycline (O4636). For use, a stock solution (100 mg/100 mL) was prepared on a daily basis and added to the milk according to IDF recommendations (ISO/IDF, 2003).

Cheeses were manufactured in the UPV pilot plant, following a traditional fresh cheese-making procedure. From each batch of cheese, 20-kg samples of raw antibiotic-free goat milk were used, these were spiked with antibiotics just before pasteurization (64±1ºC, 30 min) and cooled after heat treatment to 34±1ºC. Then, calcium chloride (Proquiga, A Coruña, Spain), and calf rennet (1:10,000 strength, Laboratorios Arroyo, Santander, Spain) were added (0.25 mL/L milk and 0.65 mL/L, respectively). When a firm curd was observed (30-40 min), the gel was gently cut into cubes (about 1 cm^3) and heated at 35-36ºC for 15-20 min whilst being stirred. Whey was drained off and the curd was placed and distributed in cylindrical molds (250 g). The cheeses were salted by rubbing salt on the surface (Organic marine salt, Intereco, Castellón, Spain) and refrigerated for 24 hours. Then, the cheeses were

weighed to calculate the cheese yield, expressed as kg of cheese obtained from 100 kg of milk, and sampled in duplicate for further analysis.

Raw goat milk used for the cheese production was analyzed at the Interprofessional Laboratory of the Valencian Community Region (LICOVAL, Valencia, Spain) to determine gross composition (MilkoScan FT6000, Foss, Hillerød, Denmark), somatic cell count (Fossomatic 5000, Foss), and total bacterial count (Bactoscan FC, Foss) The chemical composition of the fresh goat milk cheeses was also evaluated using a Food Scan Analyzer (Foss).

Drug residues in pasteurized goat milk and in the fresh cheese samples were determined by LC-MS/MS analysis at the Instituto lactológico de Lekunberri (Pamplona, Spain) to evaluate the transfer of antibiotics from milk to cheese.

2.2. Antibiotic residue quantification

Cheese samples (10±0.5 g) were placed in a stomacher bag with 20±0.01 g of trisodium citrate (20% w/w) and homogenized twice for 3 minutes at 40ºC. Then, the mixture was centrifuged for 10 min at 9,000 g. For milk, samples 10±0.5 g were directly centrifuged for 10 min at 9,000 g. In both cases, 2±0.05 g of the supernatant was extracted by solid-phase extraction (SPE) using an Oasis HLB cartridge (60 mg, 3mL, Waters Chromatography Division, Milford, MA, USA), previously conditioned with 1 mL of methanol and 1 mL of ultrapure water (generated in-house from a Milli-Q system, Millipore Corp., Billerica, MA). After the sample had passed through the cartridge, it was rinsed with 2 mL of ultrapure water, eluted with 2 mL of methanol and dried under vacuum. After evaporation, 500 µL of 0.1% formic acid were added, and homogenized in the ultrasonic bath for 5 min. Finally, the dissolved extracts were filtered into a chromatographic vial using a 0.45 µm polyvinylidene fluoride filter. 20 µL of this mixture were injected into the HPLC system.

The chromatography system consisted of an HPLC Alliance 2695 with a diode-array detector (Waters Chromatography Division) and a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters Chromatography Division). The MassLynx 4.0 software (Waters) was used for data processing. An XBridge^{TM} C_{18} column (100 x 34.6 x 2.1 mm, particle size of 3.5 µm) was used. Chromatographic separation was carried out with a mobile phase consisting of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 0.2 mL/min. The operating parameters for the mass spectrometer were as follows: voltage of the needle and lens was 3.0 kV and 0.2 V, respectively; source block temperature 140°C;

desolvation temperature 450 °C; nebulization gas (nitrogen) at a flow rate of 50 L/h. Analytes were detected using electrospray ionization in the positive ion mode.

For quantitation, the calibration curves had previously been established for each antibiotic considered. MassLynx 4.0 software (Waters) was used to calculate the antibiotic concentrations in goat milk and cheeses.

2.3. Food Safety Margin Formulation

The classical Food Safety Margins (c_FSM), proposed by Doménech and Martorell (2017) can be formulated for veterinary drug residues, *i*, in terms of the Euclidean distance between the estimated daily intake (EDI_i) and the acceptable daily intake (ADI_i), Eq. (I). This margin will always range between zero and one. A value of this metric close to one would indicate a wide safety margin, whereas a value close to zero would imply a narrow margin with a high possibility of adverse effects.

$$c\_FSM_i = \begin{cases} 0 & if\ \ HQ_i > 1 \\ 1 - HQ_i & if\ \ HQ_i \leq 1 \end{cases} \quad \text{(I)}$$

Where HQ is the hazard quotation and is calculated following Eq. (II). Where EDI_i (µg/kg bw/day) is the exposure to a chemical hazard, herein an antibiotic, *i*, and ADI_i (µg/kg bw/day) is the acceptable daily intake for the same antibiotic, *i*. Body weight (bw) refers to kg of person (children, teenagers or adults).

$$HQ_i = EDI_i / ADI_i \quad \text{(II)}$$

The ADIs considered (Table 1) were those published by WHO (2018), except for cloxacillin, which has no ADI established by this organization, using the data provided by the Australian Pesticide and Veterinary Medicines Authority (APVMA, 2017). The EDI is estimated following Eq. (III).

$$EDI_i = (H_i \times C)/bw \quad \text{(III)}$$

where H_i is the concentration of each antibiotic obtained in this study for pasteurized goat milk and fresh goat cheese (µg/kg), *i*; *C* represents the daily consumption of these both products (kg/person/day).

To complement the classical formulation, c_FSM (Hi) considering the effect of uncertainties for situations in which the EDI (Hi) exceeds the ADI, Doménech and Martorell (2017) proposed a second metric taking into account a probabilistic formulation of the food safety margin (p_FSM), Eq. (IV).

$$p\_FSM(H_i) = \int_0^{\mathrm{ADI}_i} EDI(H_i)dH = 1 - \int_{ADI_i}^{\infty} EDI(H_i)dH = 1 - EP(H_i) \quad \text{(IV)}$$

Where $EP(H_i)$ is the exceedance probability, which represents the probability that exposure to the antibiotic *i* exceeds the reference index or limit, herein the ADI established for each *i*.

Table 1. Admissible Daily Intake (ADI) of antibiotics used in Food Safety Margin (FSM) formulation.

Antibiotic	Evaluation year	ADI (µg/kg bw per day)	Toxicological study		Source
			Animal species	Effect	
Amoxicillin	2017	2	Human	Intestinal microbiota	JECFA 85 (WHO, 2018)
Penicillin G	1990	6*	Human	Hypersensitivity	TRS 799-JECFA 36/37 (WHO, 2018)
Cloxacillin	2001	200	Rats	Absence of adverse effects	Australian Government (APVMA, 2017)
Neomycin	2003	60	Guinea pig	Ototoxicity	TRS 918-JECFA 60/5 (WHO, 2018)
Erythromycin	2006	0.7	Human gut flora	Growth inhibition	TRS 939-JECFA 66/33 (WHO, 2018)
Enrofloxacin/ Ciprofloxacin	1998	2	Human gut flora	Growth inhibition	TRS 879-JECFA 48/31 (WHO, 2018)
Oxytetracycline	2002	30	Human	Resistance Enterobacteriaceae	TRS 911-JECFA 58/33 (WHO, 2018)

*Estimated maximum daily intake values at the MRLs (µg/person/day)

The data used to assess the FSM for the presence of antibiotics in pasteurized goat milk and fresh cheese is shown in Table 2. Goat milk consumption for children (3-9 years old), and adults (18-74 years old), was obtained fitting the data published by the Spanish administration (AECOSAN, 2016). To simulate the consumption of teenagers (10-18 years old), a distribution was obtained taking into account the mean consumption of milk (AECOSAN, 2016). For goat cheese consumption, the distribution for children and teenagers was also obtained from data published by the Spanish administration. For adults, the data distributions were calculated with a factor of correction (-8.6%), considering the difference in cheese consumption between teenagers and adults (AECOSAN, 2016). Finally, the body weight for children, teenagers and adults was obtained fitting the values published by López de Lara et al. (2010).

With respect to the simulation data, in order to include random uncertainty, all the input parameters, i.e. Hi, C, and *bw* have been associated with a type of probability density function (*pdf*) to account for the inherent variability of the input data. Then, a standard Monte Carlo method was used to propagate the variability from input parameters to the output. Overall, 10,000 iterations per simulation were run using Latin Hypercube sampling. The simulation procedure was built as a spreadsheet model in Microsoft Excel, with add on @Risk 5.7 software (Palisade, Middlesex, UK), yielding a *pdf* for EDI_i, for HQ_i and, consequently, for c_FSM_i estimated for each product and age group.

A sensitivity analysis was obtained using the @Risk (Palisade software), able to elucidate the dependency of the output, on the set of input parameters in Eqs. (II) to (III).

Table 2. Data used in Food Safety Margin (FSM) formulation for the presence of antibiotic in pasteurized goat milk and fresh cheese by age group (children, teenagers and adults).

Variable	Distribution type (values)	Source
Consumption goat milk-children (g/day)	Gamma *alt* (4*; 50%: 350; 95%: 600)	(AECOSAN, 2016)
Consumption goat milk-teenagers (g/day)	Normal (312±172)	(AECOSAN, 2016)
Consumption goat milk-adults (g/day)	Normal (400±247)	(AECOSAN, 2016)
Consumption goat cheese - children (g/kg bw day)	Normal (0.84±0.31)	(AECOSAN, 2016)
Consumption goat cheese-teenagers (g/kg bw day)	Normal (0.67±0.59)	(AECOSAN, 2016)
Consumption goat cheese-adults (g/kg bw day)	Normal (0.61±0.59)	(AECOSAN, 2016)
Body weight children (kg)	Gamma *alt* (10%: 18.2; 50%: 22; 98%: 32.2)	(López de Lara et al., 2010)
Body weight teenagers (kg)	Gamma *alt* (10%: 39.4; 50%: 48.2; 98%: 69.9)	(López de Lara et al., 2010)
Body weight adults (kg)	Gamma *alt* (10%: 55.4; 50%: 63.7; 98%: 90.87)	(López de Lara et al., 2010)

Gamma alt (percentile: value); Normal (mean±SD); *α= shape parameter.

2.4. Statistical analysis

Antibiotic, age group and milk product were analyzed by a multifactor analysis of variance (ANOVA) using the Statgraphics Centurion XVI.II software (Statpoint Technologies, Inc. Warrenton, Virginia, USA). A probability value of less than 5% was deemed to be significant.

3. Results and Discussion

3.1. Presence of antibiotics in pasteurized goat milk and fresh cheeses

The composition (g/100 g) of the raw goat milk presented a total solids content (mean±SD) of 14.27±0.66, fat of 5.21±0.45 and protein of 3.63±0.22. The somatic cell count and total bacterial count were 1,090±208x10^3 cells/mL (6.03±0.10 log) and 30±18x10^3 cfu/mL (4.42±0.22 log), respectively. The chemical composition of the fresh cheeses was: total solids content of 43.9±2.0, fat content 24.7±1.4, protein 15.0±1.2 and salt 1.46±0.26. In general, goat milk characteristics were similar to those reported by other authors in milk from different goat breeds (Beltrán et al., 2014; Raynal-Ljutovac et al., 2008). Likewise, the fresh cheeses produced presented a chemical composition between range values reported by other authors for unripened goat cheeses (Raynal-Ljutovac et al., 2008; Pirisi et al., 2011; Gámbaro et al., 2017).

The residual amounts of antibiotics in pasteurized goat milk and in the fresh cheeses are reported in Table 3. In general, the antibiotic concentration in raw milk was only slightly affected by the heat treatment, with erythromycin and neomycin being the least heat stable substances, showing moderate losses of 20.3 and 29.2%, respectively. Results herein are similar to those reported by other researchers when assessing the heat stability of several antibiotics in milk. According to Roca et al. (2010), enrofloxacin and ciprofloxacin are unaffected by the treatment of pasteurization showing antibiotic concentration losses in milk of 0.01%. For the penicillins (amoxicillin, benzylpenicillin, and cloxacillin) relatively low losses, ranging from 6.2 to 6.9%, were reported (Roca et al., 2011) in milk after pasteurization. Instead, Moats (1999), also indicated a moderate antibiotic concentration loss (23.6%) for oxytetracycline.

Consequently, most antibiotics initially added to raw goat milk (70.8-100%) remain in the heated milk and could, therefore, be transferred to cheese, potentially posing a risk to consumer health.

Table 3. Residual amounts of antibiotics in pasteurized milk and fresh cheese made from raw goat milk spiked at EU-MRL equivalent antibiotic concentration.

Antibiotic	Raw milk MRL[a] (µg/kg)	Pasteurized goat milk		Fresh goat cheese		
		H_m[b] (µg/kg)	Reduction[c] (%)	H_{ch}[d] (µg/kg)	CY[e]	Retention[f] (%)
β-lactams						
Amoxicillin	4	3.5±0.5	12.8±12.5	10.5±2.3	19.5±2.0	58.4±10.9
Benzylpenicillin	4	3.8±0.3	4.2±7.2	12.8±2.2	20.0±0.6	66.8±12.4
Cloxacillin	30	27±5.2	10.0±17.3	109.2±11.3	18.6±0.9	75.2±6.6
Aminoglycosides						
Neomycin	1500	1195±67.6	20.3±4.5	3916.7±495.7	19.1±1.3	62.2±4.5
Macrolide						
Erythromycin	40	28.3±3.4	29.2±8.5	98.38±5.9	19.0±0.7	64.6±3.9
Quinolones						
Ciprofloxacin	100*	100±0.0	0.0±0.0	285.8±12.8	20.1±1.2	57.3±4.5
Enrofloxacin	100*	96.6±5.8	3.4±5.8	250.9±41.5	19.7±0.5	51.1±8.8
Tetracycline						
Oxytetracycline	100	81.1±13.9	18.9±13.9	154.5±57.9	19.6±0.4	37.5±14.9

[a] MRL: Maximum Residue Limit-Commission Regulation (EU) No. 37/2010 (European Commission, 2010); [b] H_m: Antibiotic in milk; [c] Reduction: percent reduction of the antibiotic in goat milk after pasteurization treatment; [d]H_{ch}: Antibiotic in cheese; [e]CY: cheese yield, expressed as kg of cheese/100 kg of milk; [f] Retention: percentage of the antibiotic retained in fresh cheese made from pasteurized goat milk. $R = ((H_{ch} \times 100)/(H_m \times (100/CY))$; *Sum of enrofloxacin and ciprofloxacin (European Commission, 2010).

The retention of antibiotics in cheese depends fundamentally on the characteristics of solubility of these substances, and their ability to interact with the fat and/or protein fraction of this matrix (Hakk et al., 2016). The cheese-making procedure is an additional factor able to affect the transfer of antibiotics from milk to rennet curd. Thus, besides the heat treatment, technological aspects related to cheese-making such as the heating, drying off or ripening, significantly condition the residual amounts of antibiotics in the final product (Adetunji, 2011; Cabizza et al., 2017). Results herein indicate that, although a relatively large amount of antibiotics was transferred to the whey when draining off the curd, more than 50% of the antibiotics initially present in pasteurized goat milk were retained in the fresh cheese (Table 3), especially in the case of cloxacillin (75.2%), benzylpenicillin (66.8%), and erythromycin (64.6%). Although these antibiotics are highly water soluble *(*Rang et al., 2000*)*, the high whey content in this type of cheese (51.78-59.9%) is likely to favor their increased retention level.

3.2. Safety Margin of pasteurized goat milk and fresh cheese

Table 4 shows the classical formulation of FSM in goat pasteurized milk and cheese per antibiotic and age groups, obtained using Eq. (I).

Table 4. Classical formulation of Food Safety Margin (c_FSM) for pasteurized milk and fresh cheese made from raw goat milk spiked at EU-MRL equivalent antibiotic concentration according to consumer' age groups.

Antibiotic	Pasteurized goat milk			Fresh goat cheese		
	Adults	Teenagers	Children	Adults	Teenagers	Children
β-lactams						
Amoxicillin	0.988	0.988	0.971	0.999	0.995	0.995
Benzylpenicillin	0.996	0.996	0.989	0.999	0.998	0.998
Cloxacillin	0.999	0.999	0.998	0.999	0.999	0.999
Aminoglycosides						
Neomycin	0.868	0.865	0.666	0.992	0.946	0.944
Macrolide						
Erythromycin	0.737	0.731	0.353	0.984	0.885	0.881
Quinolones						
Ciprofloxacin	0.669	0.662	0.228	0.984	0.884	0.880
Enrofloxacin	0.684	0.678	0.253	0.986	0.900	0.897
Tetracycline						
Oxytetracycline	0.982	0.982	0.954	0.999	0.996	0.995

c_FSM: values between 0 and 1, indicating: 0 = low FSM; 1 = high FSM.

Results showed that children are the most sensitive group, followed by teenagers and adults. This result is coherent with the EDI formulation as exposure is inversely proportional to body weight. Statistical analysis of variance confirmed that antibiotic (p value: 0.0072), product (p value: 0.0003), and age group (p value: 0.0220), were significant. Therefore, focusing on the most affected age group, findings showed that the maximum margin in milk is obtained for β-lactams, with the maximum for cloxacillin, followed by benzylpenicillin, amoxicillin, and oxytetracycline. Neomycin showed an intermediate value (c_FSM = 0.6656). On the contrary, the minimum margin was obtained for macrolides (erythromycin) and quinolones (enrofloxacin and ciprofloxacin). It should be noted that the substances having the lowest safety margins (quinolones and macrolide) are considered "highest priority critically important antimicrobials" by WHO/FAO [Critically Important Antimicrobial for Human Medicine 5th revision (2017)]. This category includes groups of antibiotics that have a high relevance for disease treatments as there are only few therapeutic alternatives to these drugs for the treatment of specific human diseases.

Moreover, these substances are not detected at safety levels by the microbiological tests routinely applied for the screening of antibiotic residues in raw milk (Beltrán et al., 2015). Therefore, it is of utmost important to improve the analytical strategy to antibiotics control to prevent antibiotic residues from reaching the food chain.

In relation to the classical FSM for goat cheese, made from antibiotic spiked goat milk, the safety margin is close to one, for the most antibiotics in all age groups, indicanding that it is not likely to have any effects on consumer health. Slight differences in the safety margin can be observed between β-lactams and oxytetracycline (> 0.995) and other antibiotics like macrolides and quinolones (< 0.900) in the teenagers and children group.

Results obtained with the probabilistic formulation of the safety margin (p_FSM) show that the exceedance probability in pasteurized goat milk (Figure 1) was also detectable only in case of erythromycin, ciprofloxacin and enrofloxacin in the children consumer group. Regarding the exceedance probability in fresh cheese, it was zero in all cases.

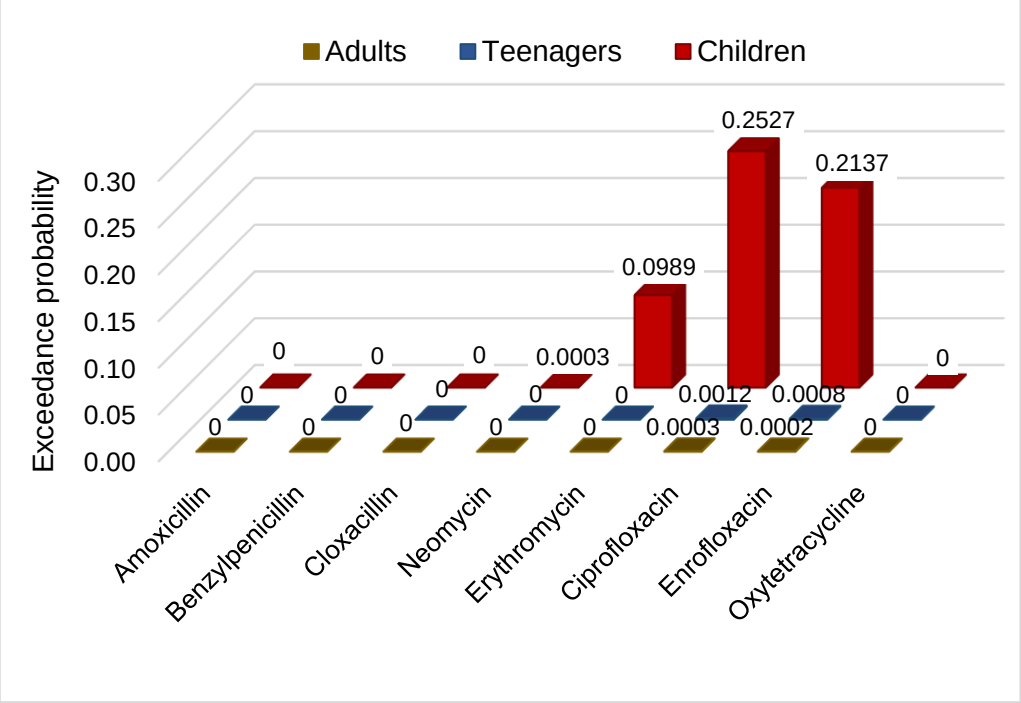

Figure 1. Probability to exceed the Food Safety Margin in pasteurized goat milk for each antibiotic and consumer age group.

A sensitivity analysis was performed to study the effects of the variation of input parameters on the final output of the FSM. The parameters with the highest relative effects are considered the most sensitive input parameters. The results show that the

most sensitive input in milk and cheese is consumption, followed by body weight and level of antibiotic contamination.

4. Conclusions

In summary, it can be concluded that pasteurization treatment affects the antibiotic residues present in goat milk only slightly, with between 70.8 and 100% of the initial concentration remaining in pasteurized milk. Furthermore, in fresh cheese, these substances might remain to a high extent, with retention above 50% in most cases. Of all the antibiotics tested, the quinolone group (enrofloxacin and its metabolite, ciprofloxacin) and macrolide drugs such as erythromycin are most likely to jeopardize consumer health as they have the lowest safety margins in milk. Moreover, for these antibiotics there is the probability that the ADI threshold in milk could be exceeded in the children consumer group, as they are much more sensitive. For fresh cheese, the lower consumption of this product compared to that of fluid milk has an increased safety margin for all the consumer groups considered. However, the large amounts of antibiotics in fresh cheese could be related to the emergence and spread of antibiotic resistance, a great concern for public health in recent years.

It is noteworthy that despite the physicochemical differences among milk from different species and also between different types of cheese, it is very likely that antibiotics would be transferred to the final products if present in raw milk, even at concentrations equal to or below the legally MRL established, posing a risk for consumer health. Therefore, it would be advisable to continue the study of the traceability of these substances in dairy products to increase the safety margin of dairy products.

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Chapter 4

General discussion

Caprine livestock is related to the economic level of different areas of the world, as goat products are a primary food source in low-income countries, but they are also present in high-income and technologically developed countries (Pulina et al., 2018). In the last decades, goat milk production has augmented considerably, as consumers have shown an increased interest in goat milk products related to their nutritional and digestive properties (Park et al, 2007). Goat milk is used to make special types of cheese, often from raw milk, and under protected designation of origin (PDO) and other recognized quality brands.

The use of antibiotics in goats to treat mastitis and other infectious diseases is a common veterinary practice that presents a high risk of contamination of the milk supply if appropriate measures are not taken. The consumption of milk or related products containing antibiotic residues can have harmful effects on human health, causing transient disturbances in the intestinal flora and allergic reactions (Dethlefsen et al., 2008; Macy, 2014). Also, the use of antibiotics at sub therapeutic dose has gained much importance in recent years related to antibiotic resistant bacteria, which has become a public health concern (Teuber, 2001; Martens and Demain, 2017).

To avoid potential risks related to drug residues in food, the control of the presence of antibiotics in milk and other products of animal origin is legally binding in many countries. In the European Union, the regulatory levels or Maximum Residue Limits (EU-MRL) for raw milk are established by Commission Regulation (EU) 37/2010 (European Commission, 2010).

The contamination of bulk milk at MRL is a remote scenario in developed countries, where control systems have been widely established. However, the screening systems routinely applied are based on the use of microbiological methods with adequate sensitivity for some groups of antibiotics such as β-lactams, but the detection of other antibiotic groups such as quinolones and tetracyclines is not up to the same standard, not being able to detect these substances at MRL concentration (Beltrán et al., 2015a, b), which implies that some antibiotics are able to reach the food chain even in amounts exceeding the legal limits. Moreover, the situation is different in many less industrialized countries, where several studies have reported antibiotic residues in milk at levels exceeding the safety limits in raw cow milk (Shitandi and Sternesjo, 2004; Abebew et al., 2014; Layada et al., 2016) even after the commercialization of heat-treated milk (Zhang et al., 2014). These residues have also been found in marketed dairy products such as cheese (Tona and Olusola, 2014), evidencing the lack of controls as well as the negligent use of antibiotics in farms.

In addition to the adverse implications on public health, the presence of antibiotic residues in milk may have negative technological effects on the activity of starters employed in the manufacture of fermented products such as cheeses (Katla et al. 2001; Berruga et al 2008a). Moreover, FAO/WHO (2004) suggested establishing MRLs of liposoluble antibiotics in milk products such as milkfat and cheese, being apprehensive that such substances might reach levels far above the initial contents in milk and, thus, possibly posing a risk for consumers. Antibiotics could be retained in milk curd to a greater or lesser extent, depending on the physicochemical properties of these substances and their ability to interact with the fat and protein fraction of the matrix (Shappell et al., 2017)

Studies on antibiotic retention during dairy manufacturing processes are crucial to prevent the negative implications related to the presence of such substances in milk products. It should be noted that related studies currently available are scarce and focus on the transfer of tetracyclines from contaminated milk to different dairy products (Cabizza et al., 2017, 2018; Gajda et al., 2018). Information about the possible retention of antibiotics belonging to other families such as β-lactams, macrolides or quinolones widely used in dairy livestock is practically unavailable. Therefore, the impact of the presence of antibiotics in raw milk on the safety of dairy products such as cheese is currently, unknown.

This thesis evaluates the effect of the most widely used antibiotics in dairy goats on the cheese-making process and the cheese characteristics as well as the transfer of these antibiotics from milk to fresh and mature cheese, assessing the potential risk for the consumer.

First, the characteristics of ripened Tronchón cheese from raw goat milk, containing an equivalent concentration of the European Union Maximum Residue Limit (EU-MRL) of antibiotics (amoxicillin, benzylpenicillin, cloxacillin, erythromycin, ciprofloxacin, enrofloxacin and oxytetracycline) were evaluated during 60 days maturation. Therefore, Tronchón cheese which is a traditional cheese made in the Maestrazgo region (northeast of Spain), was chosen as a model of the studies in matured cheese as it is representative of Spanish goat cheese (raw milk, enzymatic coagulation, uncooked, two months ripening, etc.).

The cheese-making process was unaffected by the presence of most antibiotics evaluated at MRL equivalent concentration. Only erythromycin and oxytetracycline significantly increased the time required to reach the final pH (5.30) necessary to start the maturation period of the Tronchón cheese. Results herein suggest that β-lactams

and quinolones at MRL equivalent concentration did not interfere in the cheese-making process.

Other authors studied the effect of β-lactams (Berruga et al., 2007) and quinolones (Beltrán et al., 2017) on the fermentation time for the manufacture of yogurt from sheep and goat milk, respectively. In both studies, they observed that yogurts made from milk with concentrations equal to or below than the MRL of amoxicillin, benzylpenicillin and enrofloxacin, did not present a delay in the fermentation of the yogurts. However, Berruga et al. (2008c) showed that residues of cephalosporin in milk used in yogurt production inhibited the development of starter cultures necessary in their biochemical processes.

In Manchego cheese made from raw sheep milk spiked with 5 different β-lactam (penicillin G, amoxicillin, ampicillin, ceftiofur and cephalexin) at 3 different concentrations (0.5 MRL, MRL and 1.5 MRL), Berruga et al. (2008a) showed a delay in the decrease of pH during manufacturing, only being significant when ceftiofur was present. Just as in this study, penicillines at a concentration equal to the MRL did not delay the acidification of the milk.

Cabizza et al. (2017, 2018) also observed delays in the acidification process during sheep cheese manufacture made from raw and thermized sheep milk with oxytetracycline at safety level (100 µg/kg), being 60 to 78 min longer than those found herein (108±25 min) in cheese-making of mature cheese made from goat milk also with oxytetracycline at MRL concentration. The different effects of antibiotics on the cheese-making process could be related to the distinct susceptibility of the starter microorganism cultures to antimicrobial substances. The starter culture used in Tronchón cheese-making contained *Lactococus lactis ssp. lactis, Lactococcus lactis ssp cremoris, Lactococcus lactis ssp lactis biovar diacetylactis* and *Streptococcus thermophilus.*

Katla et al. (2001) evaluated antimicrobial susceptibility of starter cultures used in Norwegian dairy products, observing that antibiotics at concentrations below their respective MRLs reduce the activity of the microorganisms such as lactobacilli or streptococci isolated from dairy products (yogurt, fermented milk, whey and cheese) and commercial starter cultures. A concentration of 16 µg/L for erythromycin was reported as able to reduce the activity of Streptococcus spp. by 50%.

The susceptibility of *Streptococcus thermophilus,* an important component of many starter cultures, to erythromycin, clindamycin, streptomycin, gentamicin, tetracycline and ampicillin was studied by Tosi et al. (2007), finding that *S.*

thermophilus was susceptible to tetracycline at concentrations somewhat higher (125 µg/L) than the MRL (100 µg/L) in milk.

The use of raw goat milk spiked with antibiotics at EU-MRL concentration did not affect the physicochemical characteristics of the cheeses, only a lower content of free fatty acid (FFA) in the cheeses with amoxicillin and cloxacillin suggesting a reduced biochemical activity in these cheeses. Regarding the color parameters evaluated in the cheeses were affected by the presence of some antibiotics in goat milk. Thus, a lower brightness (L*) value was obtained in the cheeses containing ciprofloxacin. The redness (coordinate a*) presented low values in the cheeses from milk spiked with benzylpenicillin, cloxacillin, and erythromycin when compared to control cheese. Similarly, the yellowness (coordinate b*) value was lower in the cheeses with oxytetracycline. However, differences found instrumentally could not be detected by the consumers, as the calculated ΔE value (Bodart et al., 2008) ranged from 0.88 to 2.02 for the different antibiotics considered. This is because only ΔE > 3 color differences could be detected by the human eye.

Neither, did most of the drugs used in this study affect the texture properties of the Tronchón cheeses showing similar values as those obtained for the cheeses used as reference, with only minor modifications being detected such as lower hardness and chewiness values in the cheeses from milk containing oxytetracycline, which also presented a higher cohesiveness value. These differences could be related to the interaction between this antibiotic and stable Ca^{2+} ion forming bonds leading to changes in textural properties, like hardness, of the cheeses (Everet and Auty, 2008).

All the variables evaluated in the experimental cheeses were affected by the ripening. In general, the pH and the concentration of the main cheese components showed a similar trend as that reported by other authors in different goat milk cheeses made in Spain (Delgado et al., 2011, 2012; Fresno and Álvarez, 2012; Salvador et al., 2014) with slight differences mainly related to the fat content of cheeses, possibly being related to other factors such as animal breed, lactation period, feeding as well as the specific cheese-making process applied in each type of cheese. On the whole, as ripening progressed the total solids, fat, protein and salt content of the cheeses increased, basically due to the loss of the water content along maturation.

It should be noted that proteolysis and lipolysis play a major role in the development of texture and flavor in most cheese varieties during ripening, directly contributing to flavor, via formation of peptides and FAA (Fenelon et al., 2000), as well as FFA from the lipolysis of triglycerides (Collins et al., 2003). FAA and FFA

concentrations in Tronchón cheese of the cheeses increased, as expected, throughout the ripening period, with FAA content in the experimental cheeses being in the order of those reported by other authors for cheeses of 60 days' maturation (Juan et al., 2016), and the FFA content showed a similar trend than the data presented by Buffa et al. (2001) in mature cheese made from raw goat milk.

Regarding the effect of ripening on the color properties of the cheeses, a significant reduction in the L* and in a* coordinate were observed herein, while the b* coordinate value increased along time, possibly related to proteolysis and browning reactions that occur during maturation. A similar trend in color parameters was reported by Buffa et al. (2001), Fresno and Álvarez (2012) and Salvador et al. (2014), who analyzed goat cheese under similar conditions.

Also, during ripening, the most cheese samples became significantly harder and more adhesive, while the springiness, cohesiveness and chewiness decreased significantly. In general, these changes are consistent with Delgado et al. (2011) and Salvador et al. (2014) although values vary according to the type of cheese. However, the texture of cheeses made from milk spiked with amoxicillin and cloxacillin and their references showed hardness evolved in an inverse way during maturation. These results could be related to the higher somatic cell count of goat milk used for this cheese production (1.6×10^6 cell/mL). Chen et al (2010) investigated the effect of the somatic cell count (SCC) in goat milk on yield, FFA and sensory quality of semisoft cheese. Total sensory scores as well as body and texture scores for cheeses made from high SCC milk (1,250,000 cell/mL) were lower than those for cheeses made from low and medium SCC milk (410,000 and 770,000 cell/mL, respectively). The difference in milk SCC levels also resulted in several changes in cheese texture at the beginning of the maturation (Chen et al., 2010). Also, Leitner et al. (2016) found that coagulation properties of goat milk with higher SCC resulted in a significant decrease in curd firmness.

One aspect of great interest, from a public health point of view, is the potential transfer of the different antibiotics from milk to matured cheese. In this thesis, a retention rate percentage of the antibiotic retained in the cheeses before the maturation (0-day) and the cheese yield obtained in each cheese-making trial was calculated. The retention rates for β-lactam antibiotics and erythromycin were much lower than those obtained for quinolones and oxytetracycline. The high water solubility of β-lactams and erythromycin (Giguère, 2013) could explain the lower retention rates obtained for those substances that are mostly transferred from the cheese to the whey during the draining-off. On the contrary, the high fat affinity of

quinolones and oxytetracycline (Giguère, 2013) favors their trapping in the cheese matrix, containing high concentrations of fat and protein with which oxytetracycline can also interact to form stable chelates (Lees and Toutain, 2012), likely to explain the high retention rate (68%) calculated.

In general, antibiotic residues present in the cheeses at the beginning of maturation decrease significantly along time. Thus, β-lactams and erythromycin residues are not detectable after 30 days of ripening. However, residual concentrations of oxytetracycline (20±5.7 µg/kg), relatively high concentrations of enrofloxacin (148±12 µg/kg) and of ciprofloxacin (253±24 µg/kg) residues were found in the cheeses after 60 days of maturation.

The oxytetracycline retention in cheese at the beginning of the ripening process (0-day) is similar to that shown by Cabizza et al. (2017). However, the evolution of the content of this antibiotic along maturation is different to that observed in the present study. The aforementioned authors observed a much lower degradation of the antibiotic at the end of maturation. This greater denaturation of the antibiotic could be related to the type of milk (sheep *vs* goat) and the difference in production-related factors (acidification, ripening time and conditions, surface mold growth, etc.).

Although the amounts for oxytetracycline in the present study are well below MRL admitted by the regulations (considering the MRL of milk, since dairy products have no MRL fixed), the consumer would be exposed to a certain residual quantity of the antibiotic. The sub-therapeutic dose of antibiotics has been related to antibiotic resistant bacteria, which has become a problem for public health (Teuber, 2001).

Regarding quinolones, Roca et al. (2010) indicate its high stability against the different heat treatments used in the dairy industry with a degradation percentage of quinolones in milk below 0.01% for pasteurization (72ºC-15 s) and maximum losses of concentration of 12.71% for ciprofloxacin at 120ºC-20 min. This elevated heat stability of quinolones in milk as well as the high persistence during ripening of cheese obtained here indicate that their presence in milk for cheese production could adversely affect the safety of the final products as a relatively high concentration of these substances could be retained in cheese. All this makes it necessary to study in more detail this family of antibiotics whose veterinary use is increasing, using different types of milk and technologies of manufacture of different dairy products, as well as to evaluate the potential risk of its presence for the consumer.

Also, another aspect to note is that current control systems for the detection of antibiotics in raw milk are based in the use of specific tests for β-lactam and

tetracycline antibiotics as well as microbiological methods for inhibitors with little sensitivity to quinolones, among other antibiotics, that may, consequently reach the food chain. For this reason, it would be desirable to improve the control screening system used for the detection of these molecules in milk (Beltrán et al., 2015a).

One of the most commonly broad-spectrum antibiotics used in dairy goats is oxytetracycline currently used to treat mastitis, urinary tract as well as enteric infections, among other diseases (Attaie et al., 2015). The sales of antimicrobial agents for food-producing species in Europe indicate that tetracyclines, are the most important group of antimicrobials applied, behind penicillins and sulfonamides, with the same trend present in Spain (EMA, 2017). In dairy goats, Berruga et al (2008b) reported that oxytetracycline is most commonly used in Spain for the treatment of different diseases.

Different concentrations of this antibiotic in goat milk (0, 0.5 MRL, MRL and 2 MRL) and characteristics of the cheese were evaluated (Study 2.1) to assess the impact of the presence of oxytetracycline in raw milk. The results showed that delays in the cheese-making process depend on the antibiotic concentration (21±7.5, 112±22.5 min and 212±36.8 min for 0.5, 1 and 2 MRL, respectively). Cabizza et al. (2018) reported in sheep milk cheese, made from milk spiked with concentrations of 50 and 100 µg/kg of oxytetracycline, a delay in the pH decrease (35 and 78 min, respectively), similar to results obtained. In the above-mentioned research, the delay in acidification of milk spiked with oxytetracycline provoked a dose-dependent influence of low levels of this antibiotic in milk on the starter culture development and metabolism affecting cheese fermentation.

On the other hand, the FFA concentration decreased significantly when the concentration of oxytetracycline increased. As in Study 1, characteristics of texture and color were hardly affected by the presence of oxytetracycline with small differences only in cheeses made from milk with 2 MRL. All of these minor differences remained undetected by the sensory panelists.

Cheese flavor is one of the most important organoleptic criteria for consumer acceptance, being the result of a complex balance between volatile and non-volatile chemical compounds. Biochemical processes such as glycolysis, lipolysis and proteolysis are the main pathways to produce impact-aromatic compounds like alcohols, aldehydes, carboxylic acids, esters, ketones, among others, during ripening (McSweeney and Sousa, 2000).

The presence of oxytetracycline in milk hardly modified the volatile profile of the cheese, which was affected by the ripening time only (Study 2.2). In general, the total amounts of organic acids, alcohols, and esters significantly increased during the first 45 days of maturation, whereas ketones, the group with the highest concentrations in the two first weeks of ripening, progressively decreased in this period, possibly due to a reduction of these compounds into secondary alcohols (Andiç et al., 2015). Minor changes were observed in the last two weeks of maturation, possibly related to the reduced microbial activity in the cheese and the lower water activity, which also limits the enzymatic activity in the cheeses (Beresford et al., 2001).

In 60-day ripened Tronchón cheeses, acetic, butanoic, and hexanoic acids were the most abundant volatile compounds in the cheeses, more than 50 %, being typical flavor components perceived as a goat-like smell (Castillo et al., 2007). High amounts of methyl ketones were also detected in the mature Tronchón cheese, as occurred in other Spanish goat cheeses such as Majorero (Castillo et al., 2007) and Ibores (Delgado et al., 2011). 2-pentanone linked to a smell described as orange peel and sweet, fruity (Curioni and Bosset, 2002) was the most important one along the entire maturation period. Large amounts of 2,3 butanedione (diacetyl) with an intensive creamy, buttery flavor (Le Bars and Yvon, 2008) were also detected.

Concerning oxytetracycline residues, this antibiotic was widely transferred from milk to cheese and variable amounts of oxytetracycline related with the initial concentration were detected in 60-day ripened cheese. Despite the level of oxytetracycline in ripened cheeses being lower than MRL permitted in milk, the consumer would be exposed to a certain residual amount of antibiotic, which could have relevant health implications, especially due to the development of antimicrobial resistance.

Another problem related to veterinary treatments is that, due to the low volume of business which milk production from small ruminants represents, in comparison to cow milk, there is evidently a limited availability of drugs registered for these species leading veterinarians to employ unregistered drugs. However, the exceptional use (off-label treatments) of such drugs is legally considered with a withdrawal period of at least seven days. In Spain, macrolide antibiotics are most commonly used in dairy goats for treatments of mastitis and contagious agalactia in endemic areas (Gómez-Martín et al., 2013) in an off-label manner. Hence, Study 3 was carried out with the aim to verify if the exceptional use of macrolide antibiotics in dairy goats generates residues in milk and cheeses within that period. Hence, three macrolide drugs (erythromycin, tylosin and spiramycin) were administered in an *in vivo* experiment in

dairy goats and ripened cheeses were made from bulk milk obtained before drug administration, 24 h after treatment, and at the end of the recommended withdrawal period.

Residual amounts of erythromycin (234.9±52.7 µg/kg), tylosin (198.7±57.8 µg/kg) and spiramycin (1539.8±469.4 µg/kg), widely exceeding their legal maximum residue limits (MRLs) established, were detected in milk collected 24 h after treatment. The inhibition of the starter bacteria by the erythromycin and the spiramycin residues was so pronounced that the pH values of the curd remained at 6.4-6.5 along the entire elaboration process, impeding the maturation of the cheeses. However, the presence of high concentrations of tylosin in goat milk collected 24 h after drug administration did not affect the cheese-making processes and the antibiotic retained in the cheese could represent 5-6 % of the drug initially present in the raw milk. After the seven-day period, only spiramycin was detected in goat milk (79.6±19.2 µg/kg) although no antibiotic residues were found in the cheeses.

A withdrawal time of seven days seems suitable to guarantee milk safety after the administration of erythromycin and tylosin without any negative effects neither on the milk nor on the cheese properties. However, given the rapid elimination of these substances, a shorter withdrawal period might be considered. Contrarily, spiramycin, persists for a longer period in milk. These results are in agreement with those observed by other authors when studying the pharmacokinetics of macrolide antibiotics in dairy goats. Thus, while erythromycin and tylosin, given their lipophilic nature, are rapidly eliminated from the animal's organism by excretion in milk during the first hours after their systemic administration (Ambros et al., 2007; Atef et al., 2009), spiramycin requires a longer elimination period. The lower absorption rate of spiramycin could be related to its higher pK_a value, possibly a result of the high degree of ionization in acidic conditions making the excretion in milk slower (DrugBank, 2018). Therefore, in spite of the fact that even after seven days, the antibiotic is detected below the MRL, further pharmacokinetic studies on spiramycin are recommended to establish its adequate withdrawal period to avoid negative implications on consumer health.

Finally, as information on the transfer of antibiotic residues from milk to other dairy products and their potential effect on consumer health is scarce, Study 4 was carried out with the objective to evaluate the presence of antibiotic residues in pasteurized fluid milk and fresh cheeses from goat milk containing veterinary drugs (amoxicillin, benzylpenicillin, cloxacillin, neomycin, erythromycin, ciprofloxacin,

enrofloxacin and oxytetracycline) at safety levels (MRL) and, also to assess the safety margin of these dairy products for consumers.

In general, the antibiotic concentration in raw milk was only slightly affected by the heat treatment, with erythromycin and neomycin being the least heat stable substances, showing moderate losses of 20.3 and 29.2%, respectively. Consequently, most antibiotics initially added to raw goat milk (70.8-100%) remain in the heated milk. Results herein are similar to those reported by other researchers when assessing the heat stability of several antibiotics in milk. According to Roca et al. (2010), enrofloxacin and ciprofloxacin are unaffected by the treatment of pasteurization showing antibiotic concentration losses in milk of 0.01%. For penicillins (amoxicillin, benzylpenicillin, and cloxacillin) relatively low losses, ranging from 6.2 to 6.9%, were reported (Roca et al., 2011) in milk after pasteurization. Instead, Moats (1999), also indicated a moderate antibiotic concentration loss (23.6%) for oxytetracycline.

The retention of antibiotics in cheese depends fundamentally on the characteristics of solubility of these substances. Also, the cheese-making procedure is an additional factor able to affect the transfer of antibiotics from milk to rennet curd. Results indicate that, although a relatively large amount of antibiotics was transferred to the whey when draining off the curd, more than 50% of the antibiotics initially present in pasteurized goat milk were retained in the fresh cheese especially in the case of cloxacillin (75.2%), benzylpenicillin (66.8%), and erythromycin (64.6%). Although these antibiotics are highly water soluble (Rang et al., 2000), the high whey content in this type of cheese (51.78-59.9%) is likely to favor their increased retention level. For the other antibiotics, the retention in fresh goat cheese was higher than 50% in most cases, except for oxytetracycline which presented the lowest retention (37.5 %).

Likewise, the safety margin (Doménech and Martorell, 2016, 2017) for consumers will be assessed by making a balance between the exposure to the antibiotic present in both products and the ADI established for each of the antibiotics taking into account their effect on health. The safety margin of goat milk products was calculated taking into account different age groups (children, teenagers and adults) and the negative effects of such antibiotics on consumer health. The minimum safety margin in pasteurized milk was obtained for enrofloxacin, ciprofloxacin and erythromycin in the group of children. Regarding fresh cheese, an elevated safety margin was obtained for all antibiotics and age groups considered. However, the large

amounts of antibiotics in fresh cheese could be related with the emergence and spread of antibiotic resistance, a great concern for public health in recent years.

However, considering the differences in milk from different species and the great variety of cheeses, it would be advisable to continue the study of the transfer of antibiotics including a wide range of antibiotics, different types of milk, and of technologies, in order to increase the safety margin of dairy products.

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Chapter 5

Conclusions

Antibiotic residues in raw milk for cheese production could be transferred to cheese at a high extent and reach the consumer, with the cheese making process and the physico-chemical characteristics of the antibiotics having a relevant effect on the residual amounts of these substances in the cheeses.

Thus, in the manufacture process of fresh (unripened) cheese made from goat milk containing antibiotics at EU-MRL, more than 50% of the most of these substances were transferred from milk to the cheese. However, in the production of ripened cheese, the transfer of antibiotics was highly variable, with retention rates lower than 15% for β-lactams and erythromycin, while large amounts of quinolones and oxytetracycline reaching concentrations 4 times higher than the initial drug concentration in raw milk, were detected in cheese at the beginning of maturation. In general, antibiotic concentrations decrease significantly during ripening with β-lactams and erythromycin not detected after 30 days of maturation, although large amounts of highly stable substances such as quinolones remain in the cheeses ripened for a 60-day period.

On the other hand, most antibiotics in raw goat milk at EU-MRL equivalent antibiotic concentration did not affect the cheese-making process and only slightly modified the organoleptic characteristics of the ripened Tronchón cheese, although differences remained mostly undetected by the consumers.

Considering the importance of the presence of antibiotics in foodstuff for public health, it should be noted that the use of goat milk containing antibiotics at legally admissible concentrations could lead to the obtention of cheeses with high amounts of these substances. Although the consumption of this type of cheese with the highest retention rates for all antibiotics showed an elevated safety margin, the intake of low amounts of antibiotics in foodstuffs is the cause of the emergence and spread of antimicrobial resistance, one of the most pressing public health issues nowadays. However, it would be advisable to continue the study of the transfer of antibiotics from milk to related products including a wide range of antibiotics, different types of milk, and of technologies in order to guarantee the food safety of milk and dairy products.

Dissemination of Results and Predoctoral Stay

Dissemination of Results

- Preer-reviewed scientific papers

Quintanilla P., Beltrán M.C., Molina A., Escriche M.I., Molina M.P. 2019. Characteristics of ripened Tronchón cheese from raw goat's milk containing legally admissible amounts of antibiotics. J. Dairy Sci. 102: 2941-2953.

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Quintanilla P., Hettinga K., Beltrán M.C., Escriche I., Molina M.P. 2019. Short Communication: Volatile profile of matured Tronchón cheese affected by oxytetracycline in raw goat's milk. Under review by J. Dairy Sci.

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- International Congress

Quintanilla P., Beltrán M.C., Molina A., Escriche I., Molina M.P. 2018. β-lactam antibiotics in goat's milk affecting the characteristics of mature cheeses. Annual Meeting - American Dairy Science Association (ADSA). Dairy Sci. Vol. 101, Suppl. 2, pp. 44. June 22-24. Knoxville, Tennessee, USA.

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Morai-pirlog A., Beltrán M.C., **Quintanilla P.,** Giraldo J., Escriche M.I., Molina M.P. 2015. Efecto den la presencia de ciprofloxacina en la elaboración y características del yogur de cabra" XL Congreso Nacional y XVI Congreso Internacional de la Sociedad Española de Ovinotecnia y Caprinotecnia (SEOC 2015). Pages 363-369 and oral presentation. September 16-18. Castellón, Spain.

Quintanilla P., Beltrán, M.C., Romero, T., Escriche, M.I., Molina, M.P. 2015. Presencia de quinolonas en queso fresco de cabra. XL Congreso Nacional y XVI Congreso Internacional de la Sociedad Española de Ovinotecnia y Caprinotecnia (SEOC 2015). Pages 356-362 and oral presentation. September 16-18. Castellón, Spain.

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List of publications related with this thesis

- Preer-reviewed scientific papers

Quintanilla P., Beltrán M.C., Peris B., Escriche I., Molina, M.P. 2019. Effect of the Presence of Neomycin in Goat's milk on the making process and characteristics of tronchón cheese. Accepted to Options Mediterranéennes.

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- International Congress

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Predoctoral Stay

Food Quality & Design group. Wageningen University & Research (The Netherlands). From February to July 2017 under the supervisión of Dr Kasper A. Hettinga. Desing and study of the Impact of oxytetracycline on flavour development in goat cheese. Founded by Research and Development Support Program, 'Ayudas para movilidad dentro del Programa para la Formación de Personal Investigador' (2.016) of Universitat Politècnica de València (Spain).