UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Ph.D. Thesis

Transfer of antibiotics from goat's milk to cheese and whey

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Supervisors:

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Transferencia de antibióticos de leche de cabra a queso y suero

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Departamento de Ciencia Animal
Instituto de Ciencia y Tecnología Animal
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Mª CARMEN BELTRÁN MARTÍNEZ, TÉCNICO SUPERIOR DE LABORATORIO DEL DEPARTAMENTO DE CIENCIA ANIMAL DE LA UNIVERSITAT POLITÈCNICA DE VALÈNCIA
Y
Mª PILAR MOLINA PONS, CATEDRÁTICA DE UNIVERSIDAD DEL DEPARTAMENTO DE CIENCIA ANIMAL DE LA UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INFORMAN:

Que la Tesis Doctoral titulada “Transfer of antibiotics from goat’s milk to cheese and whey” ha sido realizada por Dña. Jennifer Giraldo Gómez 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 por lo que autorizan su presentación.

Y para que así conste firman el presente informe en Valencia, a veinte de julio de 2020.

Dra. Mª Carmen Beltrán Martínez

Dra. Mª Pilar Molina Pons

Todo lo valioso lleva su tiempo,
no hay éxitos de un día a otro.
Jack Canfield

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Summary

The presence of antibiotic residues in milk and dairy products poses a risk for consumer health, as these substances could lead to toxicological effects and potentially cause antimicrobial resistance. To guarantee safety, Maximum Residue Limits (MRLs) for veterinary drugs have been established in raw milk by European legislation. However, safety levels for dairy products have not been fixed in most countries, and consumers might be exposed to significant amounts of such substances. Furthermore, the transfer of antibiotics from milk to cheese and whey fractions during cheese-making has been scarcely studied and, therefore, the impact of the use of whey containing antibiotics for the manufacture of foodstuffs for human consumption or to feed animals is, thus far, unknown.

The aim of this thesis was to evaluate the transfer of antibiotics from milk to cheese and whey fractions, as well as the validation of the performance of several methods to screen antibiotics in whey samples. To this end, different experiments were carried out grouped into two studies.

In the first study, the specific aim was to validate a multiresidue UHPLC-HRMS method using the Orbitrap Exactive™ analyser for the quantitative screening of antibiotics in fresh cheese and whey samples. The validation process was carried out according to the criteria specified in Commission Decision 2002/657/EC, using samples from two dairy matrices (fresh cheese and whey) from cows, sheep and goats spiked with 36 antimicrobial substances belonging to different antibiotic families. After validation, the UHPLC-HRMS method was applied to assess the partitioning of the antimicrobial substances during a cheese-making procedure at laboratory scale, using goat's milk spiked with five concentrations of antibiotics ranging from 0.25 to 4 times MRL.

The partitioning study indicated that most antibiotics were mainly transferred from milk to whey fraction (up to 85.9%) during cheese-making. Thus, retention rates in the rennet curd fraction were lower than 50%, except for ceftiofur (59.7%) and dicloxacillin (52.8%), and very variable between drugs. In most cases, drug distribution was unaffected by the antibiotic concentration present in milk for cheese production, and was poorly related to the drug lipophilicity, suggesting that factors other than the solubility characteristics of such substances should be considered to explain the transfer of antibiotics from milk to cheese and whey.

In the second study, the performance of different methods for screening antibiotics in whey samples was evaluated in accordance with Commission Decision

2002/657/EC, by conducting three experiments focused on commercially available screening tests, microtiter plate bioassays, and a semi-quantitative multiplate system, respectively.

Specificity (false-positive rate) and Detection Capability (CCβ) of a microbial inhibitor test (Eclipse Farm coupled to e-Reader device) and receptor-binding assays (3Aminosensor, Quinosensor, Twinsensor, and Tylosensor) were evaluated in whey samples from goats, having in general, similar results than those obtained when they are applied for milk analysis. The commercially available tests for screening antibiotics in milk, both microbial inhibitor test and receptor-binding assays, were suitable for the detection of drug residues in whey samples having pH values ranging from 6.5 to 7.0, although slight modifications in the test procedure were made in certain cases to improve test performance. Thus, a pre-diffusion of whey samples in the test tube before incubation was necessarily included for the suitable reading of the test results using Eclipse Farm coupled to e-Reader device.

Three microtiter plate bioassays with dichotomous response containing *Bacillus subtilis*, *Geobacillus thermocatenulatus* and *Geobacillus thermoleovorans*, respectively, were evaluated to be simultaneously applied with commercially available tests using *Geobacillus stearothermophilus var. calidolactis*, trying to particularly improve the detection of non β-lactam substances in whey samples. High specificity values (98-100%) were obtained when whey samples were heat treated (85ºC, 10 min) prior to analysis. *Bacillus subtilis*, having lower CCβ values for macrolides and quinolones, was the most interesting option to improve the detection profile of the Eclipse 100 test used as representative of commercial tests usually applied in milk quality control laboratories.

Regarding the multiplate system *Screening Test for Antibiotic Residues* (STAR) using five different microorganisms with complementary sensitivities (*Geobacillus stearothermophilus* for β-lactams and sulfonamides, *Bacillus subtilis* for aminoglycosides, *Kocuria varians* for macrolides, *Escherichia coli* for quinolones and *Bacillus cereus* for tetracyclines), high specificity values (≥ 98%) were obtained in most cases. The CCβ values obtained using the STAR protocol in whey samples exceed the MRL established in milk for most of the substances considered. However, this method could become an adequate tool in post-screening complementing the preliminary identification of the antibiotic residues present in whey, and hence, reduce the number of samples destined for the quantitative analysis by LC-MS/MS, which is a more complex and expensive method.

Results herein reinforce the necessity to control the presence of antibiotic residues in raw milk to prevent them from reaching to the dairy industry and, therefore, consumers. The production of cheese using milk containing antibiotics generates drug residues in the cheese and, in particular, in whey. Although in most countries, safety limits for products derived from milk, like whey, have not been set up, the suitable performance of screening methods for the detection of veterinary drug residues in this cheese-making by-product will allow the establishment of an adequate control strategy to prevent the risk of the presence of antibiotic residues in whey, with negative effects on public and animal health, and the environment.

Resumen

La presencia de residuos de antibióticos en la leche y los productos derivados representa un riesgo para la salud del consumidor, ya que estas sustancias podrían dar lugar a efectos toxicológicos y ser la causa potencial de la aparición de resistencias antimicrobianas. Para garantizar la seguridad alimentaria, la legislación europea ha establecido Límites Máximos de Residuos (LMRs) para los medicamentos veterinarios en la leche cruda. Sin embargo, en la mayoría de los países no se han fijado niveles de seguridad para los productos lácteos y los consumidores podrían estar expuestos a cantidades significativas de esas sustancias. Además, la transferencia de antibióticos de la leche a la cuajada y al lactosuero durante el proceso de elaboración del queso apenas ha sido estudiada y, por tanto, hasta el momento se desconoce el impacto de la utilización de lactosuero con residuos de antibióticos en la fabricación de diferentes alimentos destinados al consumo humano o en su empleo en la alimentación animal.

El objetivo de esta tesis fue evaluar la transferencia de antibióticos de la leche a las fracciones queso y lactosuero, así como la validación de las características de varios métodos de cribado para la detección de antibióticos en muestras de lactosuero. Para ello, se llevaron a cabo diferentes experimentos agrupados en dos estudios.

En el primer estudio, el objetivo específico fue validar un método UHPLC-HRMS multi-residuo utilizando el analizador Orbitrap Exactive™, para el cribado cuantitativo de antibióticos en muestras de queso fresco y lactosuero. El proceso de validación se llevó a cabo de acuerdo con los criterios especificados en la Decisión 657/2002/CE de la Comisión, utilizando muestras de las dos matrices lácteas procedentes de vacas, ovejas y cabras, fortificadas con antibióticos pertenecientes a diferentes familias de antimicrobianos. Después de la validación, se empleó el método UHPLC-HRMS para evaluar la distribución de 36 antibióticos, entre la cuajada y el lactosuero, durante un procedimiento de elaboración de queso a escala de laboratorio, utilizando leche de cabra fortificada con cinco concentraciones de antibióticos, comprendidas entre 0,25 y 4 veces el LMR.

El estudio de distribución indicó que la mayor parte de antibióticos se transfirieron principalmente de la leche a la fracción lactosuero (hasta el 85,9%) durante la elaboración de queso. Por tanto, los porcentajes de retención de antibióticos en la cuajada fueron inferiores al 50%, excepto en el caso del ceftiofur (59,7%) y la dicloxacilina (52,8%), y muy variables entre los distintos antimicrobianos. En la mayor parte de los casos, la distribución de medicamentos no se vio afectada por la concentración de antibióticos presente en la leche para la producción de queso, y

estuvo escasamente relacionada con la lipofilicidad de los antibióticos, lo que sugiere que se deben considerar factores distintos a las características de solubilidad de estas sustancias para explicar su transferencia en estas matrices lácteas.

En el segundo estudio, se evaluaron las características de diferentes métodos de detección de antibióticos en muestras de lactosuero de acuerdo con la Decisión 657/2002/CE de la Comisión, mediante la realización de tres experimentos centrados en la validación de métodos de cribado comercializados para la leche, en el desarrollo de bioensayos en placas microtiter con respuesta dicotómica y en la aplicación de un sistema multiplaca semicuantitativo, respectivamente.

La especificidad (porcentaje de falsos positivos) y la capacidad de detección (CCβ) de un método de detección de inhibidores (Eclipse Farm provisto del dispositivo e-Reader) y de métodos de unión a receptores (3Aminosensor, Quinosensor, Twinsensor y Tylosensor) se evaluaron en muestras de lactosuero de leche de cabras, obteniendo, en general, resultados similares a los obtenidos cuando se aplican para el análisis de la leche. Los métodos comerciales para el cribado de antibióticos en la leche, tanto el método de inhibición microbiana como los test de unión a receptores, fueron adecuados para la detección de residuos de antibióticos en muestras de lactosuero con valores de pH comprendidos entre 6,5 y 7,0, aunque en algún caso se realizaron ligeras modificaciones en el procedimiento de ensayo con objeto de mejorar el rendimiento del método. Así, se incluyó necesariamente una predifusión de las muestras de lactosuero en los viales del método antes de la incubación para obtener una lectura más adecuada de los resultados del análisis con Eclipse Farm provisto del dispositivo e-Reader.

Se evaluaron tres bioensayos en placa microtiter y respuesta dicotómica, que contenían *Bacillus subtilis*, *Geobacillus thermocatenulatus* y *Geobacillus thermoleovorans*, respectivamente, para ser aplicados simultáneamente con los métodos comercialmente disponibles basados en la utilización de *Geobacillus stearothermophilus var. calidolactis*, con objeto de tratar de mejorar especialmente la detección de antibióticos no betalactámicos en muestras de suero. Elevados valores de especificidad (98-100%) se obtuvieron cuando las muestras de lactosuero fueron tratadas térmicamente (85ºC, 10 min) antes de realizar el análisis. *Bacillus subtilis*, con menores valores de CCβ para macrólidos y quinolonas, fue la opción más interesante para mejorar el perfil de detección del Eclipse 100 utilizado como representante de los métodos comerciales que generalmente se utilizan en los laboratorios de control de calidad de la leche.

En cuanto al sistema multiplaca *Screening Test for Antibiotic Residues* (STAR) que utiliza cinco microorganismos diferentes con sensibilidades complementarias (*Geobacillus stearothermophilus* para betalactámicos y sulfonamidas, *Bacillus subtilis* para aminoglucósidos, *Kocuria varians* para macrólidos, *Escherichia coli* para quinolonas y *Bacillus cereus* para tetraciclinas) aplicado al lactosuero, presentó una elevada especificidad (≥ 98%) en la mayor parte de casos. Los valores de CCβ obtenidos con el protocolo STAR en muestras de lactosuero superan el LMR establecido en la leche para la mayor parte de las sustancias consideradas. Sin embargo, este método podría representar una adecuada herramienta en la etapa de post-cribado para la identificación preliminar de los residuos de antibióticos presentes en el lactosuero y, por tanto, reducir el número de muestras destinadas al análisis cuantitativo por LC-MS/MS, que es un método mucho más complejo y costoso.

Los resultados obtenidos reafirman la necesidad de controlar la presencia de residuos de antibióticos en la leche cruda para evitar su llegada a la industria láctea y, por tanto, a los consumidores. La producción de queso a partir de leche con antibióticos genera residuos en el queso y, especialmente, en el lactosuero. Aunque en la mayor parte de países no se han establecidos límites de seguridad para los productos derivados de la leche como el lactosuero, las adecuadas características de los métodos de cribado ensayados para la detección de residuos de antibióticos en este importante subproducto de la elaboración del queso permitiría el establecimiento de una estrategia de control para evitar la presencia de antibióticos en el lactosuero y sus posibles efectos negativos sobre la salud pública, la sanidad animal y el medio ambiente.

Resum

La presència de residus d'antibiòtics en la llet i els productes derivats representa un risc per a la salut del consumidor ja que aquestes substàncies podrien donar lloc a efectes toxicològics i causar potencialment resistències als antimicrobians. Per a garantir la seua seguretat, la legislació europea ha establit Límits Màxims de Residus (LMRs) per als medicaments veterinaris en la llet crua. No obstant això, no s'han fixat nivells de seguretat per als productes lactis en la majoria dels països i els consumidors podrien estar exposats a quantitats significatives d'aquestes substàncies. A més, la transferència d'antibiòtics de la llet al formatge i al sèrum durant el procés d'elaboració del formatge quasi no s'ha estudiat i, per tant, fins al moment es desconeix l'impacte de l'ús de sèrum de llet amb antibiòtics per a la fabricació d'aliments destinats al consum humà o per a l'alimentació animal.

L'objectiu d'aquesta tesi va ser avaluar la transferència d'antibiòtics de la llet a les fraccions formatge i sèrum, així com la validació de la resposta de diversos mètodes per a la detecció d'antibiòtics en mostres de sèrum de llet. Per a això, es van dur a terme diferents experiments agrupats en dos estudis.

En el primer estudi, l'objectiu específic va ser validar un mètode UHPLC-HRMS multi-residu utilitzant l'analitzador Orbitrap Exactive^{TM}, per al garbellat quantitatiu d'antibiòtics en mostres de formatge fresc i sèrum. El procés de validació es va dur a terme d'acord amb els criteris especificats en la Decisió 657/2002/CE de la Comissió, utilitzant mostres de les dos matrius làcties procedents de vaques, ovelles i cabres, fortificades amb antibiòtics pertanyents a diferents famílies d'antimicrobians. Després de la validació, es va emprar el mètode UHPLC-HRMS per a avaluar la partició de 36 antibiòtics durant un procediment d'elaboració de formatge a escala de laboratori, utilitzant llet de cabra fortificada amb cinc concentracions d'aquestes substàncies, compreses entre 0,25 i 4 vegades el LMR.

L'estudi de partició va indicar que la major part d'antibiòtics es van transferir principalment de la llet a la fracció sèrum de llet (fins al 85,9%) durant l'elaboració de formatge. Per tant, els percentatges de retenció d'antibiòtics en la quallada van ser inferiors al 50%, excepte en el cas del ceftiofur (59,7%) i la dicloxacilina (52,8%), i molt variables entre els diferents fàrmacs. En la majoria dels casos, la distribució de medicaments no es va veure afectada per la concentració d'antibiòtics present en la llet per a la producció de formatge, i va estar escassament relacionada amb la lipofilicitat dels antibiòtics, el que suggereix que s'han de considerar factors diferents

de les característiques de solubilitat d'aquestes substàncies per a explicar la transferència d'antibiòtics en aquestes matrius làcties.

En el segon estudi, es van avaluar les característiques de diferents mètodes de detecció d'antibiòtics en mostres de sèrum d'acord amb la Decisió 657/2002/CE de la Comissió, mitjançant la realització de tres experiments centrats en la validació de mètodes de garbellat comercialitzats per a la llet, en el desenvolupament de bioassajos en plaques microtiter amb resposta dicotòmica i en l'aplicació d'un sistema multiplaca semiquantitatiu, respectivament.

L'especificitat (percentatge de falsos positius) i la capacitat de detecció (CCβ) d'una prova d'inhibició microbiana (Eclipse Farm acoblat al dispositiu e-Reader) i d'assajos d'unió a receptors (3Aminosensor, Quinosensor, Twinsensor i Tylosensor) es van avaluar en mostres de sèrum de llet de cabra, obtenint, en general, resultats similars als obtinguts quan s'apliquen per a l'anàlisi de la llet. Els mètodes comercials per al garbellat d'antibiòtics en la llet, tant les proves d'inhibició microbiana com els assajos d'unió a receptors, van ser adequats per a la detecció de residus d'antibiòtics en mostres de sèrum amb valors de pH compresos entre 6,5 i 7,0, encara que en determinats casos, es van realitzar lleugeres modificacions en el procediment d'assaig a fi de millorar el rendiment del mètode. Així, es va incloure necessàriament una pre-difusió de les mostres de sèrum de llet en el tub d'assaig abans de la incubació per a obtindre una lectura adequada dels resultats de la prova Eclipse Farm acoblat al dispositiu e-Reader.

Es van avaluar tres bioassatjos en placa microtiter i resposta dicotòmica, que contenien *Bacillus subtilis, Geobacillus thermocatenulatus* i *Geobacillus thermoleovorans*, respectivament, per a ser aplicats simultàniament amb els mètodes disponibles comercialment basats en la utilització de *Geobacillus stearothermophilus var. calidolactis*, a fi de tractar de millorar especialment la detecció d'antibiòtics no betalactàmics en mostres de sèrum. Elevats valors d'especificitat (98-100%) es van obtindre quan les mostres de sèrum van ser tractades tèrmicament (85°C, 10 min) abans de realitzar l'anàlisi. *Bacillus subtilis*, amb menors valors de CCβ per a macròlids i quinolones, va ser l'opció més interessant per a millorar el perfil de detecció del mètode Eclipse 100 utilitzat com a representant de les proves comercials que generalment s'utilitzen als laboratoris de control de qualitat de la llet.

Respecte al sistema multiplaca *Screening Test for Antibiotic Residues* (STAR) que utilitza cinc microorganismes diferents amb sensibilitats complementàries (*Geobacillus stearothermophilus* per a betalactàmics i sulfonamides, *Bacillus subtilis* per a

aminoglucòsids, *Kocuria varians* per a macròlids, *Escherichia coli* per a quinolones i *Bacillus cereus* per a tetraciclines), va presentar una elevada especificitat (≥ 98%) en la major part de casos. Els valors de CCβ obtinguts amb el protocol STAR en mostres de sèrum superen el LMR establit en llet per a la major part de les substàncies considerades. No obstant això, aquest mètode podria convertir-se en una eina adequada en el post-garbellament per a la identificació preliminar dels residus d'antibiòtics presents en el sèrum de llet i, per tant, reduir el nombre de mostres destinades a l'anàlisi quantitativa per LC-MS/MS, que és un mètode més complex i car.

Els resultats obtinguts reforcen la necessitat de controlar la presència de residus d'antibiòtics en la llet crua per a evitar la seua arribada a la industria làctia i, per tant, als consumidors. La producció de formatge a partir de llet amb antibiòtics genera residus en el formatge i, especialment, en el sèrum. Malgrat que en la major part de països no s'han establit límits de seguretat per als productes derivats de la llet com el sèrum, l'adequada prestació dels mètodes de garbellat per a la detecció de residus d'antibiòtics en aquest subproducte d'elaboració del formatge permetria l'establiment d'una estratègia de control per a evitar el risc derivat de la presència d'aquestes substàncies en el sèrum, amb efectes negatius sobre la salut pública, la sanitat animal i el medi ambient.

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Chapter I. Introduction

I.1. USE OF ANTIBIOTICS IN DAIRY LIVESTOCK

I.1.1. General Considerations

The use of antimicrobials, especially antibiotics, constitutes a fundamental tool to keep animals healthy and guarantee their well-being and, therefore, has a positive effect on livestock productivity and sustainability, in order to obtain safe and quality animal-based food. Although its rational use is necessary to maintain its clinical efficacy and decrease the development and spread of antimicrobial resistance (AMR).

Currently, AMR is an increasingly important and urgent concern for both human and animal health because resistance can spread from animals to humans through the food chain or direct contact. Combating the threat of antimicrobial resistance, particularly resistance to antibiotics, is a high priority of the European Medicines Agency (EMA) and other medicines regulatory agencies. In veterinary medicine, EMA promotes the prudent use of antimicrobials in animals, collecting data on their use in the European Union (EU) and providing scientific recommendations. The latest report (EMA, 2019) detailed the sales of antibiotics for use in animals along 2017 in Europe expressed as mg/PCU (Population Correction Unit), PCU refers to the unit of mass that can be treated and corresponds to the total census of food-producing animals multiplied by the estimated weight of each species. Figure 1 shows the distribution of sales of antimicrobials by European countries, where we can observe that Cyprus (423.1 mg/PCU), Italy (273.8 mg/PCU) and Spain (230.3 mg/PCU) are the three countries with the highest sales of antimicrobials.

However, the use of antibiotics for the prevention and treatment of bacterial diseases in livestock is totally necessary. The recommendations on the prudent use of drugs in veterinary medicine, considering adequate management, biosecurity, and hygiene, have led to reduce their consumption by more than 32% between 2011 and 2017 (EMA, 2019).

Regarding total veterinary sales by antimicrobial class in 2017, the largest amounts were registered for tetracyclines (30.4%), penicillins (26.9%) and sulfonamides (9.2%) accounting for the 66.5% of the total sales in 31 European countries (Figure 2).

In Spain, sales of antimicrobials have decreased by 45% from 2014 to 2017 (418.8 vs 230.3 mg/PCU). The REDUCE Programs, created within the framework of the development of the first National Plan against Resistance to Antibiotics (PRAN) of the Ministry of Health, Consumer Affairs and Social Welfare (2014-2018), recently expanded to a second stage (2019-2021), have directly contributed to decrease the antibiotics sales in different livestock sectors.

Figure 1. Spatial distribution of antimicrobials sales for food-producing animals, in mg/PCU, for 31 European countries in 2017

Source: EMA (2019)

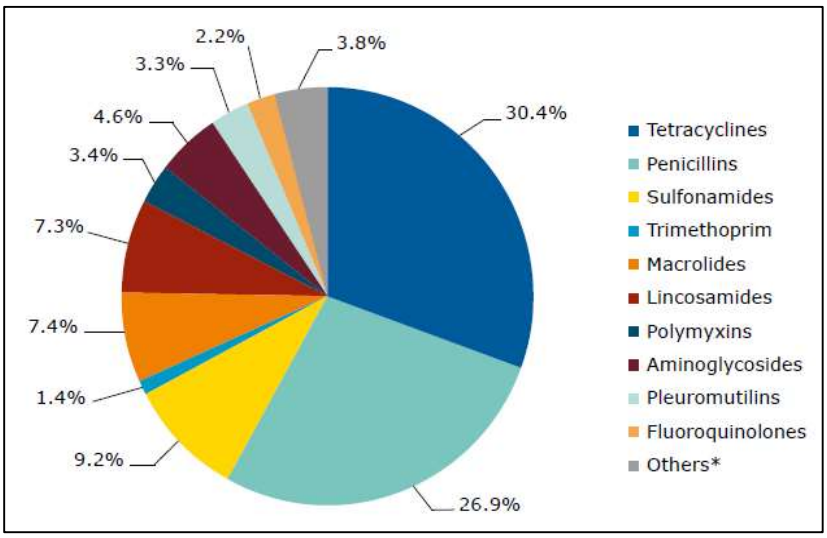


Figure 2. Percentage of the total sales of veterinary products by antimicrobial class for food-producing animals, for 31 European countries in 2017

Source: EMA (2019)

The purpose of the REDUCE Programs is to reveal and analyse the consumption of antibiotics on the farms, propose objectives to reduce the consumption and implement management and treatment guidelines to promote the prudent use of antibiotics to avoid the proliferation of antimicrobial resistance. However, Spain

continues to be among the countries with the highest consumption of antibiotics in the European Union, both in human and veterinary medicine.

For an adequate use of antimicrobials, it is essential to know the characteristics of the different classes of substances used in veterinary treatments. The different groups or classes of antimicrobials have been described in detail by several authors in compendia on antimicrobial therapy in veterinary medicine (Botsoglou and Fletouris, 2001; Menzies and Ramanoon, 2001; Mavrogianni *et al.*, 2011; Landers *et al.*, 2012; De Briyne *et al.*, 2014; OIE, 2015; Obaidat *et al.*, 2017; Prats-Van der Ham *et al.*, 2017). Table 1 summarizes the antimicrobial substances most commonly used in veterinary medicine grouped according to their chemical structure and including the main bacterial, physicochemical and pharmacological properties and their applications in dairy livestock.

The irresponsible use of drugs in dairy livestock is mainly related to the presence of antibiotics residues in milk. To avoid the risk of the presence of antibiotics in milk, the treatments should be applied following the veterinary prescription related to the dose, route of administration, and, particularly, the withdrawal period (Daeseleire *et al.*, 2017). The application of Good Farming Practices (GFP) to the veterinary treatments in livestock is crucial to prevent the presence of antibiotic residues in milk and dairy products, potentially supposing a health hazard to consumers (IDF, 2013).

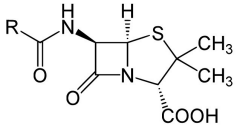
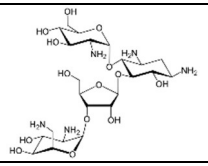
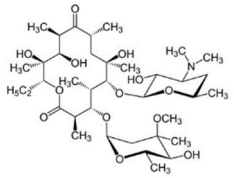
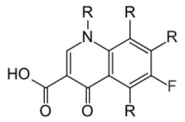
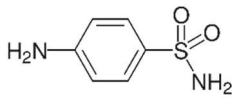
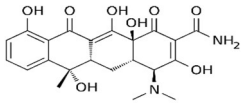
I.1.2. Consequences of the Presence of Antibiotic Residues in Milk

The benefits of antibiotic therapy in dairy animals are counteracted by the presence of residues of these substances in milk and dairy products that can be related to potential repercussions for the food safety and dairy industry. Veterinary residues in milk could promote harmful consequences for human health, causing disturbances in the intestinal flora (Jeong *et al.*, 2009) and/or allergic reactions, in extreme cases, leading to anaphylaxis (Graham *et al.*, 2014).

Numerous cases of drug hypersensitivity reactions (DHRS) have been reported to β-lactams, ranging from 0.7% to 10% of the general population, i.e. amoxicillin in combination with clavulanic acid, achieving around 50% of DHRS in Europe (Torres *et al.*, 2019); and with an incidence of about 8% for penicillins in the USA (Macy, 2014).

In the case of tetracyclines, allergic reactions, skin rashes and phototoxic dermatitis have been reported, and for aminoglycosides, specifically streptomycin, problems of hypersensitivity and toxicity effects (neurotoxicity) were indicated by Bacanli and Başaran (2019).

Table 1. Classification of the most commonly used antimicrobials in veterinary medicine

Antimicrobial groups	Structure	Bacterial effect/ Spectrum and Mechanism of action	Physicochemical and pharmacological properties	Therapeutic Area	Substances
β-lactams		Bactericide/ Broad-spectrum and cell wall synthesis inhibitors.	Natural and semisynthetic antibiotics, β-lactam ring with antibacterial activity and side chains with variable chemical and pharmacological properties, moderate to strongly acid character (pK_a 2.7), polar organic acids and non-lipophilic nature.	Gastrointestinal diseases, mastitis, prophylaxis, reproductive disorders, respiratory (pneumonia) and urinary tract infections, septicaemias.	Amoxicillin, ampicillin, benzylpenicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, cefacetrile, cefalexin, cefapirin, cefazoline, cefoperazone, cefquinome, ceftiofur, desfuroylceftiofur.
Aminoglycosides		Bactericide/ Narrow-spectrum and protein synthesis inhibitors.	Natural and semisynthetic antibiotics, one or more sugar units (glycosamide) connected by a glycosidic linkage to a central aglycon fraction, highly polar and poor lipophilicity.	Abortion, gastrointestinal diseases, mastitis and dry cow therapy, respiratory (pneumonia) and urinary tract infections, septicaemias.	Gentamicin, neomycin, streptomycin.
Macrolides Lincosamides		Bacteriostatic/ Narrow-spectrum and protein synthesis inhibitors.	- Macrolides: natural antibiotics, macrocyclic lactone ring linked to amino sugars and lipophilic weak organic bases. - Lincosamides: natural and semisynthetic antibiotics, monoglycosides with an amino acidlike side chain. Moderate to high lipophilicity.	Gastrointestinal diseases, liver abscesses, mastitis, mycoplasmosis, prophylaxis, respiratory (pneumonia) infections.	Erythromycin, spiramycin, neo spiramycin, tilmicosin, tylosin. Lincomycin.
Quinolones		Bactericide/ Broad-spectrum and acid nucleic synthesis inhibitors.	Synthetic antimicrobials, amphoteric compounds: carboxylic acidic and basic amino groups (pK_a: 5-6.5 and 7.5-9.3), moderate to high lipophilicity.	Colibacillosis, enteric and respiratory infections, gastrointestinal diseases, mastitis, septicaemias.	Danofloxacin, enrofloxacin, ciprofloxacin, flumequine
Sulfonamides		Bacteriostatic/ Broad-spectrum and acid nucleic synthesis inhibitors.	Synthetic antimicrobials, chemical nucleus with antibacterial activity, insoluble in water.	Bacterial, coccidial, protozoal and respiratory (pneumonia) infections, foot rot, gastrointestinal diseases, mastitis, metritis.	Sulfacetamide, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfathiazole.
Tetracyclines		Bacteriostatic/ Broad-spectrum and protein synthesis inhibitors.	Natural and semisynthetic antibiotics, tetracyclic nucleus (ring of four atoms) linked to functional groups, moderate to high lipophilicity.	Bacterial and chlamydial diseases, gastrointestinal disorders, mastitis and dry cow therapy, nervous system pathologies, prophylaxis, respiratory (pneumonia) and urinary tract infections.	Chlortetracycline, 4-epi-chlortetracycline, doxycycline, oxytetracycline, 4-epi-oxytetracycline, tetracycline, 4-epi-tetracycline.

Moreover, the use of antibiotics in livestock has largely contributed to the evolution of microorganism resistant strains, which can be easily transferred to humans (Sharma *et al.*, 2018) promoting AMR (Figure 3).

Figure 3. Causes of Antibimicrobial Resistance (AMR)

Source: PAHO and WHO (2015)

AMR to several microorganisms is a global threat to public and animal health, increasing mortality and prolonging illnesses in humans and animals, and also impacting food safety and food production, with production losses in agriculture, livestock, and aquaculture (FAO, 2017). Around 700,000 human deaths each year are related to AMR, and a rate of ten million people death per year by 2050 has been estimated (O'Neill, 2014; WHO, 2019). The global impact of antimicrobial resistance on human health is summarized in Figure 4.

Recently, numerous studies on the presence of antimicrobial resistant bacteria and/or genes in milk or dairy products and the possibility to act as vehicles in the generation of resistance to certain antibiotics in humans have been published. Akindolire *et al.* (2015) indicated in raw and pasteurised milk samples a large proportion (60-100%) of *Staphylococcus aureus* resistant to benzylpenicillin, ampicillin, oxacillin, vancomycin, and erythromycin, and to minor extent (8.3-40%) to gentamicin, kanamycin and sulfamethoxazole. Jamali *et al.* (2015) also discovered *S. aureus* isolates resistant to tetracycline and benzylpenicillin in raw cow and sheep milk and in traditional cheese, with a percentage of about 50%. Other studies focused on cheese also reported staphylococcus strains mainly resistant to β-lactams and tetracyclines

(Spanu *et al.*, 2014; Casaes *et al.*, 2016). Concerning other pathogenic bacteria, Kevenk and Gulel (2015) and Bedasa *et al.* (2018) indicated resistant *Listeria monocytogenes* and *Escherichia coli* isolates, respectively, in raw milk and cheese. The majority of the described studies registered high percentages of bacteria with multi-drug resistance.

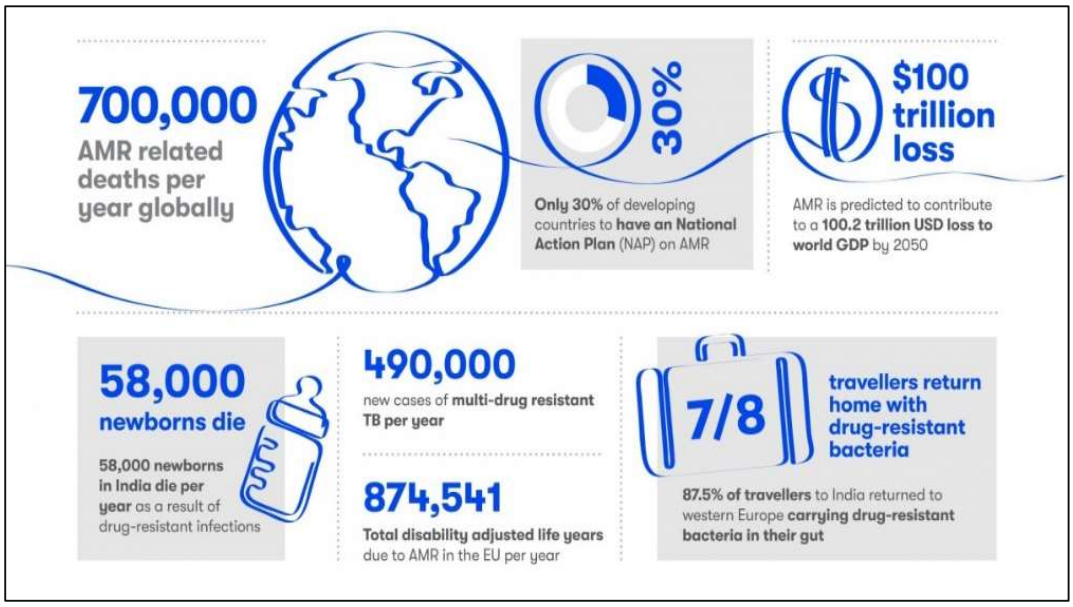


Figure 4. Global impact of AMR on human health

Source: Centrient Pharmaceuticals (2019)

On the other hand, the presence of antibiotics in milk is able to generate technological failures on dairy products that require fermentative processes, like yogurt and cheese. In the manufacture of fermented products, starter cultures containing different lactic acid bacteria (LAB) could be partial or totally inhibited by antibiotic residues even at or below safety levels.

In this sense, studies in sheep milk yogurt indicated delays in the pH decrease rate for penicillins (Berruga *et a*l., 2007a; Berruga *et al.*, 2011) and cefalosporins (Berruga *et a*l., 2008a; Novés *et al.*, 2015), even below their corresponding MRLs. Berruga *et al.* (2007b) also observed in cheese from sheep milk spiked with β-lactams at corresponding safety levels delays in the pH evolution during manufacture process (5-295 minutes), only significant in the case of ceftiofur.

For the other antibiotic families, the kinetics of acidification of ripened cheeses was considerably affected by the presence of oxytetracycline at safety level (Cabizza *et al.*, 2017), with a prolongation of 60 min in the time required for cheese production. Also, the presence of erythromycin and oxytetracycline, at corresponding Maximum Residue Limits (MRLs), delayed the acidification phase (122±29 and 108±25 min, respectively)

until reaching the final pH of 5.30±0.05 (Quintanilla *et al.*, 2019a). Paba *et al.* (2019) showed a statistically significant ($P< 0.05$) lower acid lactic concentration in thermised sheep milk spiked with oxytetracycline at the MRL concentration after 6 and 7 hours of incubation.

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 involved lower firmness values in sheep milk yogurts. However, only slight or no differences on the quality characteristics of ripened cheese in the presence of different antibiotics (amoxicillin, benzylpenicillin, cloxacillin, erythromycin, enrofloxacin, ciprofloxacin and oxytetracycline) at MRL concentration were found by Quintanilla *et al.* (2019a).

Moreover, the elimination of veterinary residues present in milk destined to dairy products by the different heat-treatments commonly applied in dairy industry is very limited and, therefore, residual concentrations of antibiotics could be found in derived products. It is well-known that pasteurization and sterilization processes reduce to a greater or less extent the concentration of antibiotics in milk (Zorraquino *et al.*, 2008, 2009, 2011), with some families of antibiotics like quinolones having a high stability to heat treatments (Roca *et al.*, 2010). Thus, in fresh cheese made from pasteurized goat's milk, Quintanilla *et al.* (2019b) found a retention of over 50% for most classes of antibiotics. In mature cheese from raw goat's milk (Quintanilla *et al.*, 2019a), the retentions were lower since the maturation process denatures most antibiotics; residues in the cheese were found after 60 days of maturation only for oxytetracycline (20.0±5.7 µg/kg) and at relatively high concentrations for quinolones (enrofloxacin: 148±12 µg/kg and ciprofloxacin: 253±24 µg/kg).

Other aspects to be considered are the economic losses both for farmers and dairies incurred by the presence of antibiotics in milk. According to current legislation, raw milk with antibiotics is considered "unfit for human consumption" and can be banned by the corresponding authorities, with the consequent commercialization restrictions of milk together with its storage and elimination costs.

Finally, antibiotics are not metabolized entirely by animals and are eliminated by milk and/or excreted through urine and/or feces (Kemper, 2008), contaminating the topsoil where they can accumulate or seep into the groundwater (Martínez-Carballo *et al.*, 2007) affecting the microflora, the microfauna, and the groundwater quality, with serious environmental implications. Furthermore, the presence of antibiotics in the environment could accelerate the spread of bacteria that carry antibiotic resistant genes

through multiple pathways that include effluents from human, agricultural, aquaculture and animal production waste (Figure 5).

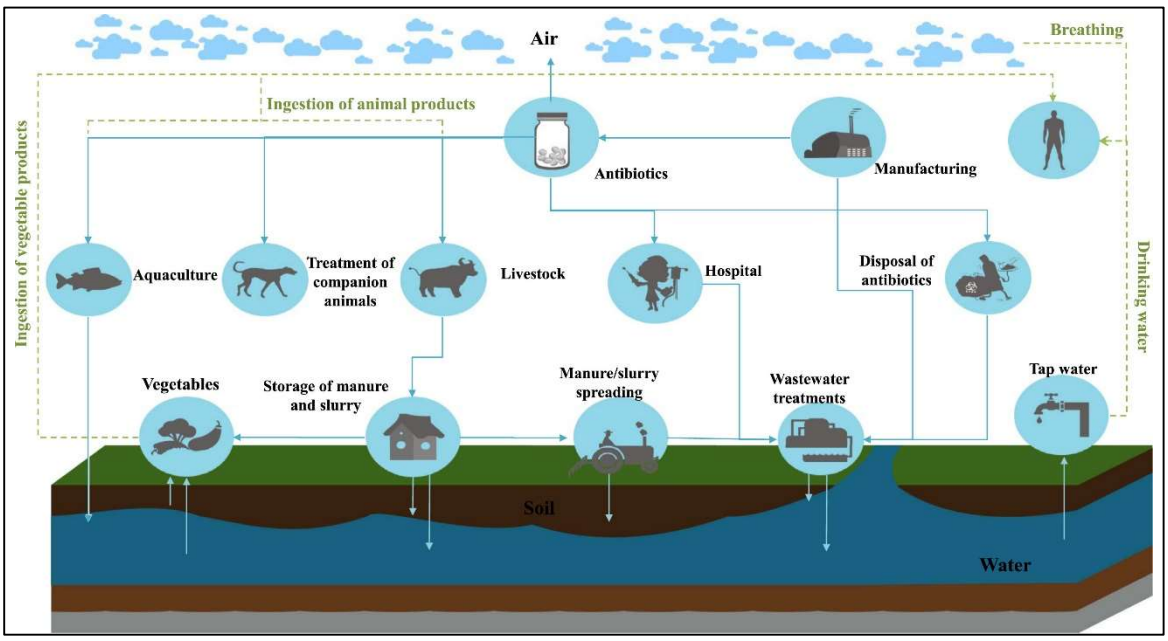


Figure 5. Human exposure to antibiotic resistance associated with antibiotics residues in the environment

Source: Ben *et al.* (2019)

I.1.3. Legislative Aspects and Quality Control of Antibiotics

In order to protect public health, Maximum Residue Limits (MRLs) of pharmacologically active substances in foodstuffs of animal origin have been established in accordance with procedures provided by Regulation (EC) Nº 470/2009 (EC, 2009), that 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 following the concept of acceptable daily intake (ADI), which corresponds to the amount of a residue that a human being may ingest on a daily basis with food along their lifetime without any toxicological hazard for human health. The ADI concept to establish the MRLs depends on pharmacokinetics and depletion of residues, as well as the marker residue and target tissues.

Pharmacologically active substances, including their metabolites, classified as allowed and prohibited/banned substances according to MRLs in foodstuffs of animal origin, are set in the Annex to Commission Regulation (EU) Nº 37/2010 (EU, 2010). Currently, the MRL has been fixed for raw milk from different species (Table 2) but not for all dairy products.

Table 2. European Union Maximum Residue Limits (EU-MRLs) for antibiotics in raw milk

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

BOC: Bovine, ovine, caprine

Source: Regulation (EU) Nº 37/2010 (EU, 2010)

For substances prohibited or not regulated by the EU, Regulation (EC) Nº 470/2009 (EC, 2009) has also set up a procedure to establish “the reference values for purposes of intervention”, defined as the lowest concentration of a residue that can be detected and confirmed by an official laboratory, previously known as Minimum Required Performance Limits (MRPLs).

The responsibility of the operators of the food chain in the production of safe food is defined by Regulation (EC) Nº 852/2004 (EC, 2004a) on the hygiene of foodstuffs, and Nº 853/2004 (EC, 2004b), which lays down specific rules of hygiene of foodstuffs

of animal origin. To ensure compliance with these hygienic rules, the European Union also published Regulation (EC) Nº 854/2004 (EC, 2004c) setting up specific rules for the organisation of official controls on products of animal origin intended for human consumption.

Regarding the control of the traceability of foodstuffs at all stages of production, processing and distribution by food companies, the EU established Regulation (EC) Nº 178/2002 (EC, 2002a). In Spain, to comply with this regulation in milk, the Ministry of Agriculture, Fisheries and Food (MAGRAMA) issued Real Decreto 217/2004 (BOE, 2004), responsible for identifying and registering all the operators involved in the dairy sector, as well as the recording of the movements of milk, creating the web application "Letra Q database" module (Leche, TRAzabilidad, Qualidad).

Additionally, Real Decreto 1728/2007 (BOE, 2007) and Real Decreto 752/2011 (BOE, 2011) fix the mandatory minimum controls to be performed *in situ* in farms and dairy industries (Figure 6) and standardize the conditions required from laboratories for the analysis of raw milk from cows, goats and sheep. Monitoring the presence of antimicrobials in raw milk includes the application of tests, to be carried out in farms before loading milk into the collection tank and prior to the discharge of milk into the storage silos of dairy factories (Figure 6).

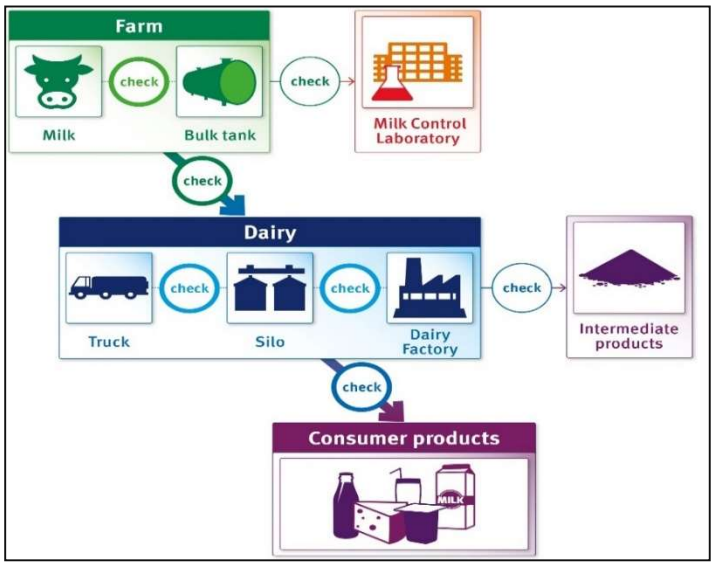


Figure 6. Quality checks in raw milk

Source: Branco (2016)

The "Letra Q database" can generate alarms to the competent authorities about infractions in somatic cell counts, bacteriology and/or test of antibiotic residues. In case of defaults, especially in the test of antibiotic residues, immobilization of the milk and its subsequent destruction will be carried out.

I.2. CHEESE-MAKING AND WHEY

I.2.1. Cheese and Whey Production

Cheese is a dairy product derived from buffalo, cow, goat, or sheep milk that is produced by enzymatic or acid coagulation of the milk protein casein. After coagulation, milk whey or cheese whey, hereinafter referred to as whey, is separated from curd, that is molded and pressed into its final form. The cheese properties are intrinsically related to original milk chemical composition. However, the manufacturing process (Figure 7) affects the nutritional and sensory characteristics of the finished product (Raynal-Ljutovac *et al.*, 2011).

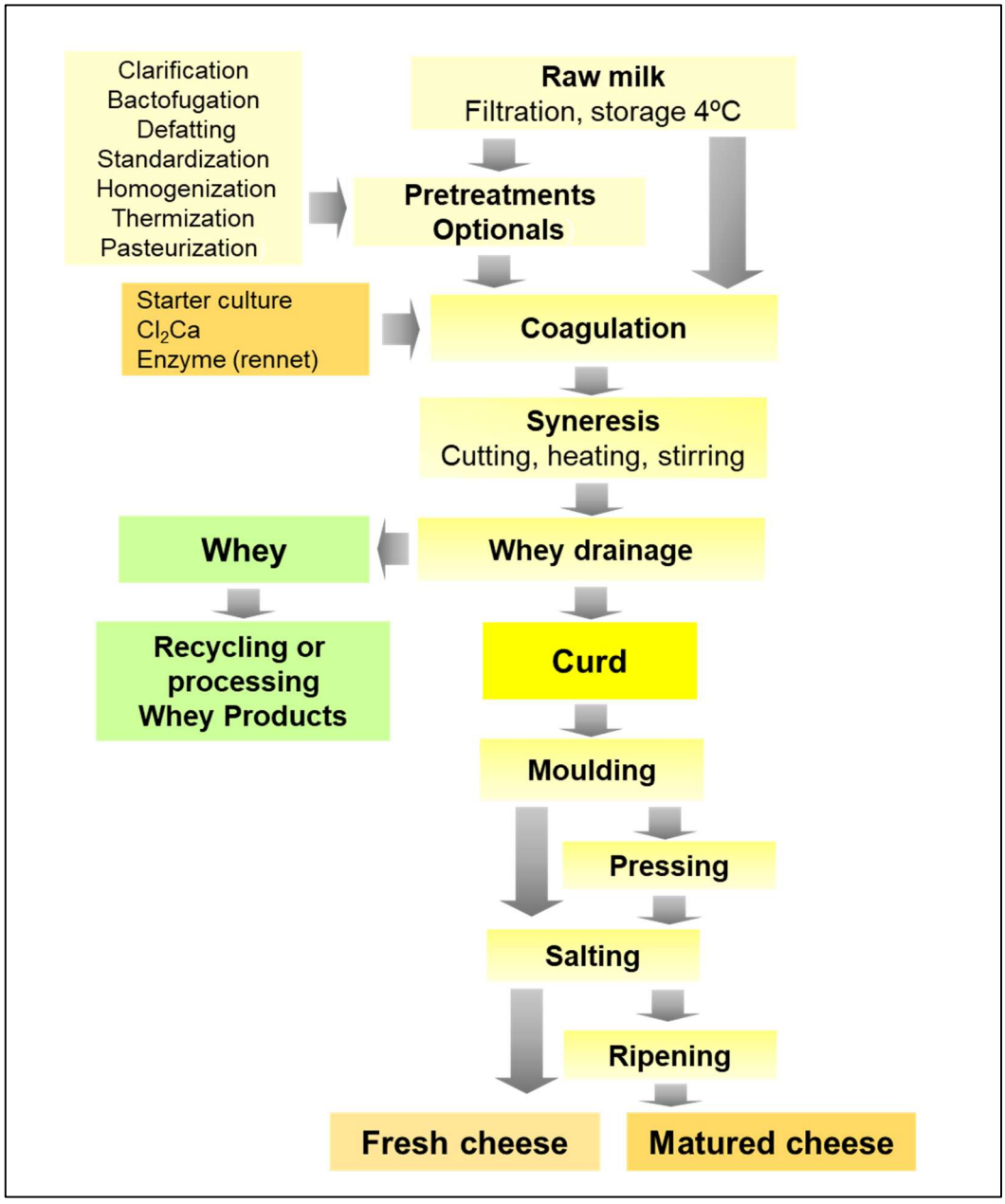


Figure 7. Cheese-making process for enzymatic cheese

The classification of cheeses is related to various factors: milk origin, raw or heat-treated milk, type of coagulation (enzymatic, lactic, mixed coagulation), ripening time, fat content of the cheese, internal and external appearance of the cheese, among others (IDF, 1981). Overall, cheeses can be divided in different groups, i.e. fresh (unfermented) cheeses, soft cheeses that undergo lactic fermentation and mold surface flora, semi-hard and hard cheeses.

Cheese production in the world has significantly increased in recent years, reaching a total of 23 million tons according to the last data from FAOSTAT (2020). Europe represents the most important part of the cheese production in the world (around 50%), followed by America, especially the United States (EEUU) accounting for the 25% of the total world production (Figure 8).

Although the cheese production from goat's milk (2%) is considerably lower in comparison to the production of cow cheese, as it is observed in Figure 8, this production is characterized by a great tradition in Mediterranean and eastern European countries, including France (46%), Greece (23%) and Spain (19%) (FAOSTAT, 2020). In Spain, goat's milk production is mainly intended for the manufacture of cheeses (approx. 33.6 tons x 10^3 annually), many of them under the protected designation of origin (PDO) such as Ibores, Murcia, Palmero or Majorero, and other brands of quality of international recognition.

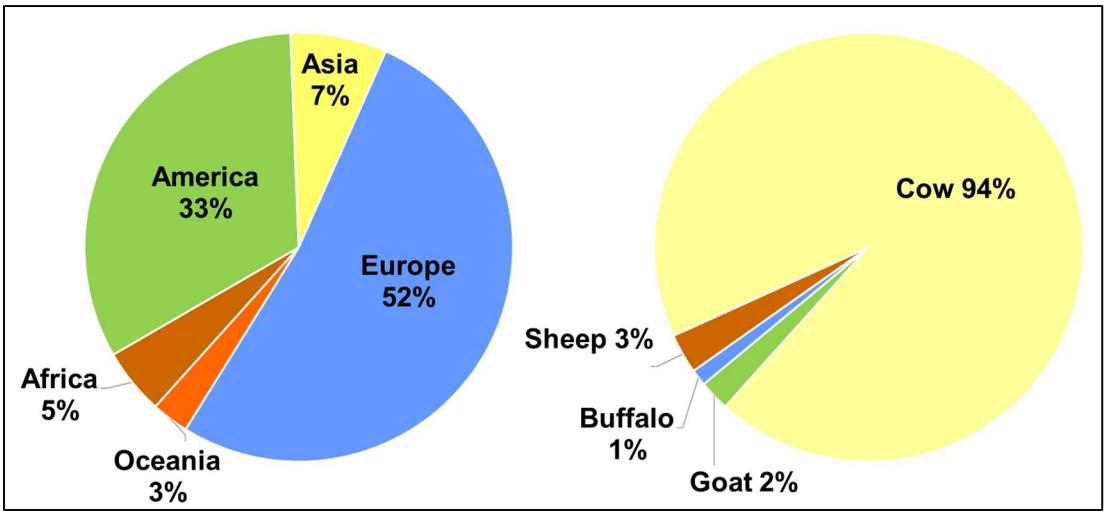


Figure 8. World cheese production in 2014

Source: FAOSTAT (2020)

One of the problems associated with cheese production is the generation of a large volume of whey. The effluents produced in the dairy industry depend directly on cheese yield. Thus, about 10 kg of cow milk are necessary for the production of 1 kg of cheese, 8 kg for goat and 5 kg in the case of sheep, and, consequently, originating

approximately 9 kg, 7 kg and 4 kg of whey residue, respectively (Prazeres *et al.*, 2012; López *et al.*, 2018; Quintanilla *et al.*, 2018). In addition, it has been considered that cow cheese production is between 30 and 50 times higher than sheep and goat's milk cheese production, with a considerable volume of whey. Therefore, the amount of whey generated is approximately 7 to 10 times higher than the cheese produced, depending on its variety (Callejas *et al.*, 2012).

As can be seen in Figure 9, the majority of whey production, taking into account condensed and dry whey data, is attributed to Europe, with high percentages for France, Italy and Germany, corresponding to the most important cheese-producing countries in Europe.

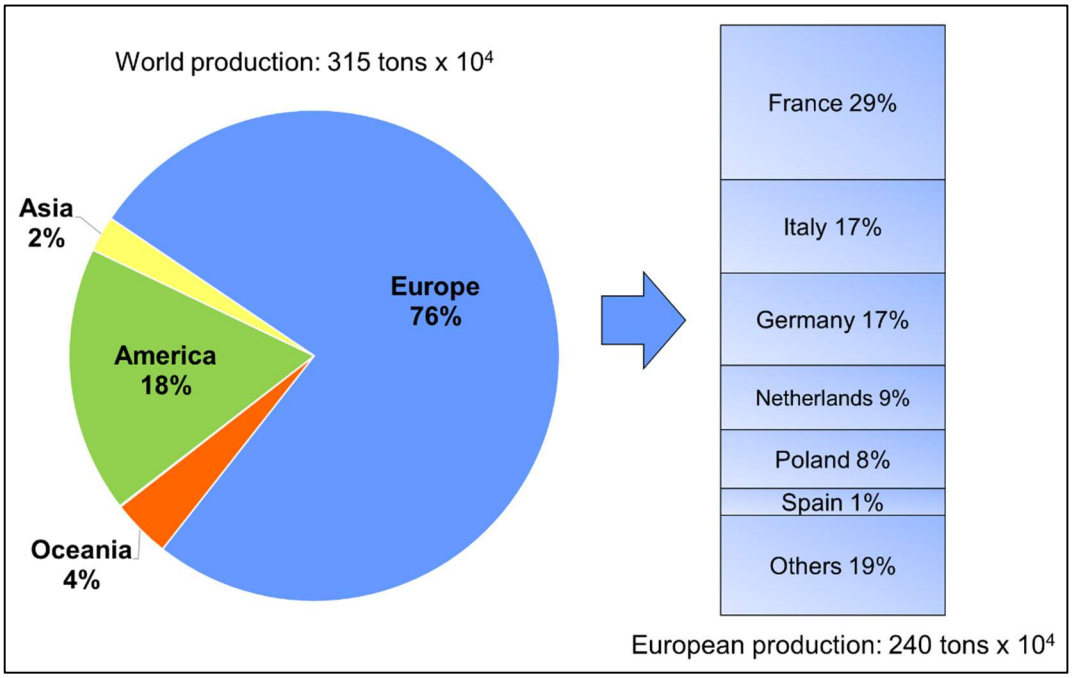


Figure 9. World whey production in 2014

Source: FAOSTAT (2020)

The considerable whey production rate, which increases by approximately 3% per annum for the last 21 years (Lappa *et al.*, 2019), involves significant environmental and health public problems due to its high organic content. In past, the effluents of most of the cheese factories were directly discharged into the receiving waters (rivers, lakes, ocean, etc.), or municipal sewage system, without any pre-treatment. Currently, about 50% of the whey production is treated and transformed into different foodstuffs for human and animal consumption, a large percentage of whey production is not however being reused (Prazeres *et al.*, 2012).

The disposal of whey into water reservoirs causes a potential pollution due to the high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), 40-48,000 g/L and 89-95,000 g/L, respectively, with a strong impact on the environment, such as depleting of the dissolved oxygen (DO), toxicity for specific types of fish and algae, or the risk of the eutrophication phenomenon (Ahmad *et al.*, 2019). Furthermore, the discharge of whey from cheese-making into municipals sewers has been legally prohibited since it interrupts biological treatments developed in these plants (Yadav *et al.*, 2015). In this sense, whey is considered a contaminant residue and its discharge was prohibited in Europe by Council Directive 96/61/EC (EC, 1996) concerning integrated pollution prevention and control.

Alternatively, whey could be treated by constructing wetlands, which use hydophyte plants and rhizosphere microbials to remove chemical contaminants and constitute a more low-cost and environmentally friendly technology. However, wetlands require large areas, which suppose a significant risk for surface and ground water (Carvalho *et al*., 2013; Ahmad *et al.*, 2019).

Regarding the use of whey for food industry and feed animal, of the total of processed whey volume 50% is used directly in its liquid form, mainly for the manufacture of whey cheeses (Requesón, Riccotta, Mizithra…), and other whey-based fermented beverages, 30% as dried whey powder, 15% for lactose and by-products, and 5% in the production of whey protein concentrates (Rama *et al.*, 2019). In addition, whey constitutes an alternative to replace some of the conventional sources of protein (soy, flour fish, skim milk, etc.) in feed for animal consumption (Fresno *et al.*, 2015).

Another use of whey has been the application to the soil as a fertilizer in agriculture, especially for the correction of acid or calcareous soils. However, this dairy by-product could alter the physical and chemical composition of soils, the presence of suspended solids decreasing soil's permeation and gas exchange, in addition to its high salinity content affects the availability of water in detriment of plant growth and fruit production (Prazeres *et al.*, 2012).

The significant amount of whey (approx. 50%) that remains still unutilized makes convenient to consider new reusing alternatives of this effluent, to maximize profits and reduce potential pollutant effect. From the appraisal point of view, whey is a nutrient-rich effluent that contains the main ingredients of the original milk such as lactose, soluble proteins, minerals, lactic acid and fats and is, therefore, considered as a potential resource for the production of various value-added products.

I.2.2. Characteristics and Use of Whey

Whey is the product obtained after the precipitation and removal of milk casein from cheese manufacturing and represents about 80–90% of milk volume. Its organic value is principally derived from lactose, fat and protein content, highlighting β-lactoglobulin (more than 50% of the total of whey proteins), α-lactoglobulin, immunoglobulins, serum albumin and lactoferrin, with a significant BOD/COD ratio, normally above 0.5, being an adequate substrate to be treated by biological processes. Whey also contains mineral salts, principally NaCl and KCl and phosphates, and other minor components such as citric and lactic acids, non-proteinic nitrogen compounds and vitamins (Prazeres *et al.*, 2012).

According to Yadav *et al.* (2015), two types of whey are produced depending on the cheese-making process implicated, and the main differences between them concern acidity, the mineral content, and the whey protein fraction. Sweet whey, being the most frequently produced type, results from the coagulation by means of proteolytic enzymes such as chymosin at around pH 6.5, while acidic whey (pH< 5.0) is obtained after addition of organic or mineral acids to achieve the isoelectric point of casein (pH= 4.6). Some examples of chemical composition of liquid whey from cow, sheep and goat species are showed in Table 3.

Table 3. Composition of sweet and acid whey from different animal species

Constituent (g/L)	Cow Whey		Sheep Whey	Goat Whey
	Sweet	Acid	Sweet	Acid
Dry matter	70.84	65.76	83.84	62.91
Protein	9.24	7.80	18.71	9.35
Nitrogen	1.45	1.22	2.93	1.47
Non-protein nitrogen	0.37	0.54	0.80	0.67
Ammonium nitrogen	0.04	0.14	0.13	0.18
Fat	5.06	0.85	6.46	0.40
Lactose	51.81	45.25	50.98	39.18
Ash	5.25	7.56	5.65	8.36
Calcium	0.47	1.25	0.49	1.35

Source: Pintado *et al.* (2001)

Considering the huge volume of whey produced annually and its significant organic value, it is advisable to recycle or to convert this cheese industry effluent into valuable products through the implementation of various technologies that limit the impact of environmental pollution.

Only the half of the global whey production is destined to animal feed and food applications, including the elaboration of whey cheeses with a long tradition in countries such as Greece (Manouri and Mizithra), Italy (Riccota), Norway (Gjetost), Spain (Requesón) and Switzerland (Ziger). For that, to maximize the benefits of whey in greater extent, in recent decades the management of whey has focused on its fractionation in protein concentrates and lactose permeates by means of direct physical and thermal treatments or biotechnological processes or even using combinations of both types (Figure 10).

The physicochemical treatments have the aim to obtain different protein contents: whey powder, reduced lactose whey, whey protein concentrate (WPC) and whey protein isolate (WPI) and the main types of processes used are precipitation with coagulants/flocculants, thermal/isoelectric precipitation, and membrane separation such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Prazeres *et al*., 2012; Carvalho *et al*., 2013; Yadav *et al*., 2015).

Demand for whey proteins is increasing due to their various functional and nutritional properties (Yadav *et al*., 2015; Kareb and Aïder, 2018; Lappa *et al.*, 2019). Thus, whey proteins have numerous food applications like sports beverages, baked products, infant foods, dairy products, etc., and in the pharmaceutical industry. Whey is characterized by a high content of essential and branched amino acids (isoleucine, leucine, and valine), which engage in different metabolic functions, regulate blood glucose homeostasis and induce sleep, among others. Furthermore, whey proteins are associated to specific bioactivities: antibacterial, antitumoral, antiviral, and immunomodulatory, enhancement of gut health, regulation of fatty acids metabolism, synthesis of glutathione (GSH), etc. These functions of whey proteins could be extended with the release of non-allergenic bioactive peptides by enzymatic hydrolysis or fermentation processes, which has become an interesting approach to add functional and nutraceutical value to whey.

Biotechnological processes are applied to the biotransformation of lactose contained in whey permeates (Prazeres *et al*., 2012; Carvalho *et al*., 2013; Yadav *et al*., 2015). Normally lactose products are destined to food industry in the production of baby foods, reconstituted dairy products as well as confectionary and bakery products, have pharmaceutical applications, and other uses are related to the production of biogas, bioplastics or even electricity; and, additionally, lactose derived prebiotics have been synthesized to be used in functional foods and for pharmaceutical applications (Yadav *et al*., 2015; Kareb and Aïder, 2018; Ahmad *et al.*, 2019; Lappa *et al.*, 2019).

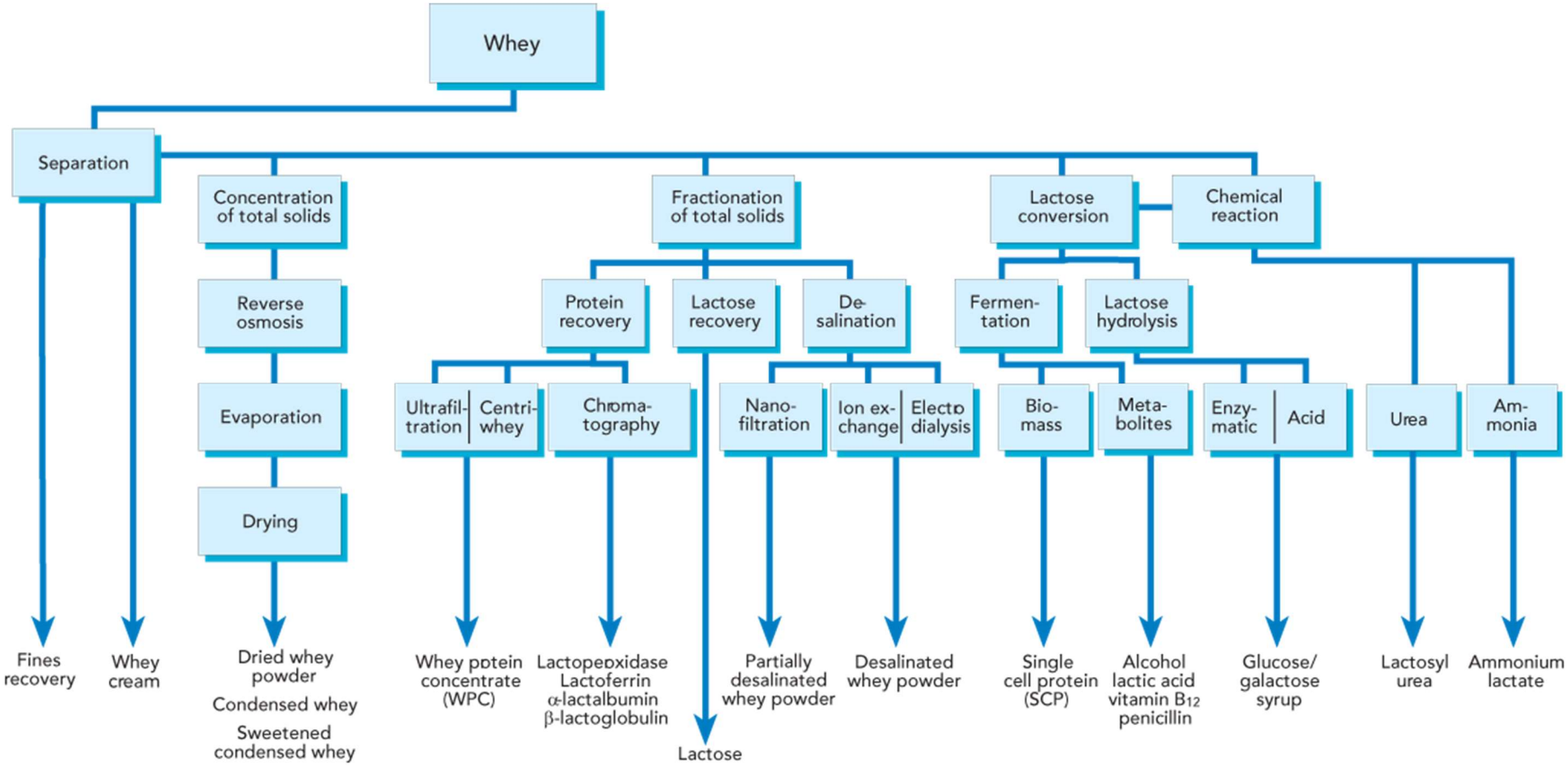


Figure 10. Whey processing alternatives

Source: Bylund (2015)

Some of the most well-known biotechnological alternatives are aerobic fermentation that is used for the obtention of proteinaceous biomass (bioprotein) such as bacteria, yeast, fungi and algae (Pesta *et al.*, 2007; Rama *et al.*, 2019), which are used in animal feeding or as a protein source for humans; anaerobic fermentation in the production of acetic, citric and propionic acid (Soriano-Pérez *et al.*, 2012; Zotta *et a.*, 2020), hydrogen, ethanol (Pesta *et al.*, 2007; Das *et al.*, 2016) and even biopolymers such as polyhydroxyalcanoates (PHAs) or bacterial cellulose (Bosco and Chiampo, 2010; Carreira *et al*. 2011; Zotta *et al.*, 2020); and, anaerobic digestion is associated to methane or biogas production (Pesta *et al.*, 2007; Dinuccio *et al.*, 2010). Moreover, another additional application is the direct production of electricity through microbial fuel cells (MFCs) (Yadav *et al.*, 2015).

Although whey has been mainly managed from a technological point of view to obtain valuable products, in most of cases destined to food applications, the pharmaceutical industry and animal feed, a significant amount of this cheese by-product is still unused, and, additionally, small and medium factories must assume the disproportionate costs associated to the existent valorization technologies. Hence, novel integrated whey biorefinery approaches, within the context of a circular economy, are being implemented to obtain functional foods with improved physicochemical and sensory characteristics, and higher nutritional value (Lappa *et al.*, 2019).

I.2.3. Transfer of Antibiotics from Milk to Cheese

The potential transfer of antibiotics from milk to dairy products is determined by numerous factors like the drug concentration in raw milk, the nature of the antibiotic, and the type of technological process, among others. Antibiotics remain in one or more fractions of milk, depending on the physicochemical properties such as lipophilicity and hydrophobicity, protein binding capacity, drug-drug and drug-nutrient interactions, etc. In the case of cheese made from milk containing antibiotics, drugs may be concentrated together with casein or fat globules in curd and/or are released in whey.

Several studies have reported the presence of different veterinary residues in commercial dairy products. Adetunji (2011) found benzylpenicillin (5.4±0.1 μg/kg), streptomycin (3.6±1.9 μg/kg) and tetracycline (2.12±0.08 μg/kg) in commercial soft cheeses made from cow milk. Another study (Tona and Olusola, 2014) also found the presence of tetracycline in all the types of dairy products analysed (cow milk, goat milk, butterfat, soft cheese and yogurt), with the highest concentration of the antibiotic being detected in the case of cheese, which revealed that the different processing used for the transformation of milk into its derived products were unable to remove tetracycline.

Furthermore, Darko *et al*. (2017) detected chloramphenicol, oxytetracycline, sulfamethoxazole and sulfathiazole in milk, cheese, and yogurt from Kumasi's market, at very low antibiotic concentrations, without supposing a risk to consumers. Some studies even indicated drug residues in milk after pasteurization and ultra high temperature (UHT) treatments, especially in the case of β-lactams (Schlemper and Sachet, 2017) and quinolones (Zhang *et al*., 2014).

Data concerning the traceability of antimicrobial substances in the different milk fractions is scarce and insufficient in scientific literature. There are few studies on the transfer of antibiotics from milk to cheese and most of them focus on a limited number of veterinary drugs.

Sniegocki *et al*. (2015) revealed that chloramphenicol was transferred from milk contaminated at 10 µg/kg to dairy products with higher fat content such as butter (4.86 µg/kg), sour cream (3.5 µg/kg) and white cheese (2.36 µg/kg).

Regarding tetracyclines, Cabizza *et al*. (2017) indicated that 61% (388 µg/kg) of oxytetracycline added to milk at the MRL concentration (100 µg/kg) was concentrated in mature sheep cheese, while only 21% remained free in sweet whey (29 µg/kg). Similarly, Gajda *et al*. (2018) determined that tetracycline antibiotics were mainly concentrated in fresh cheese (280-561 µg/kg) made from spiked milk at the corresponding MRL concentration (100 µg/kg).

Giraldo *et al*. (2017), through cheese manufacturing at laboratory scale, assessed the antimicrobial activity in whey from milk spiked with different antibiotics using a microbial screening test for the detection of antibiotics in milk (Eclipse 100). Antimicrobial activity variation (AAV%) between whey and milk was used to estimate the potential retention of antibiotics in curd. Thus, similar antimicrobial activity in both matrices (AAV%= 0%) suggests that antibiotics are completely transferred from the milk to whey. However, an increase in the AAV% indicates the retention of antibiotics in the curd. Figure 11 shows that β-lactams are preferably released into the whey, while non β-lactam groups such as aminoglycosides, quinolones and tetracyclines, present a higher AAV%, and, therefore, a greater retention in curd.

Some studies demonstrated that the technological process of the type of cheese may influence on the partitioning of antibiotics in the different milk fractions. Quintanilla *et al.* (2019b) studied the retention of different groups of antibiotics in fresh cheese made from goat's milk at MRL concentration, finding that most of them were retained in cheese with percentages ranging between 37.5% for oxytetracycline and 75.2% for cloxacillin. Despite the fact that some antibiotics such as penicillins and erythromycin

are characterized by a high solubility in water (Rang *et al*., 2000), the study showed considerable percentages of retention in cheese for these substances (58.4-75.2%), attributing this behaviour to the high whey content in this type of cheese (51.78-59.9%) that could have favoured a greater retention in curd.

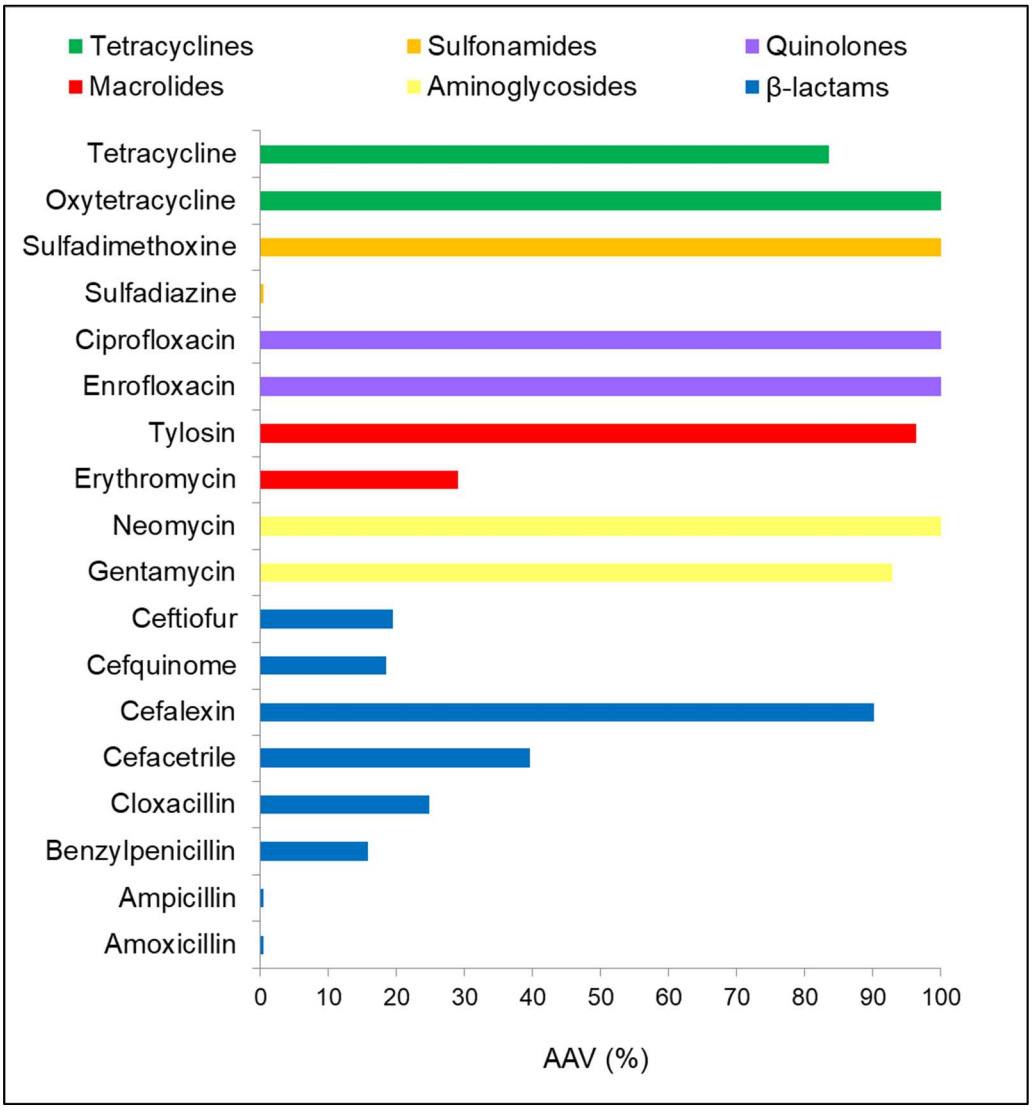


Figure 11. Antimicrobial activity variation (AAV%) as indicator of the antibiotic drugs transfer from goat's milk to whey

Source: Giraldo *et al.* (2017)

In the previously cited study (Quintanilla *et al*., 2019b), the Food Safety Margin (FSM), which is a metric that allows assessing whether the margin established between the exposure to an estimated daily intake (EDI) and the safety threshold as the ADI for the food chemical of concern is sufficient, was reported in pasteurized goat's milk and fresh cheese. While the lowest FSMs (least safe) were obtained in pasteurized milk for enrofloxacin, ciprofloxacin and erythromycin in the group of children, the FSM calculated in fresh cheese for the total of antibiotics in all age groups

was significantly more elevated, being related to the lower consumption of cheese in comparison to milk. However, although the presence of macrolides and quinolones in fresh cheese did not generate significant toxicological effects on consumer health, the continued intake of small amounts of antibiotics could contribute to the development and spread of antimicrobial resistances, which implies serious hazards for public health.

In mature cheeses, Quintanilla *et al*. (2019a) studied the retention of antibiotics in milk at MRL and showed that β-lactams (amoxicillin, benzylpenicillin and cloxacillin) and erythromycin were largely released in whey, including retention percentages in curd below 20%, possibly due to the high water-solubility of these substances. However, naturally lipophilic groups such as quinolones (39.4-56.4%) and oxytetracycline (68%) showed a higher retention percentage in curd than the rest of families studied.

Additionally, as shown in Table 4, the aforementioned authors reported that antibiotic residues in cheese decreased during ripening up to the point that β-lactams and erythromycin were not detectable after 30 days of ripening. In contrast, quinolones and oxytetracycline were found even after a period of 60 days in the ripening chamber under controlled conditions, with relatively high concentrations for enrofloxacin and ciprofloxacin.

Table 4. Antibiotic residues during ripening (Mean±SD) in Tronchón cheese made from goat's milk spiked with antibiotics at EU-MRL concentration

Antibiotics	EU-MRL (µg/kg)	Antibiotic concentration in cheese (µg/kg)		
		Ripening time (days)		
		0	30	60
Amoxicillin	4	traces	nd	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	362.5±36.5	309.4±19.6	252.9±23.7
Enrofloxacin	100	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

EU-MRL: European Union Maximum Residue Limit in raw milk (EU, 2010); traces (LOD< result< LOQ); nd: not detected (result< LOD); LOD: Limit of Detection; LOQ: Limit of Quantification.

Source: Quintanilla *et al.* (2019a)

As a consequence of the different characteristics of the veterinary substances and the wide variety of type of cheese-making process, some authors have tried to develop

theoretical models about the partitioning of drugs in milk fractions (milk fat and skimmed milk, whey and curd, etc.), associating the results obtained only to the physicochemical properties of the studied compounds. Hakk *et al*. (2016) calculated the distribution of radioactively marked antibiotics between skimmed milk and the fat fraction, reporting that benzylpenicillin, erythromycin, sulfadimethoxine and oxytetracycline were transferred to the skimmed milk fraction with a high percentage of 90%. Also, Shappell *et al*. (2017), based on microscale cheese-making with radiochemical analysis, found that the distribution of antibiotics in the curd ranged from 12% for benzylpenicillin to approximately 28% for sulfadimethoxine, attributing this partition to a greater affinity of the studied veterinary substances to the aqueous fraction constituted by whey.

Considering the lack of information about the transfer of antibiotics from milk to cheese and whey, it is, therefore, of great interest to investigate the behaviour of a wide-range of antibiotic families with different physicochemical properties using an accurate quantitative methodology for the analysis.

I.3. METHODS FOR THE DETECTION OF ANTIBIOTICS IN MILK AND DAIRY PRODUCTS

I.3.1. General Aspects

The presence of antibiotics in milk poses a risk to food safety and, consequently, within the control programs for raw milk, there is a section dedicated to monitoring residues of antimicrobial drugs. In order to carry out an effective detection of antibiotics, it is necessary to apply the most appropriate analytical strategy depending on the control stage that combines currently available methodologies to detect the greatest number of substances at an acceptable economic cost.

To ensure the safety of milk and dairy products in the EU, an integrated control system with shared responsibilities for farmers, processors and food inspection is used. The control program for antibiotic residues in milk is usually carried out in two steps: a primary screening to detect potentially non-compliant samples and a second confirmation phase to identify the molecule present in the sample and to quantify it (Figure 12). In this sense, the International Dairy Federation (IDF) published the Guidance on the application of screening and confirmatory methods in integrated dairy chain management for the detection of antibiotic residues in milk (IDF, 2014a).

The first methods for the detection of antibiotic residues in milk were used around the 1950s and consisted of evidence of microbial growth inhibition (Bishop and White, 1984). Since then, the performance characteristics of these methods such as the

rapidity of response, accuracy and sensitivity have been improved and, also, several screening methods based on microbiological and immunological techniques, or protein receptors have been developed, that greatly reduce trial times. Currently, new technologies, electrochemical and optical immunosensors, flow cytometry-based immunoassays and biochip array technology applications have emerged (Suárez-Pantaleón *et al*., 2014), offering a very promising future in the detection of residues in food.

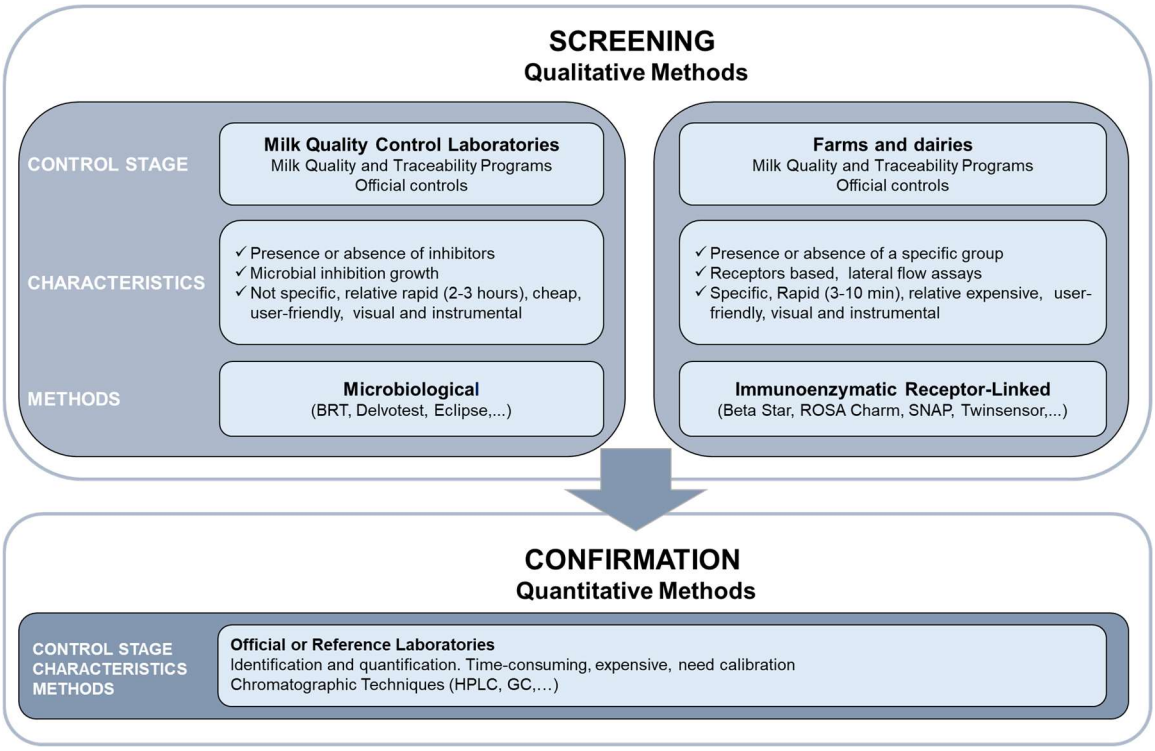


Figure 12. Classification of analytical methods to detect antibiotics in milk

Source: Berruga *et al.* (2016)

Commission Decision 2002/657/EC (EC, 2002b) provides rules for the analytical methods (routine and reference methods) to be used in the testing of official samples and specifies common criteria for the interpretation of results. The Decision also classifies the analytical methods for the detection of inhibitory substances in foodstuffs such as milk into qualitative methods and quantitative methods to be applied in the screening and confirmatory steps.

Moreover, the Community Reference Laboratories for residues (CRLs) have published the “Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines" (CRLs, 2010), that supplements Commission Decision 2002/657/EC (EC, 2002b) and describes two distinct phases in the validation process:

the initial validation of screening methods in the originating laboratory and the shortened or 'abridged' validation of these methods in the receptor laboratory.

Qualitative methods are used for screening antibiotic residues in milk, that allow the detection of the presence/absence of any antibiotic residues in milk above the legally fixed safety limits. These screening tests are largely immunological, microbiological, and receptor-binding assays commercially available. For the confirmation phase, there are physicochemical methods able to identify unequivocally and, if necessary, quantify drugs at the level of interest. These quantitative methods are based mostly on the chromatographic separation of residues, particularly liquid chromatography coupled to mass spectrometry (LC-MS/MS, LC-HRMS).

Currently, most of the screening and confirmatory methods have been developed for cow milk and subsequently validated in other species, but hardly studies have been carried out in dairy products such as whey.

I.3.2. Characteristics of Methods for the Detection of Antibiotics

Table 5 summarizes the different types of existing antibiotic analytical methods classified according to their principle, techniques involved, precision, specificity, and practical details on time, cost, equipment and operator skills required.

Microbial screening methods or inhibitor tests are preferably used in quality control laboratories. Currently, most commercial microbial screening methods use *Geobacillus stearothermophilus var. calidolactis* as bacteria test, being highly sensitive in the detection of β-lactams. These inhibitor tests have been developed and optimized for their use in cow milk (Stead *et al*., 2008; Perme *et al*., 2010), and later evaluated in sheep and goat's milk (Sierra *et al*., 2009a,b; Beltrán *et al*., 2015; Romero *et al*., 2016). Additionally, Mata *et al*. (2016) coupled the microbial inhibitor test Eclipse Farm 3G to an e-Reader device, to be used in farms and dairies for the detection of antibiotics in cow milk. Giraldo *et al*. (2019) assessed this method in sheep and goat's milk.

The specificity of inhibitor tests may be affected by the presence of natural inhibitors in milk such as immunoglobulins, lactoferrin, or lysozyme (Crosson *et al*., 2010), Somatic Cell Counts (SCCs) (Beltrán *et al*., 2015), and other compounds from its chemical composition (fat, protein, lactose, total solids) (Althaus *et al*., 2003). Moreover, the use of preservatives in milk, principally potassium dichromate and acidiol (Molina *et al*., 2003) and the residues of detergents or disinfectants (Romero *et al*., 2014), increase the percentage of false-positive results.

Table 5. Antibiotic analytical methods: principles and results delivered

	Screening					Confirmation
Result	**Qualitative (positive/negative)**		**Semi-quantitative. Estimated concentration**		**Quantitative. Accurate concentration**	**Confirms antibiotic identity. Accurate concentration**
Detection type	Biological	Biochemical	Biological	Biochemical	Physico-chemical	Physico-chemical
Principle	Cellular metabolic response	Molecular antibiotic and ligand interactions	Cellular metabolic response	Molecular antibiotic and ligand interactions	Separation individual antibiotics-physical detection	Separation individual antibiotics-physical detection
Typical techniques	Bacterial growth inhibition	Immunoassay	Bacterial growth inhibition	Immunoassay	Chromatography + Spectrometry	Chromatography + Mass spectrometry
Methods	Incubation: solution, plates, ampoules	Lateral flow, ELISA, biochip, radioimmunoassay	Plate test/inhibition zone	Specific ELISA	LC-UV, LC-FL, LC-ECD, LC-MS, GC-FID	LC-MS/MS or LC-HRMS
Interpretation	Visual-colorimetric readers	Visual-colorimetric readers	Visual, size of inhibition zone	Colorimetric with calibration curve	UV or FL-spectrometry, with calibration curve	Mass spectrometry with calibration curve
Analysis time	1-3.5 h	2-10 min to 3 h	Several hours	2-4 h	1-2 h	1-2 h
Precision	-	-	Low	Medium	High	High
Specificity	Not specific: antibiotics-families	Specific: antibiotics-families	Not specific: antibiotic families	Specific: single antibiotic	Identification/determination individual antibiotics	Identification/determination individual antibiotics
Range of antibiotics analysed	Large range	One or more antibiotics	Medium/large range	Single antibiotic	Small/medium range	Medium/large range
Cost	Cheap	Cheap/medium	Cheap	Medium	Medium/expensive	Expensive
Sample preparation	None or simple	None or simple	Medium	Simple to complex	Complex	Complex
Equipment/complexity	Simple	Simple or medium	Simple	Medium	Medium	High
User skills / training	Low	Low/Medium	Low	Medium	Medium/high	High
Typical application	From farm to dairy	From farm to dairy	Collection centre to dairy	Dairy silo	Dairy silo	Finished product

Source: IDF (2014a)

Some of prior described studies demonstrated the lack of sensitivity of *Geobacillus stearothermophilus* bacteria for non β-lactams antibiotics and these tests were, therefore, not able to detect aminoglycosides, macrolides and quinolones, at or below MRLs fixed in milk. Thus, for the improvement of the sensitivity of inhibitor tests, some authors proposed the combination of different bacteria tests in microtiter plates as an alternative to enhance the detection rate of potential drug residues in milk, in a simple, relatively fast (6 hr.), and economical manner.

Nagel *et al*. (2013a) developed a microbial system in microtiter plates of *Geobacillus stearothermophilus*, *Bacillus cereus* and *Bacillus subtilis* as bacteria test to achieve detection limits close to MRLs established in milk for β-lactams, quinolones, sulfonamides and tetracyclines. In sheep milk, Nagel *et al*. (2012) combined *Geobacillus stearothermophilus* and *Bacillus* subtilis to improve the sensitivity of macrolides and quinolones. Also, new combinations of mesophilic bacteria have been reported by Tumini *et al*. (2019) using *Geobacillus stearothermophilus* together *Bacillus licheniformis* and *Bacillus megaterium*, reaching levels close to MRLs for β-lactams, macrolides, quinolones, sulfonamides and tetracyclines, in 4-5.5 hours of incubation.

Recently, some authors have researched the use of thermophilic bacteria to reduce the total incubation period. Thus, Nagel *et al*. (2013b, 2014) using bioassays with *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*, respectively, reached suitable results (CCβ≤ MRLs) for the detection of β-lactams in milk, in less than 2.5 hours.

Other efforts to increase sensitivity of *Geobacillus stearothermophilus*, in particular towards non β-lactam drugs, were related to the addition of chelating agents or antifolates, like trimethoprim, into the culture medium, to enhance the detection of tetracyclines and sulfonamides in milk, respectively (Adriany *et al*., 1995; Langeveld *et al*., 2005; Nagel *et al*., 2013c).

Regarding biochemical qualitative methods, receptor-binding assays are most commonly applied for screening individual or simultaneous antibiotics in farms and dairies as they are easy and fast to handle (less than 10 minutes). They are based on the union of the antibiotic present in milk to specific protein receptors, conjugated to an enzyme, to include a detection spectrum normally limited to one specific group of antibiotics. The results can be interpreted visually by comparison of colored lines or spots appearing as consequence of the interaction of the analyte and the receptors contained in the test, but they also offer the possibility of using automatic equipment which provides more objective readings.

Rapid tests were initially validated in cow milk showing high specificity, low false-positive rate, and CCβs at or below MRLs for most antibiotics (Perme *et al*., 2010; Reybroeck *et al*., 2010; Salter *et al*., 2011), some of these commercial assays have also been evaluated in sheep and goats (Beltrán *et al*., 2014a,b). The information about the performance of receptor-binding assays in dairy products like whey is rather scarce or even non-existent.

As previously mentioned, screening microbial inhibition tests are very sensitive to β-lactam antibiotics, but they are not as sensitive to other groups of substances, like aminoglycosides, macrolides, quinolones, sulfonamides and tetracyclines. In addition, the information on the identity of the residue obtained with the screening method is insufficient to know which specific antibiotic or antimicrobial family was used. This fact makes it necessary to use a post-screening technique that links the presumptive study with the final confirmation and subsequent quantification. Currently, microbiological semi-quantitative methods (Table 5) can be used as a post-screening technique, which have several advantages for their simplicity, low cost and detection capacity. These methods, called "Multiresidue Bioassays" or "Multiple or Multiplate Microbiological Systems", are based on the diffusion of the sample under study, on an agar with different pH conditions and where different microorganisms that are sensitive to a specific residue groups have been inoculated. Sensitivity manifests itself with the appearance of a halo of inhibition around the sample, which can be quantified.

IDF bulletin no. 258 (IDF, 1991) describes one of the multiresidue bioassasys known as "3 plates": *Geobacillus stearothermophilus var. calidolactis* (β-lactams and tetracyclines), in another *Bacillus subtilis* (aminoglycosides and macrolides), and in the third *Bacillus megaterium* (chloramphenicol and sulfonamides). Also, the IDF describes a “6 plates” system (IDF, 1991), in which the sample is placed in cylindrical perforations in the agar or on discs, in 6 plates containing *Bacillus cereus*, *Bacillus subtilis* at pH 6, *Bacillus subtilis* at pH 8, *Sarcina lutea*, *Escherichia coli*, and *Geobacillus stearothermophilus var. calidolactis*. Each plate is incubated at the optimum temperature and time. If in any of the plates an inhibition zone of more than 1 mm is observed around the discs or perforations, the result is positive.

In recent years, EU Reference Laboratories for veterinary drug residues have developed different multiresidue bioassays. Thus, the RIKILT EU Reference Laboratory (Wageningen, The Netherlands) developed a multiplate diffusion bioassay, specific for cow milk, which uses 6 or 7 plates. This bioassay is a very complete technique that allows the detection of almost all types of antimicrobial families in milk with great sensitivity (Nouws *et al*., 1999). More recently, studies validated this

multiplate microbial method (NAT-screening) in kidney (Pikkemaat *et al*., 2008) and also in kidney and muscle as post-screening alternative (Pikkemaat *et al*., 2009).

In turn, the Community Reference Laboratories for residues (CRLs) (ANSES Fougères Laboratory, Fougères, France) also developed the Screening Test for Antibiotic Residues (STAR) constituted by five different plates using *Geobacillus stearothermophilus var. calidolactis* pH 7.4 for β-lactams and sulfonamides, *Bacillus subtilis* pH 7.2 for aminoglycosides, *Kocuria varians* pH 8 for macrolides, *Escherichia coli* pH 8 for quinolones, and *Bacillus cereus* pH 6 for tetracyclines. The STAR method has been validated in accordance with Commission Decision 2002/657/EC (EC, 2002b) criteria for the detection of antibiotics in milk by Gaudin *et al*. (2004) and meat (Gaudin *et al*., 2010). It is the official technique for the detection of antibiotics and sulfonamides in milk intended for human or animal nutrition and also for animal tissue. Although the STAR protocol is less sensitive for β-lactam antibiotics than microbial commercial tests, it is able to detect macrolides, lincosamides, quinolones, sulfonamides, tetracyclines and miscellaneous drugs (trimethoprim and baquiloprim) at lower concentrations and it has been applied in official control laboratories with the aim of reducing the number of antibiotics to be tested for confirmation, being less time-consuming as well as more economical.

Finally, the quantitative analysis of veterinary substances is based on the chromatographic separation of drugs, especially Liquid Chromatography (LC), followed by Diode Array Detectors (DAD), Fluorescence (FLD), Ultraviolet (UV), or Mass Spectrometry (MS). Recently, the LC-MS methodology has become the most common due to its greater sensitivity and selectivity in the multiresidue analysis.

One of the important limitations of mass spectrometry is the matrix effect (ion suppression or signal enhancement phenomenon) that can be made worse by the lack of specificity in sample treatment (Freitas *et al*., 2015). However, for multiresidue analysis, the tendency is towards using more generic sample preparations as very different physico-chemical substances are considered in this type of extraction, and the use of isotopically labelled Internal Standard (IS) as well as the application of matrix-matched calibration curves are an alternative for the reduction of matrix effect.

Concerning the extraction, combinations of acetonitrile (ACN), which allows to the precipitation of proteins and extraction with fewer interferences than methanol (Cepurnieks *et al*., 2015), with other aqueous solvents are frequently used for the analysis of antibiotics in milk. For example, the EDTA solution prevent chelation complexes with cations present in solution increasing recovery percentages for

tetracyclines, fluoroquinolones and macrolides (Aguilera-Luiz *et al.*, 2008; Wang *et al.*, 2015); water in combination with acetonitrile provides higher extraction efficiency for both polar and non-polar compounds (Wang *et al*., 2015); and acetate buffer allows keeping the acidity of the medium stable and favours salting-out stratification with acetonitrile (Li and Wu, 2017). On the other hand, generic clean-up alternatives combining existing purification procedures have been investigated. For example, in recent years, the use of Dispersive Solid Phase Extraction (DSPE) with C18, which combines rapidity of QuEChERS and the principle of binding co-extractives components from matrix onto the sorbent of SPE, has been applied in a multiresidue study in dairy products (Schwaiger *et al*., 2018).

The unspecificity of a multiclass multiresidue method could be compensated for instrumental analysis selectivity. High Performance Liquid Chromatography (HPLC) and Ultra High Performance Liquid Chromatography (UHPLC) reduce run times through the use of columns with stationary phase particles smaller than 2.0 μm and improve resolution and sensitivity, especially when the analysis includes several compounds with different physicochemical properties, increasing separation and narrow peak widths.

Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS), particularly triple quadrupole (QQq), has become the most popular technique for the screening and confirmation of veterinary residues in biological matrices like milk or dairy products due to its elevated sensitivity and selectivity (Gómez Pérez *et al*., 2013; Zhao *et al*., 2017; Schwaiger *et al*., 2018). However, a laborious SRM (Simple Reaction Monitoring) or MRM (Multiple Reaction Monitoring) method set-up is required for the selection of precursor and product ions and the number of analytes that can be detected in a one injection is limited.

Currently, the use of High Resolution Mass Spectrometry (HRMS) in milk, mainly Time of Flight (TOF) mass spectrometers (Wang *et al*., 2007; Stolker *et al*., 2008) and Orbitrap (Exactive^{TM}) technology (Cepurnieks *et al*., 2015; Wang *et al*., 2015; Moretti *et al*., 2016), has emerged as an alternative to tandem mass spectrometry. HRMS allows full scan approaches with exact mass measurement and the simultaneous detection of a wide-range of veterinary residues, with an enhancement of selectivity as it resolves isotope peaks and isomeric ions in complex matrices. In the case of the Orbitrap analyser, it offers resolutions up to 100,000 full width at half maximum (FWHM) at 2-5 parts per million mass error (ppm error) of mass accuracy. Furthermore, the use of full scan allows the retrospective analysis of non-preselected and unknown analytes (“post-targeted” analysis) (Wang *et al*., 2018). However, few studies have been conducted on

the detection of antibiotics in dairy products by HRMS, particularly using Orbitrap technology (Igualada et al., 2017).

I.3.3. Criteria for Validation of Screening Methods for Detection of Antibiotics

Commission Decision 2002/657/EC (EC, 2002b) establishes the performance characteristics that should be verified for the validation of qualitative and quantitative analytical screening and confirmation methods to be used in the testing of samples by official control laboratories and specifies which criteria should be commonly used for the interpretation of the analytical results obtained (Table 6).

Table 6. The performance characteristics for the validation of analytical methods

Parameter	Analytical methods			
	Qualitative		Quantitative	
	Screening	Confirmatory	Screening	Confirmatory
Detection Capability (CCβ)	✔	✔	✔	✔
Decision Limit (CCα)		✔		✔
Trueness/Recovery				✔
Precision			✔	✔
Selectivity/ Specificity	✔	✔	✔	✔
Applicability/ Ruggedness/ Stability	✔	✔	✔	✔

✔ : determination is mandatory.

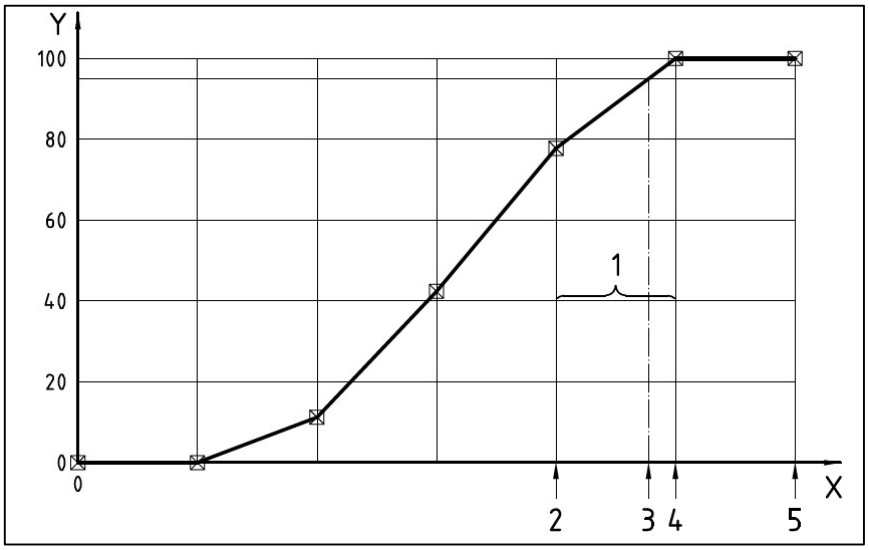
Source: Commission Decision 2002/657/EC (EC, 2002b)

The performance characteristics that should be evaluated for the validation of qualitative and quantitative screening methods used in the present thesis are explained as follows.

Detection Capability (CCβ) means the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β. In the case of substances with an established permitted limit, the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1-β (EC, 2002b).

For the calculation of the CCβ of a microbial test or receptor-binding assay for screening antibiotic residues in milk, the International Dairy Federation (ISO/IDF 2003a,b) recommends a calculation based in a dose-response curve (Figure 13) from the positive frequencies for each concentration assessed, making a total of 10-20 replicates if the interpretation of the test results is made visually, and 3-5 replicates if it is photometric. Test concentrations must include a negative control (antibiotic-free milk

sample), a concentration of at least 1.5 to 2 times higher than the concentration that is expected to be positive and a concentration equivalent to the MRL. The CCβ is calculated as the concentration which corresponds to the intersection of the dose-response curve with the line that represents the 95% of positive results.



X: antimicrobial content (µg/kg); Y: positive results (%); 1: range of detection limit; 2: expected positive; 3: detection limit; 4: EU-MRL; 5: 1.5 x expected positive.

Figure 13. Dose-response curve model for the calculation of the detection limit of the screening methods

Source: ISO/IDF (2003a,b)

Similarly, Community Reference Laboratories for residues (CRLs, 2010), which supplements Commission Decision 2002/657/EC (EC, 2002b), defines CCβ as the concentration at which only ≤ 5% false compliant results remain. For authorized analytes, the concentration at which a screening test categorizes the sample as screen positive (potentially non-compliant) is called Screening Target Concentration (STC) and it must be at or below MRL, and the total number of milk samples to be analysed depends on its relationship with the corresponding MRL (Table 7).

Table 7. Guidelines for the calculation of the Detection Capability (CCβ) according to Community Reference Laboratories for residues (CRLs, 2010)

STC (µg/kg)	Number of replicates	False complaints permitted (≤ 5%)	CCβ (µg/kg)
0.5 EU-MRL	20	1	≤ 0.5 EU-MRL
0.5 EU-MRL< STC≤ 0.9 EU-MRL	40	2	0.5- 0.9 EU-MRL
0.9 EU-MRL< STC≤ 1 EU-MRL	60	3	> 0.9- 1 EU-MRL

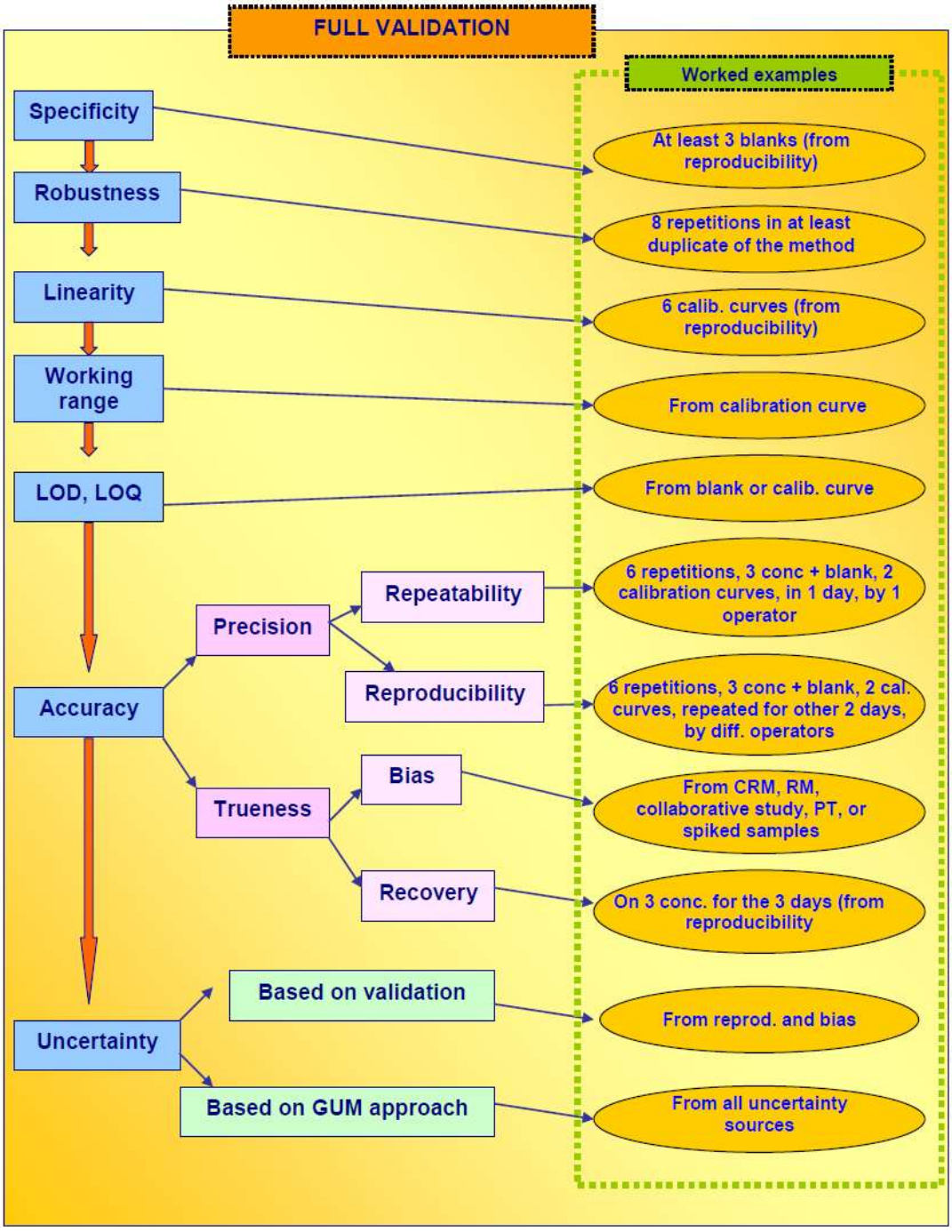
STC: Screening Target Concentration; CCβ: Detection Capability; EU-MRL: European Union-Maximum Residue Limit (EU, 2010).

In the case of semi-quantitative screening tests, CRLs describes two different approaches for the calculation of the cut-off, that is defined as the response or signal from a screening test which indicates that a sample contains an analyte at or above the Screening Target Concentration. The first defines cut-off as the lowest response for the spiked samples that does not overlap with the highest response for the blank samples, while the second is a statistical approach that considers β error of 5% and it requires the calculation of the Threshold value (T) and the cut-off factor (Fm). T and Fm are calculated according to the following equations: $T = B + 1.64 \times SD_b$ (*B* is the mean response of the blank samples and SD_b the standard deviation of blank samples) and $Fm = M - 1.64 \times SD$ (*M* is the mean response of the spiked samples and *SD* the standard deviation of spiked samples). Fm > B is required to validate the screening method and Fm > T to determine an acceptable false-positive (FP) rate < 5%.

Another performance characteristic of analytical methods is Precision that is determined by within-laboratory reproducibility, obtained in the same laboratory under predetermined conditions (method, test materials, operators, environment) over justified long time intervals; and by repeatability, under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment (Figure 14). The within-laboratory reproducibility and repeatability are evaluated by calculating the percentage of Relative Standard Deviation (RSD_R and RSD_r, respectively), in comparison to Horwitz Equation (EC, 2002b).

Regarding Specificity of the methods, this parameter refers to the ability of a test to distinguish between the analyte being measured and other substances. The specificity is predominantly a function of the measuring technique described but can vary according to class of compound or matrix (EC, 2002b). Specificity is associated with the presence of false-positive results and for its determination a large number of milk samples (at least 20 samples) from animals not treated with veterinary products should be analysed to detect the presence of possible interferences and to estimate their effect on the compounds of interest (ISO/IDF, 2003a,b; CRLs, 2010).

Finally, Ruggedness means the susceptibility of an analytical method to changes in experimental conditions. Minor modifications (stability of reagents, composition of the sample, pH, temperature) and major changes (species, matrices, or sampling conditions) that could affect the analytical result should be indicated (EC, 2002b).



LOD: Limit of Detection; LOQ: Limit of Quantification.

Figure 14. Main steps for the validation of analytical methods according to Commission Decision 2002/657/EC (EC, 2002b)

Source: Bratinova *et al*. (2009)

According to Commission Decision 2002/657/EC (EC, 2002b) and Community Reference Laboratories for residues (CRLs, 2010), ruggedness could be evaluated by means of the analysis of at least 10 different blank samples and 10 different samples spiked at the level of interest, to evaluate the Detection Capability (CCβ) and the specificity for this analyte at different experimental conditions. When it has been

demonstrated that one factor influences the performance of the method, the performance characteristics (specificity and CCβ) should be determined for this factor.

The evaluation of performance characteristics of milk qualitative and quantitative screening methods by Commission Decision 2002/657/EC (EC, 2002b) in dairy products allows to assess the transfer of antibiotics from milk to cheese and whey and, at the same time, to know the suitability of these methods to screen antibiotic residues in these matrices. After that, it would be possible to establish the most appropriate analytical strategy whether necessary the inclusion of new quality control steps in the dairy industry and even the establishment of MRLs for such dairy products to guarantee consumer safety and to avoid health hazards.

Chapter II. Objectives

The presence of veterinary drug residues in milk and dairy products is a great concern for food safety may led to allergic reactions, disturbances in the intestinal flora and the development of antimicrobial resistance.

In the case of the cheese-making process, antibiotics present in milk could be transferred to whey, which is the major by-product in the dairy industry. Whey is principally destined to animal feed, the food industry and pharmaceutical applications. In recent years, the valorization of whey has increased from a technological point of view to obtain valuable biotechnological products. However, whey containing antibiotics poses a potential risk for human and animal health. Moreover, the presence of antimicrobials in whey has not been regulated, and neither is there an analytical strategy for its control in place.

Therefore, the aim of this thesis was to evaluate the transfer of antibiotics from milk to cheese and whey by means of the quantification of an elevated number of antibiotics, commonly used in livestock, and establish the most appropriate quality control strategy for their screening in whey.

With this aim in mind, the following specific objectives were defined:

1. To develop a new multiresidue method for the detection and quantification of a wide range of antimicrobial families in fresh cheese and whey.

2. To study the transfer of antibiotics from goat's milk to rennet curd and whey.

3. To assess microbial and receptor-binding commercial tests for the screening of antibiotics in whey.

4. To establish the performance of microbial bioassays in microtiter plates with a dichotomous response for the detection of antibiotics in whey.

5. To characterize a large number of antibiotic groups in whey using a semi-quantitative detection method based on a microbiological multiplate system.

These objectives were reached through various experiments presented in Chapter III and Chapter IV corresponding to each one of the five objectives established.

Chapter III. Distribution of Antibiotics from Raw Goat's Milk to Cheese and Whey

III.1. VALIDATION OF A MULTIRESIDUE METHOD BY UHPLC-HRMS FOR ANTIBIOTICS DETECTION IN FRESH CHEESE AND WHEY

III.1.1. Introduction

The safety limits for antibiotic residues have not been established for dairy products, and consumers might be exposed to significant amounts of these residues, even higher than those indicated for milk in concentrated milk products like cheese (Cabizza *et al.*, 2017; Quintanilla *et al.*, 2019a).

Liquid Chromatography-Mass Spectrometry (LC-MS) is commonly used for the screening of antibiotics in milk. The determination of a wide number of drugs with different physicochemical properties requires non-specific extraction and clean up procedures. Overall, organic and aqueous solvent combinations, mainly acetonitrile with water or with different type of buffers, are commonly used for the extraction of veterinary residues from food. Recently, Dispersive Solid Phase Extraction (DSPE) has been proposed to purify antibiotics from dairy samples, resulting in the detection of a larger number of antibiotics from different families (Schwaiger *et al.*, 2018).

The lack of specificity of sample treatments is compensated by the selectivity of the instrument. High and Ultra High Performance Liquid Chromatography (HPLC/UHPLC) improve resolution, obtaining greater separation and narrow peaks widths. Triple Quadrupole Mass Spectrometer (QQq) has been frequently used for antibiotics analysis in milk, cheese and whey (Gómez Pérez *et al*., 2013; Rossi *et al*., 2017; Zhao *et al*., 2017) due to its high sensitivity and selectivity, but the number of substances that can simultaneously be analysed is limited by working at unit-resolution scanning. Alternatively, in the last decades, the inclusion of full scan approaches using High Resolution Mass Spectrometry (HRMS) by means of Time of Flight (TOF) (Wang *et al*., 2007; Stolker *et al*., 2008) and Orbitrap (Exactive^{TM}) (Romero-González *et al*., 2011; Konak *et al*., 2017) analysers has allowed the unlimited determination of veterinary substances in one injection, with accurate exact mass measurement and resolution of isotope peaks and isomeric ions.

To the best of our knowledge, studies regarding the use of HRMS for the detection of veterinary substances in dairy products have been scarcely reported. Therefore, the aim of this study is to develop a new quantitative approach for multiresidue antibiotics screening in fresh cheese and whey complying with the specifications established by the Commission Decision 2002/657/EC (EC, 2002b), using an Orbitap Exactive^{TM} analyser.

III.1.2. Material and Methods

III.1.2.1. Experimental Design

This experiment was conducted entirely in the facilities of Public Health Laboratory of Valencia-FISABIO. In order to evaluate the UHPLC-HRMS methodology using the Orbitrap Exactive™ analyser, samples of fresh cheese and whey from cow, goat and sheep species were used. The validation was carried out according to the criteria specified in Commission Decision 2002/657/EC (EC, 2002b) and, more specifically, the "Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines" (CRLs, 2010). The performance characteristics to be determined in screening quantitative methods are Detection Capability (CCβ), selectivity/specificity, and precision (repeatability and reproducibility) Furthermore, data about trueness (recovery) of the method were also reported.

For the validation, thirty six antibiotics (Table 8) from β-lactam, macrolide and lincosamide, quinolone, sulfonamide and tetracycline families were evaluated at three different concentrations considering Maximum Residue Limit established in milk: 0.25 or 0.50 MRL, MRL or 2 MRL, and 4 MRL or 8 MRL, corresponding to Low Quality Control (LQC), Medium Quality Control (MQC) or High Quality Control (HQC).

An optimization of the extraction procedure was carried out with four different solvent combinations: acetonitrile (ACN), water with ACN (20:80, v/v), McIlvaine EDTA-buffer with ACN (20:80, v/v), and acetate buffer at pH 5.2 with ACN (20:80, v/v). Spiked cheese and whey samples were fortified at 0.5 MRL fixed in milk and analysed with the proposed solvents four times in two days.

Prior to the validation process, to confirm whether isotopically labelled Internal Standard (IS) could correct the influence from the extraction procedure and the matrix, percentages of matrix effect and recovery percentages were evaluated with and without the IS at the interest levels (LQC, MQC and HQC) following the systematic proposed by Matuszewski (2006), cited by León *et al.* (2012). Three different types of samples: A: antibiotics and IS in redissolve solution (water/methanol (90/10, v/v) with 0.1% of formic acid), B: blank fresh cheese and whey extracts obtained by the optimized extraction and clean-up method and redissolved with 300 µL of the standards solution used in sample A, and C: fresh cheese and whey samples spiked before the extraction procedure, were prepared for each matrix (fresh cheese and whey) and were analysed in triplicate in two days. Matrix effect (ME) and absolute recovery (RE) percentages were obtained by comparing the absolute peak areas (area analyte) of A, B and C: (ME%= B/A × 100 and RE%= C/B × 100).

Table 8. Antibiotics used for validation parameters in fresh cheese and whey

Antibiotics	Reference	EU-MRL (µg/kg)	LQC - MQC - HQC (µg/kg)	
			Cheese	Whey
β-lactams				
Ampicillin	59349[1]	4	1-4-16	1-4-16
Benzylpenicillin	46609[1]	4	2-8-32	2-8-32
Cloxacillin	46140[1]	30	7.5-30-120	7.5-30-120
Dicloxacillin	46182[1]	30	15-60-240	15-60-240
Nafcillin	32071[1]	30	15-60-240	15-60-240
Oxacillin	46589[1]	30	7.5-30-120	7.5-30-120
Cefalexin	33989[1]	100	25-100-400	25-100-400
Cefoperazone	32426[1]	50	25-100-400	25-100-400
Ceftiofur	34001[1]	100	25-100-400	25-100-400
Desfuroylceftiofur	D289980[2]	100	50-200-800	50-200-800
Penicillin G-D7*	32985[1]		100	100
Macrolides				
Erythromycin	46256[1]	40	10-40-160	20-80-320
Spiramycin	46745[1]	200	50-200-800	50-200-800
Neo Spiramycin	N390040[2]	200	50-200-800	50-200-800
Tilmicosin	33864[1]	50	12.5-50-200	12.5-50-200
Tylosin	33847[1]	50	12.5-50-200	25-100-400
Lincosamides				
Lincomycin	15443869[3]	150	37.5-150-600	37.5-150-600
Roxithromycin*	15599483[3]		100	100
Quinolones				
Danofloxacin	33700[1]	30	7.5-30-120	7.5-30-120
Enrofloxacin	33699[1]	100	25-100-400	25-100-400
Ciprofloxacin	33434[1]	100	25-100-400	25-100-400
Flumequine	45735[1]	50	12.5-50-200	12.5-50-200
Norfloxacin-D5*	CH001[4]		100	100
Sulfonamides				
Sulfacetamide	46770[1]	100	50-200-800	25-100-400
Sulfadiazine	35033[1]	100	25-100-400	25-100-400
Sulfadimethoxine	46794[1]	100	25-100-400	25-100-400
Sulfamerazine	46826[1]	100	25-100-400	25-100-400
Sulfamethazine	46802[1]	100	25-100-400	25-100-400
Sulfamethoxypyridazine	46858[1]	100	25-100-400	25-100-400
Sulfapyridine	31738[1]	100	25-100-400	25-100-400
Sulfaquinoxaline	45662[1]	100	25-100-400	25-100-400
Sulfathiazole	46902[1]	100	25-100-400	25-100-400
Sulfadimethoxine-D6*	SA001[4]		100	100
Tetracyclines				
Chlortetracycline	C4881[1]	100	50-200-800	25-100-400
4-epi-Chlortetracycline	268231000[5]	100	25-100-400	25-100-400
Doxycycline	33429[1]	100	25-100-400	25-100-400
Oxytetracycline	46598[1]	100	25-100-400	25-100-400
4-epi-Oxytetracycline	257711000[5]	100	50-200-800	25-100-400
Tetracycline	31741[1]	100	25-100-400	25-100-400
4-epi-Tetracycline	233121000[5]	100	50-200-800	25-100-400
Demeclocycline*	46161[1]		100	100

EU-MRL: European Union Maximum Residue Limit in raw milk (EU, 2010). LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control. *Isotopically labelled Internal Standard (IS). [1]Sigma-Aldrich Química, S.A. (Madrid, Spain); [2]Toronto Research Chemicals, Inc. (Toronto, Canada); [3]Honeywell Riedel-de Haën, AG. (Seelze, Germany); [4]WITEGA Laboratorien Berlin-Adlershof GmbH (Berlin, Germany); [5]Acros Organics (Geel, Belgium).

Moreover, percentages of matrix effect and recovery with IS correction (ME/IS and RE/IS) were calculated by using area ratios (area analyte/area IS). In this study, the same 5 Internal Standards were selected for fresh cheese and whey, according to a previous study on the detection of antimicrobials in milk carried out by Laboratory of Public Health of Valencia (Igualada *et al*., 2017).

Figure 15 schematizes the type and number of samples used for the optimization of extraction procedure, the evaluation of matrix effect and recovery percentages and for the validation parameters.

III.1.2.2. Fresh Cheese and Whey samples

Fresh cheese and whey samples were obtained from commercial cheese factories of the Comunitat Valenciana region (Spain). The samples were kept frozen at -20°C throughout the experiment until analysis.

The pH of fresh cheese and whey samples was measured by a conventional pH-meter Basic 20 (Crison, Barcelona, Spain). Whey composition (fat, protein, lactose, and total solids) was determined by MilkoScan 6000 (Foss, Hillerød, Denmark) and for cheese composition (fat, protein, salt and total solids content) a FoodScan infrared device (Foss, Foss Iberia, Barcelona, Spain) was used. In the case of whey, the fat and protein content was significantly lower than the percentages typically attributed to milk (Marques *et al*., 2011; Beltrán *et al*., 2015), and fresh cheese composition was according to commercial label data (Table 1, Annex). Whey composition analysis was carried out in the Interprofessional Laboratory of the Valencian Community (LICOVAL, UPV).

III.1.2.3. Antibiotics and Spiked Samples

In total, 36 antibiotics, frequently used for prophylaxis and treatment of bacterial diseases in dairy livestock, and 5 Internal Standards (IS) from six different antibiotic families (β-lactams, macrolides and lincosamides, quinolones, sulfonamides and tetracyclines) of high purity (≥ 90%) were studied and provided by different distributors. As shown in Table 8, the antibiotic concentrations ranged from 0.25 to 4 or from 0.50 to 8 times the MRL established for veterinary residues in milk (EU, 2010). In some cases, it was convenient to reduce quantification range to 0.25-2 MRL or 0.5-4 MRL in order to keep the linearity of matrix-matched calibration curves.

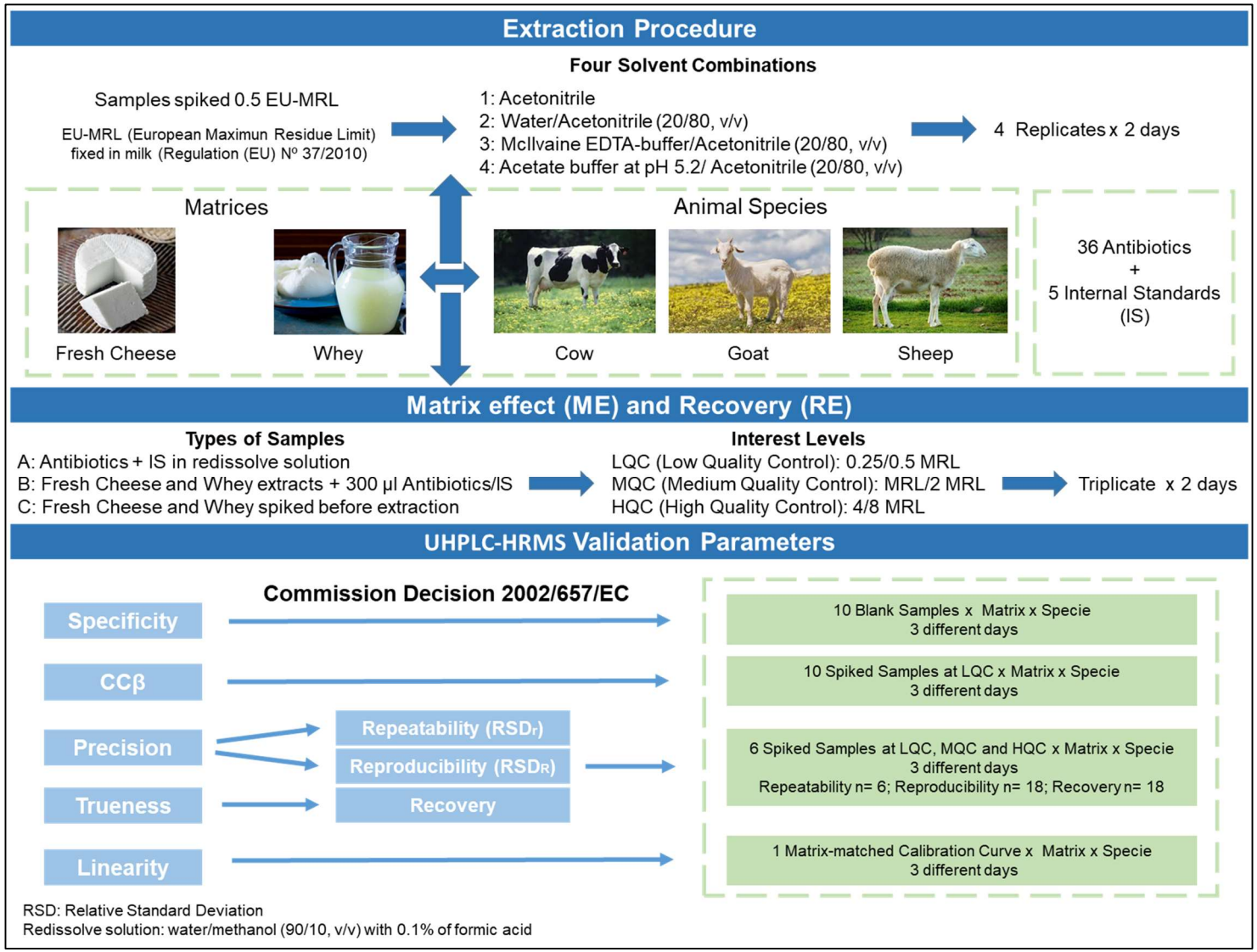


Figure 15. Experimental Design of the UHPLC-HRMS methodology validation for antibiotics detection in fresh cheese and whey

Individual stock solutions containing 250-1,500 µg/mL of the analytes were prepared by weighing each compound and dissolving it in methanol. They were stored at -20°C for a maximum of 6 months. Intermediate mixture solutions were also prepared in methanol at concentrations of 0.5-50 µg/mL and were stored at -20°C, with an expiration date of 2 months. Working solutions were prepared daily by diluting different volumes of the intermediate solution in 1 mL of water for each of the five levels of the calibration curve.

As isotopically labelled Internal Standard (IS), penicillin G-D7 was used for β-lactam antibiotics, roxithromycin for macrolides and lincosamides, norfloxacin-D5 for quinolones, sulfadimethoxine-D6 for sulfonamides and demeclocycline for tetracyclines. Stock solutions, which included 250-1,000 µg/mL of IS, and intermediate mixture solution, which was equivalent to 30 µg/mL, were also prepared in methanol. Working solutions for internal standards were made in water and contained 15 µg/mL.

Aminoglycosides were excluded from the validation process because this type of antibiotic is normally not detected in a multiresidue analytical method (Wang *et al.*, 2015; Schwaiger *et al.*, 2018), as their high hydrophilicity makes difficult the extraction by generic treatments and also their intrinsic polar character impedes the retention on conventional reversed-phase C18 columns. Additionally, aminoglycoside group requires mobile phases with volatile ion-pair reagents, such as heptafluorobutyric acid (HFPA) or pentafluoropropionic acid (PFPA), which are incompatible with the determination of other veterinary residues (Bogialli and Di Corcia, 2009).

III.1.2.4. Sample Preparation, Extraction, and Clean-Up Procedure

In a similar way to the validation process previously carried out in milk (Igualada *et al.*, 2017), the performance of four extraction procedures was assessed and the selection of the most appropriate strategy was carried up according to the number of extracted and identified analytes and by comparison of the highest absolute peak areas (analyte area).

Acetonitrile used for sample preparation was HPLC grade from Merck® (KGaA, Darmstadt, Germany) and HPLC grade water was in-house produced using a MilliQ system (Millipore).

The McIlvaine EDTA-buffer was prepared diluting separately 28.4±0.1 g of disodium phosphate anhydrous and 21±0.1 g of citric acid monohydrate, both from Sigma-Aldrich Química, S.A. (Barcelona, Spain) in 1 L of HPLC grade water. After 625 mL of the phosphate solution and 1 L of the citric acid solution were mixed, its pH was

verified at 4.0±0.1. Afterwards, 60±0.1 g of NA_2-EDTA (Sigma-Aldrich Química, S.A., Barcelona, Spain) were added to the combined solution and the mixture was thoroughly homogenized by stirring with a magnetic bar.

Also, 0.2 mol/L acetate buffer solution at pH 5.2 was prepared diluting 16.4 g of sodium acetate 3-hydrate in 1L of HPLC grade water, and the pH was adjusted with acetic acid glacial. Sodium acetate 3-hydrate was bought from Panreac® (Barcelona, Spain) and acetic acid was 100% Suprapur® quality from Merck® (KGaA, Darmstadt, Germany).

Finally, acetate buffer at pH 5.2 was adopted as the extracting solvent in this method in accordance to the number of analytes detected and the number of them that improved their signal, as described in III.1.3.1 The extraction and clean-up procedure are summarized in Figure 16.

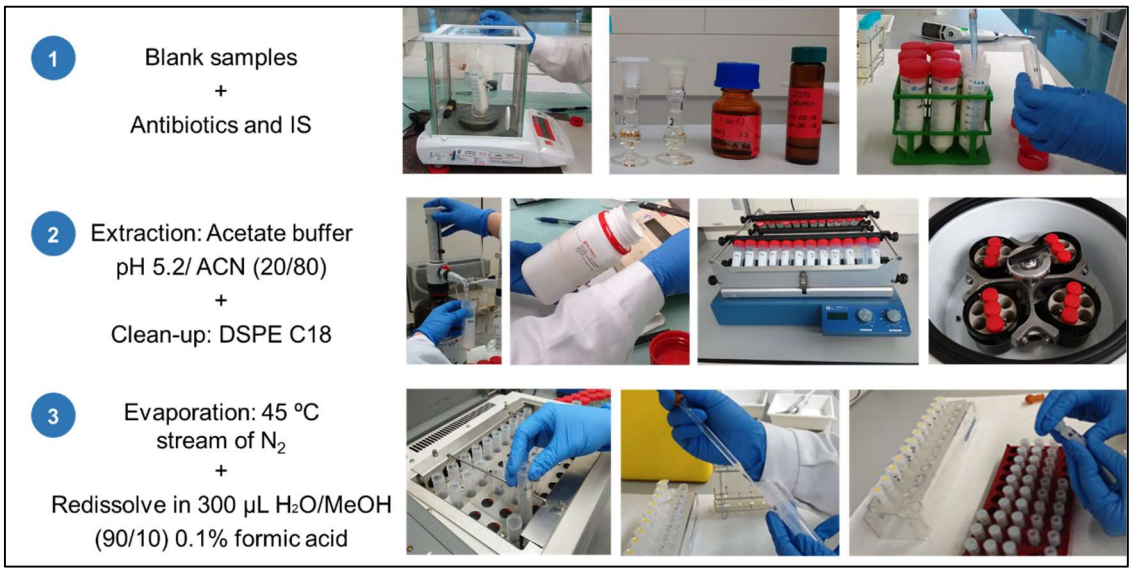


Figure 16. Sample Preparation: Extraction and Clean-up Procedure

Fresh cheese was minced and prior to the extraction process, both fresh cheese and whey were homogenized using a vortex for some seconds. For the preparation of spiked samples (C), 20 µL of working internal standards solution containing penicillin G-D7, roxithromycin, norfloxacin-D5, sulfadimethoxine-D6 and demeclocycline at 15 µg/mL were added to 3 g of blank samples. Then, 2 mL of 0.2 M acetate buffer at pH 5.2 and 8 mL of acetonitrile were added. The mixture was thoroughly homogenized using a vortex for 20 seconds. In the case of samples B, which are used in the matrix effect study, IS solution was added after blank extraction and clean-up. The extraction was performed after mixing for 10 minutes (Digital Roller Shaker. IKA, Staufen, Germany) and centrifuging (Allegra X-15 Centrifuge Performance. Beckman Coulter, Barcelona, Spain) at 5,000 rpm for 5 minutes at 15°C.

A Dispersive SPE (DSPE) clean up procedure was carried out: the supernatant was transferred into a 50 mL conical centrifuge tube (Merck®, KGaA, Darmstadt, Germany) that contained 0.5 g of octadecyl (C18) bonded silica adsorbent (Sigma-Aldrich Química, S.A., Barcelona, Spain) and after mixing for 5 minutes, it was centrifuged again in the same conditions.

The extracts were transferred into a 15 mL polypropylene tube (Merck®, KGaA, Darmstadt, Germany) and evaporated to dryness in a TurboVap® (Zymark®. LabX Media Group, Midland, Ontario, Canada) under a nitrogen stream at 45°C and next, reconstituted with 300 µL of water/methanol (90/10, v/v) containing 0.1% of formic acid and introduced in an ultrasonic bath (Letslab delivering solutions S.L.U., Barcelona, Spain) for 1 min. The redissolved extracts were placed in a microcentrifuge tubes of 1.5 mL and ultracentrifuged at 13,000 rpm for 10 minutes at 4°C (Centrifuge 5415-R. Eppendorf Ibérica S.L.U., Madrid, Spain). Finally, the supernatant from the eppendorf tube was placed in an ultrafree® MC centrifugal filter with microporous membranes of 0.2 µm and ultracentrifuged in the same conditions. The final extracts were placed into conical vials of 250 µL and 10 µL were injected into the UHPLC-HRMS system. All eppendorf tubes and vials were provided from Merck® (KGaA, Darmstadt, Germany).

III.1.2.5. UHPLC-HRMS Instrumentation and Parameters

Instrument conditions adopted for fresh cheese and whey in the present study were previously optimized for the same antibiotic families in animal tissue (PEE/LSPV/275), and milk (unpublished data) by Public Health Laboratory of Valencia.

Chromatographic separation was performed on an Accela UHPLC system (ThermoFisher Scientific, Bremen, Germany) equipped with a Kinetex C18 XB column (50 x 3.00 mm, 2.6 µm) (Phenomenex, Madrid, Spain). This chromatographic column is based on reserved-phase C18 with iso-butyl side chains (XB), being slightly more hydrophobic than only C18 phase, and, additionally, allows better peak shape and enhances separation of basic compounds under neutral and acidic conditions.

Separations were performed using a linear gradient programme: 0-8 min 98% A, 8-8.10 min 80% B and 8.10-9 min 1% A, 9-9.5 min 98% A, 9.5-15 min 98% A. Solvent A consisted of 0.1% formic acid aqueous solution, while solvent B consisted of methanol containing 0.1% formic acid. Formic acid was 98-100% Suprapur® quality, while methanol and water used as mobile phase were hypergrade quality for LC-MS from Merck® (KGaA, Darmstadt, Germany). Flow rate was set at 400 µL/min, at a temperature of 25°C and the injection volume was 10 µL. Data acquisition was performed by the Thermofisher Scientific's Xcalibur 2.1.0 software.

Mass analysis was performed on the Orbitrap mass spectrometer Exactive™ analyser (Thermofisher Scientific, Bremen, Germany) (Figure 17). The system was equipped with a heated electrospray ionization interface (HESI-II) and operated in positive and negative mode alternatively depending on the compound. The ESI source parameters were optimized by direct infusion of standard solutions of all the analytes (10 µg/mL) at a flow rate of 10 µL/min. The different parameters were manually varied to obtain the maximum total ion current signal (TIC) both in positive and negative operation mode within the mass range of 80-1,200 m/z.

Figure 17. Orbitrap Exactive™ Instrument

For the detection of the analytes in the Orbitrap Exactive™ analyser the elemental composition and the monoisotopic mass of each adduct, both in positive ($[M+H]^+$, $[M+2H]^{+2}$) and negative ionization mode ($[M-H]^-$), were calculated (Table 9).

Parameters of the ion source were as follows: spray voltage: 3.0 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow rate: 40 au; auxiliary gas flow rate: 10 au; skimmer voltage: 30 V; heater temperature: 300°C; capillary temperature: 260°C; capillary voltage: 50 V and tube lens voltage: 110 V.

The system operated in full-scan mode (65-500 Da) at a resolving power of 50,000 FWHM. The value of mass tolerance was set at 5 ppm error to find a compromise between a good sensitivity and appropriate selectivity, and no specific lock mass was used for internal mass axis correction (external mass calibration). For the Automatic Gain Control (AGC) the "Balanced" (10^6) setting was selected.

Each analyte was confirmed using relative retention time (RTT) calculated for corresponding internal standard and the accurate mass. To identify the different analytes, a retention time deviation of ±2.5% and a mass tolerance within 5 ppm error were established.

Table 9. Antibiotics, molecular formula, theoretical m/z, diagnostic ion, and retention time

Antibiotics	Molecular formula	Theoretical m/z	Diagnostic ion	Retention time (min)
β-lactams				
Ampicillin	$C_{16}H_{19}N_3O_4S$	349.11018	$[M+H]^+$ 350.11690	4.38
Benzylpenicillin	$C_{16}H_{18}N_2O_4S$	334.09928	$[M-H]^-$ 335.10600	6.94
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	435.06612	$[M-H]^-$ 436.07285	7.69
Dicloxacillin	$C_{19}H_{17}Cl_2N_3O_5S$	469.02715	$[M-H]^-$ 470.03387	8.03
Nafcillin	$C_{21}H_{22}N_2O_5S$	414.12549	$[M-H]^-$ 415.13222	8.05
Oxacillin	$C_{19}H_{19}N_3O_5S$	401.10509	$[M-H]^-$ 402.11182	7.50
Cefalexin	$C_{16}H_{17}N_3O_4S$	347.09453	$[M+H]^+$ 348.10125	4.17
Cefoperazone	$C_{25}H_{27}N_9O_8S_2$	645.14239	$[M-H]^-$ 644.13512	5.17
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	523.02846	$[M+H]^+$ 524.03629	6.03
Desfuroylceftiofur	$C_{14}H_{15}N_5O_5S_3$	429.02408	$[M+H]^+$ 430.03081	4.38
Penicillin G-D7*	$C_{16}H_{11}D_7N_2O_4S$	341.14266	$[M-H]^-$ 342.14994	6.90
Macrolides				
Erythromycin	$C_{37}H_{67}NO_{13}$	733.46124	$[M+H]^+$ 734.46852	6.81
Spiramycin	$C_{43}H_{74}N_2O_{14}$	842.51455	$[M+2H]^{+2}$ 422.26427	5.14
Neo Spiramycin	$C_{36}H_{62}N_2O_{11}$	698.43481	$[M+2H]^{+2}$ 350.22496	4.77
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	868.56658	$[M+H]^+$ 869.57332	5.81
Tylosin	$C_{46}H_{77}NO_{17}$	915.51970	$[M+H]^+$ 916.52643	6.91
Lincosamides				
Lincomycin	$C_{18}H_{34}N_2O_6S$	406.21376	$[M+H]^+$ 407.22104	3.52
Roxithromycin*	$C_{41}H_{76}N_2O_{15}$	836.52457	$[M+H]^+$ 837.53185	7.55
Quinolones				
Danofloxacin	$C_{19}H_{20}FN_3O_3$	357.14942	$[M+H]^+$ 358.15615	4.41
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.16507	$[M+H]^+$ 360.17180	4.38
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.13322	$[M+H]^+$ 332.14050	4.31
Flumequine	$C_{14}H_{12}FNO_3$	261.08067	$[M+H]^+$ 262.08740	6.81
Norfloxacin-D5*	$C_{16}H_{13}D_5FN_3O_3$	324.16460	$[M+H]^+$ 325.17188	4.19
Sulfonamides				
Sulfacetamide	$C_8H_{10}N_2O_3S$	214.04176	$[M+H]^+$ 215.04849	2.59
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.05245	$[M+H]^+$ 251.05972	3.11
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	310.07412	$[M+H]^+$ 311.08085	5.55
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264,06864	$[M+H]^+$ 265.07537	3.64
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	278.08429	$[M+H]^+$ 279.09102	4.12
Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	280.06356	$[M+H]^+$ 281.07029	4.29
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.05775	$[M+H]^+$ 250.06447	3.44
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	300.06865	$[M+H]^+$ 301.07537	5.72
Sulfathiazole	$C_9H_9N_3O_2S_2$	255.01417	$[M+H]^+$ 256.02089	3.37
Sulfadimethoxine-D6*	$C_{12}H_8D_6N_4O_4S$	316.11124	$[M+H]^+$ 317.11851	5.50
Tetracyclines				
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.11429	$[M+H]^+$ 479.12157	4.99
4-epi-Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.11429	$[M+H]^+$ 479.12157	4.48
Doxycycline	$C_{22}H_{24}N_2O_8$	444.15381	$[M+H]^+$ 445.16054	5.69
Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.14818	$[M+H]^+$ 461.15546	4.18
4-epi-Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.14818	$[M+H]^+$ 461.15546	3.92
Tetracycline	$C_{22}H_{24}N_2O_8$	444.15327	$[M+H]^+$ 445.16054	4.07
4-epi-Tetracycline	$C_{22}H_{24}N_2O_8$	444.15327	$[M+H]^+$ 445.16054	3.62
Demeclocycline*	$C_{21}H_{21}ClN_2O_8$	464.09864	$[M+H]^+$ 465.10592	4.47

*Isotopically labelled Internal Standard (IS).

III.1.3. Results and Discussion

III.1.3.1. Optimization of the Extraction and Clean-Up Procedure

The results of the different solvent extractions evaluated before the performance of the quantitative multiresidue screening method are summarized in Figure 18, according to the percentage of analytes not detected and those with the highest analytical response (peak area) by each sample treatment.

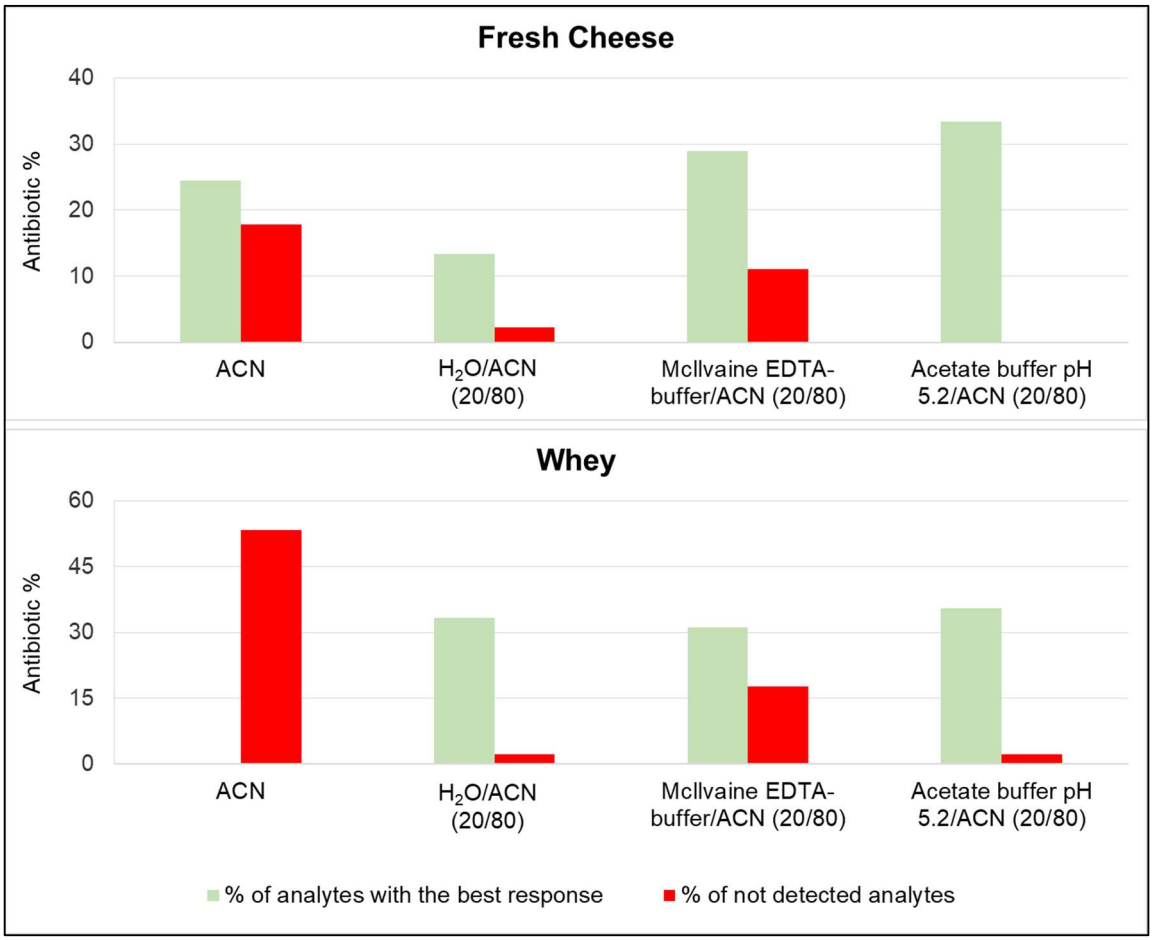


Figure 18. Percentage of antibiotics detected by the different extraction procedures in fresh cheese and whey

The use of acetonitrile as the only extraction solvent was not a recommendable option for most veterinary drugs since 17% and 51% of the antibiotics studied in fresh cheese and whey, respectively, were not detected. In the case of McIlvaine EDTA-buffer, some analytes were not extracted either, 12% and 17% in the same order. On the contrary, the water/ACN (20/80) (v,v) and acetate buffer pH 5.2/ACN (20/80) (v,v) solvent combinations allowed the detection of almost all studied antibiotics, except for benzylpenicillin for the first option and tylosin for the second in whey and desfuroylceftiofur for water/acetonitrile in the case of fresh cheese.

Regarding the best response of analytes, the highest percentage of antibiotics was obtained by acetate buffer pH 5.2 for the two matrices assessed. In the case of tetracyclines, their extraction was increased using McIlvaine EDTA-buffer as it could prevent the chelation with Ca^{2+} ions.

Finally, considering that the percentage of analytes with highest peak area, especially for β-lactam family that are essential for the treatment of mastitis in livestock, was obtained with the acetate buffer pH 5.2/ACN (20/80) (v,v): 39% for whey and 34% of the total of antibiotics in the case of fresh cheese, this last extraction method was finally selected. As a drawback, tylosin presented poor sensitivity and reproducibility using acetate buffer pH 5.2/ACN combination in whey and, thus, the macrolide had to be excluded for this matrix.

Definitely, in a multiresidue method, the determination of compounds with different physicochemical characteristics is a challenge that could be overcome by minimizing the specificity of sample extraction to avoid the loss of relevant target analytes, although the recovery of some of them will always be more favoured than others.

III.1.3.2. Matrix Effect and Recovery parameters

The isotopically labelled Internal Standard (IS) corresponding to every antibiotic family should present a matrix effect (ME) and recovery (RE) percentages similar to the antibiotics for which it is selected in order to confirm the suitability for correcting the signal changes derived from extraction procedure and matrix (fresh cheese and whey).

The results obtained in the matrix effect (ME) study have been categorized similarly to the study conducted by Ferrer *et al*. (2011), cited by León *et al.* (2012). The ME was classified into three different categories according to the calculated values. There was no matrix effect when the ME factor was below or equal to 20% (between 80% and 120%) as repeatability of the results would be close to this range. A medium ME was considered when the values ranged between 40% and 80% or 120% and 150%. Finally, percentages below 40% or above 150% were classified as high ME.

As shown in Figure 19 and Figure 20, the ME results for fresh cheese and whey are classified into the three interest levels studied (LQC, MQC and HCQ). Overall, when the ME was evaluated without the IS correction, percentages of antibiotics equal or lower than 5% in whey and 10% in fresh cheese were not affected by matrix. Furthermore, analytes with high ME ranged from 27% to 39% in fresh cheese and 55-63% in whey; and moderate ME was observed mainly in cheese (54-71%).

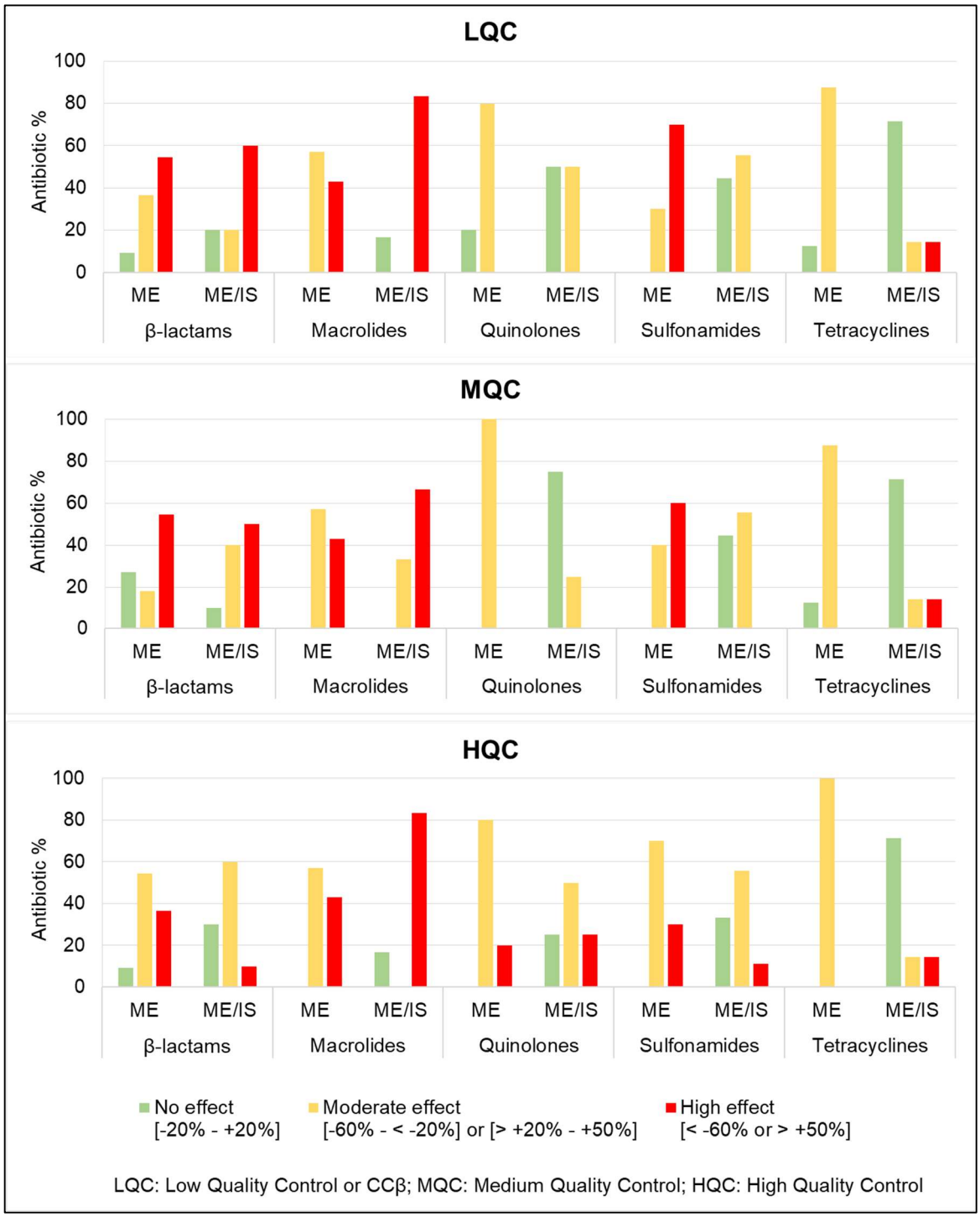


Figure 19. Percentage of the number of antibiotics corresponding to their percentages of Matrix Effect (ME) and Matrix Effect corrected with Internal Standard (ME/IS) by antibiotic family in fresh cheese

However, when ME was corrected by using an appropriate Internal Standard (ME/IS), the percentage of analytes with high ME reduced up to 60% in some interest levels and matrices (HQC in whey). Overall, when the correction with isotopically labelled internal standards was applied, the highest increase of the number of analytes without ME was found at high quality control. Thus, the number of compounds without

ME improved significantly considering calculated area ratios (area analyte/area internal standard), with a high percentage of antibiotics with matrix effect ≤ 20% for quinolones (25-75%), sulfonamides (33-56%) and tetracyclines (57-100%).

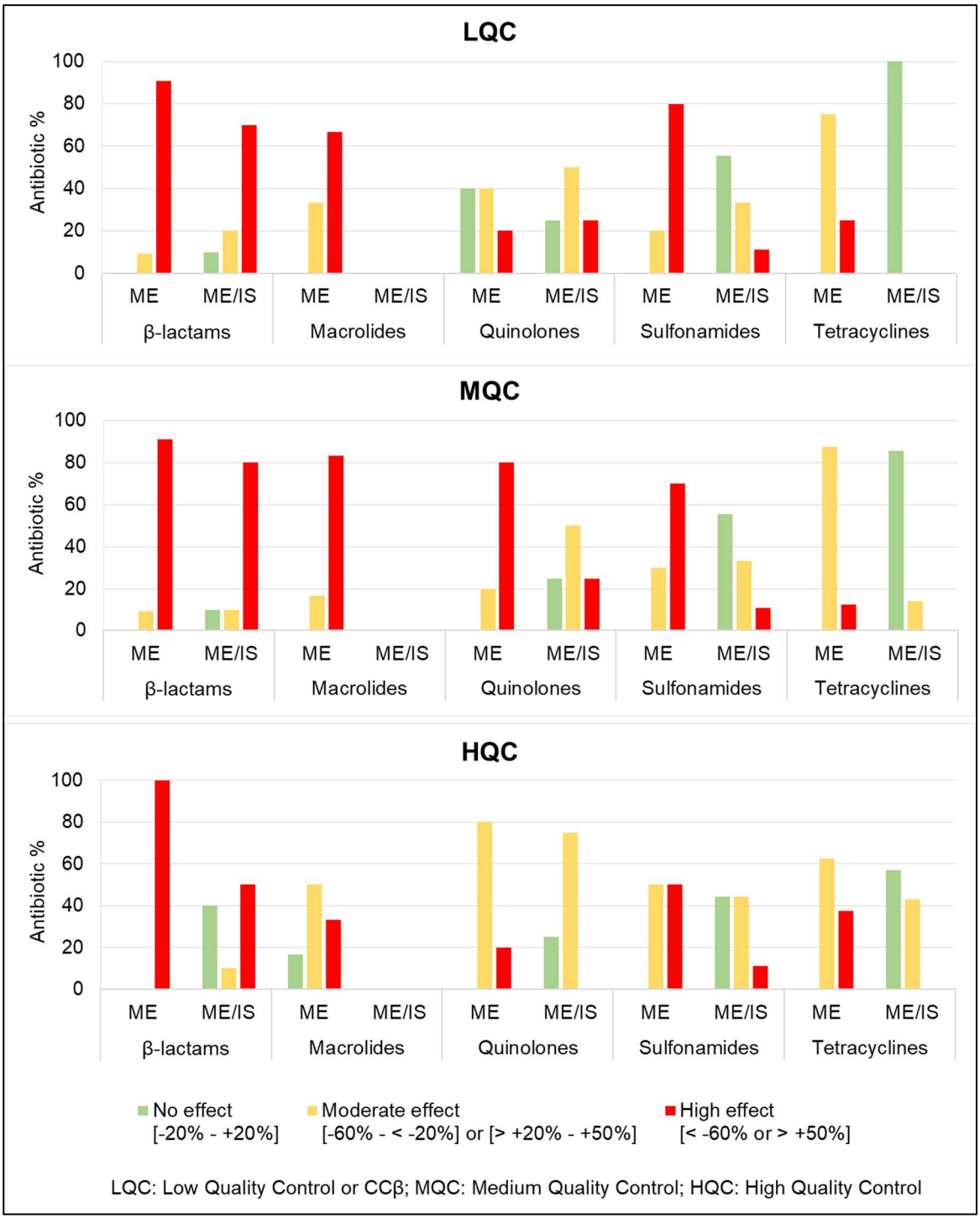


Figure 20. Percentage of the number of antibiotics corresponding to their percentages of Matrix Effect (ME) and Matrix Effect corrected with Internal Standard (ME/IS) by antibiotic family in whey

In contrast to the ME results obtained in the present study, other authors indicated an enhancement phenomenon > 20% for tetracyclines in milk (Wang *et al.*, 2015), in addition to quinolones (Moretti *et al.*, 2016). For sulfonamides, while studies conducted in milk by Wang *et al.* (2015) and in cheese by Gómez Pérez *et al.* (2013) reported a positive matrix effect > 20%, Moretti *et al.* (2016) found for this antimicrobial group an ion suppression effect (24-36%) working with milk, according to this thesis.

Regarding β-lactams, this antibiotic group was more affected by matrix, showing in most cases an ion suppression effect above 60%, even after correction with IS, in accordance with a study reported by Moretti *et al.* (2016), who also indicated ion suppression effect for β-lactams ranging from 22% to 82%.

In the case of macrolides and lincosamides, it should be noted that matrix effect of roxithromycin was significantly different to the values obtained for these antibiotic families for which it corrects. While internal standard presented a high ion suppression effect around 90%, even this compound not being detected in whey samples, overall, response of macrolides and lincomycin decreased moderately (ME: -60% - < -20%) in the case of fresh cheese and in a higher percentage (ME: < -60%) for whey. A negative matrix effect (ME: ≤ -30%) for roxithromycin was also indicated in milk by Wang *et al.* (2015). Additionally, erythromycin included a pronounced positive matrix effect (ME: > +50%) for the two types of dairy samples, which decreased as the concentration of the antibiotic studied increased, in contrast of the studies conducted by Gómez Pérez *et al*. (2013) in cheese and Moretti *et al*. (2016) in milk, who reported an ion suppression effect for this macrolide, using concentrations (10-200 µg/kg and 2-150 µg/kg) similar to those in this thesis.

More than 83% and 98% of analysed compounds included a reduction of the analytical response for fresh cheese and whey, respectively. However, applying the correction with IS, the number of compounds negatively affected by matrix ranged between 50-60%, similarly for both matrices.

The percentages of absolute and corrected recovery (RE and RE/IS) were also calculated (Figure 21). Overall, absolute recovery average was between 62% and 97%, while recoveries after correction with IS achieved higher values ranging from 73% to 132%. The lowest recovery percentages (RE) were associated to Low Quality Control (LQC), while the highest values, close to or above 100%, were obtained with an increase of the antibiotic concentration (MQC and HQC).

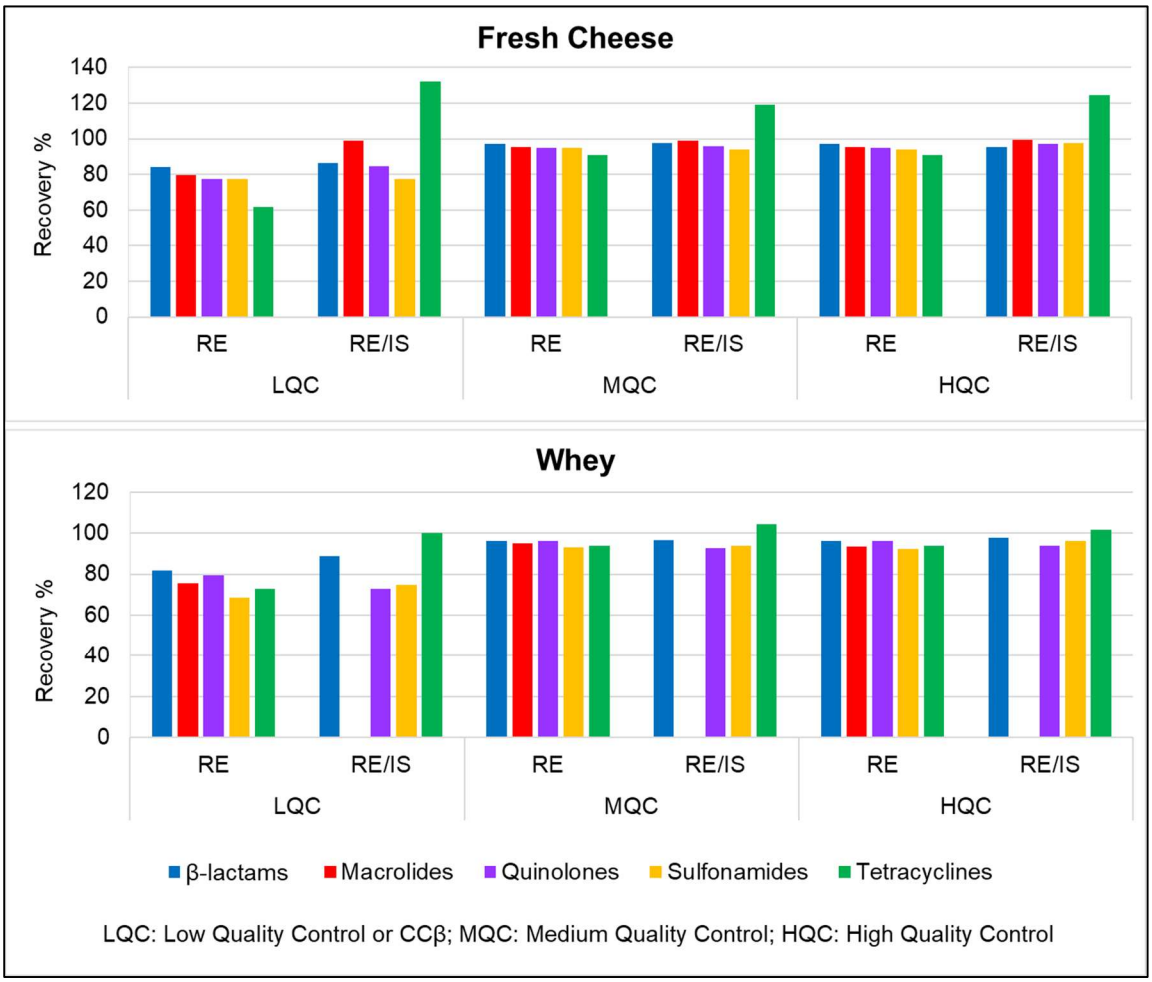


Figure 21. Percentages of Recovery (RE) and Recovery corrected with Internal Standard (RE/IS) by antibiotic family in fresh cheese and whey

The lowest RE percentages were reported for sulfonamides and tetracyclines at LQC level, with values between 60-77%, in agreement with the recoveries obtained for some sulfonamides in previous studies in milk (Moretti *et al.*, 2016) and cheese (Gómez Pérez *et al.*, 2013). However, for tetracyclines, Moretti *et al* (2016) indicated higher recovery averages (RE: > 85%) applying a concentration range between 2-150 µg/kg. Regarding RE/IS, some analytes presented high values for all interest levels evaluated such as 4-epi-chlortetracycline, doxycycline, oxytetracycline and tetracycline in fresh cheese (122-180%), and desfuroylceftiofur in whey (121-153%). For these antibiotic groups, the corrected recovery percentages tended to lower values when the evaluated antibiotic concentration was increased (MQC and HQC). Additionally, desfuroylceftiofur showed a high percentage of RE and RE/IS at LQC in whey, and ceftiofur similarly in cheese.

Considering recovery percentages by antibiotic family (Figure 21), tetracyclines achieved high corrected recoveries (RE/IS) at all interest levels studied (LQC, MQC and HQC) in fresh cheese, up to 132% at low quality control. Differences between the

three quality controls (LQC, MQC and HQC) were observed in the case of the sulfonamides, with absolute and corrected recoveries at LQC significantly lower (RE and RE/IS: < 80%) than those obtained at higher antibiotic concentrations.

In the case of tetracyclines, although their RE were similar to that obtained for demeclocycline in fresh cheese, the inadequate RE/IS percentages above 110% (EC, 2002b) can be attributed to an improper handling of the samples during the extraction procedure, since tetracyclines are characteristically photosensitive and could have been degraded before injection into the chromatograph. Also, this behaviour might be consequence of the keto-enol tautomeric equilibrium between tetracyclines and their epimers that generates differences on the analytical response by the influence of several factors, mainly temperature and pH (Cherlet *et al*., 2006).

When these experiments are observed altogether, it can be seen that matrix-matched standard calibration with isotopically labelled internal standards resulted in more adequate percentages of matrix effect (from 93% to 63% for fresh cheese and from 98% to 56% in the case of whey) and it also improved recovery values at LQC.

However, for macrolides and lincosamides, as aforementioned, roxithromycin did not improve ME of these compounds. None of the other studied internal standards presented a similar behaviour to these antibiotic families and, finally, a quantification by via external matrix-matched calibration was applied since adequate absolute recovery percentages (RE: ≥ 80%) were obtained for the most of macrolides considered as well as for lincomycin.

III.1.3.3. Validation Parameters

III.1.3.3.1. Specifity and Detection Capability

Blank fresh cheese and whey samples (n= 30) were free of interferences at the Retention Time (RT) of the compounds.

The final CCβ were selected for a mass assignment < 5 ppm error. Chromatograms with 50,000 FWHM resolution showed an adequate mass accuracy (< 5 ppm error) for blank fresh cheese and whey samples fortified at CCβ (Figure 1 and Figure 2, Annex).

Although the number of false positives is minimized using a narrow mass-extraction window and low tolerance for mass accuracy, values < 5 ppm error of mass accuracy could involve the presence of false negative results, a challenge essential in a screening method.

The results of B, T, Fm and CCβ obtained are presented in Table 10 and Table 11 for fresh cheese and whey, respectively. The totality of antibiotics considered achieved

the criterion Fm > B established in Commission Decision 2002/657/EC (EC, 2002b). Also, Fm was above T for all the substances under study, which means that the rate of false-positive is below 5%.

For most of substances analysed, the detection capabilities were 0.25 MRL established in milk, with the exception of certain analytes such as benzylpenicillin, dicloxacillin, nafcillin, cefoperazone, desfuroylceftiofur, sulfacetamide, chlortetracycline, 4-epi-oxytetracycline and 4-epi-tetracycline in fresh cheese, and benzylpenicillin, dicloxacillin, nafcillin, cefoperazone, desfuroylceftiofur and erythromycin in whey, which were detected at a concentration equivalent to 0.50 MRL, complying with Commission Decision 2002/657/EC (EC, 2002b). In this study, CCβ corresponds to the limit of quantification (LOQ).

Studies in cheese using UHPLC-MS/MS indicated limits of detection (LODs) and limits of quantification (LOQs) lower than the CCβs obtained in the present study for different antibiotic families such as macrolides and sulfonamides (Gómez Pérez *et al*., 2013). Also, Schwaiger *et al*. (2018), using C18 DSPE sample treatment, achieved LODs and LOQs for curd and soft cheese below or equal to 0.1 of MRL fixed in milk. However, erythromycin was not detected for both matrices, and neither ampicillin, benzylpenicillin, cloxacillin and ceftiofur in the case of cheese, contrary to the results obtained in this study for fresh cheese.

Concerning whey, studies have been focused on different types of powder derives, liquid whey matrix not having been evaluated in any study by LC-MS. Zhao *et al*. (2017) studied 150 veterinary drugs belonged to β-lactams, aminoglycosides, lincosamides, macrolides, quinolones, sulfonamides, tetracyclines and others, in whey protein isolate (WPI) by UHPLC-MS/MS, reporting LOQ values significantly lower than those obtained in this study, between 1 and 10 μg/kg for most of substances. Ampicillin and desfuroylceftiofur were detected at the same concentrations than those for liquid whey, 1 μg/kg and 50 μg/kg respectively, while benzylpenicillin (LOQ= 5 μg/kg) and oxacillin (LOQ= 10 μg/kg) were above the limits of quantification obtained in this study.

Another multiresidue analysis conducted by Wittenberg *et al*. (2017) in various milk-based powders for the detection of antimicrobials by UHPLC-MS/MS, indicated LODs< 0.5 μg/kg and LOQs< 1.4 μg/kg. However, in contrast to the results obtained in this study for liquid whey, ampicillin, ceftiofur, oxytetracycline and tetracycline had to be eliminated as the validation criteria were not met. Only the lack of sensitivity of tylosin accords with the results of the present study.

Table 10. Mean ratio response of the Blank samples (B), Threshold value (T), Cut-off factor (Fm) and the Detection Capability (CCβ) for fresh cheese

Antibiotics	B	T	Fm	EU-MRL (μg/kg)	CCβ (μg/kg)
β-lactams					
Ampicillin	0.000	0.000	0.033	4	1
Benzylpenicillin	0.000	0.000	0.010	4	2
Cloxacillin	0.000	0.000	0.030	30	7.5
Dicloxacillin	0.000	0.000	0.024	30	15
Nafcillin	0.000	0.000	0.204	30	15
Oxacillin	0.000	0.000	0.018	30	7.5
Cefalexin	0.000	0.000	0.581	100	25
Cefoperazone	0.000	0.000	0.014	50	25
Ceftiofur	0.000	0.000	0.058	100	25
Desfuroylceftiofur	0.004	0.013	0.015	100	50
Quinolones					
Danofloxacin	0.000	0.000	0.098	30	7.5
Enrofloxacin	0.000	0.000	0.402	100	25
Ciprofloxacin	0.000	0.000	0.221	100	25
Flumequine	0.000	0.000	0.274	50	12.5
Sulfonamides					
Sulfacetamide	0.000	0.000	0.026	100	50
Sulfadiazine	0.000	0.000	0.053	100	25
Sulfadimethoxine	0.000	0.000	0.146	100	25
Sulfamerazine	0.000	0.000	0.098	100	25
Sulfamethazine	0.000	0.000	0.182	100	25
Sulfamethoxypyridazine	0.000	0.000	0.149	100	25
Sulfapyridine	0.000	0.000	0.092	100	25
Sulfaquinoxaline	0.000	0.000	0.085	100	25
Sulfathiazole	0.000	0.000	0.031	100	25
Tetracyclines					
Chlortetracycline	0.000	0.000	0.411	100	50
4-epi-Chlortetracycline	0.000	0.000	2.269	100	25
Doxycycline	0.000	0.000	1.580	100	25
Oxytetracycline	0.007	0.023	1.455	100	25
4-epi-Oxytetracycline	0.000	0.000	0.160	100	50
Tetracycline	0.000	0.000	1.140	100	25
4-epi-Tetracycline	0.000	0.000	0.423	100	50
Macrolides[*]					
Erythromycin	0.000	0.000	839,587	40	10
Spiramycin	163,239	444,507	4,074,008	200	50
Neo Spiramycin	0.000	0.000	11,918,522	200	50
Tilmicosin	0.000	0.000	452,151	50	12.5
Tylosin	15,083	31,573	54,529	50	12.5
Lincosamides[*]					
Lincomycin	30,363	78,464	21,685,551	150	37.5

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). [*]Calculations without isotopically labelled Internal Standard (IS) correction.

Table 11. Mean ratio response of the Blank samples (B), Threshold value (T), Cut-off factor (Fm) and the Detection Capability (CCβ) for whey

Antibiotics	B	T	Fm	EU-MRL (μg/kg)	CCβ (μg/kg)
β-lactams					
Ampicillin	0.000	0.000	0.026	4	1
Benzylpenicillin	0.000	0.000	0.005	4	2
Cloxacillin	0.000	0.000	0.029	30	7.5
Dicloxacillin	0.000	0.000	0.008	30	15
Nafcillin	0.000	0.000	0.020	30	15
Oxacillin	0.000	0.000	0.056	30	7.5
Cefalexin	0.000	0.000	0.671	100	25
Cefoperazone	0.000	0.000	0.030	50	25
Ceftiofur	0.000	0.000	0.300	100	25
Desfuroylceftiofur	0.000	0.000	0.028	100	50
Quinolones					
Danofloxacin	0.000	0.000	0.048	30	7.5
Enrofloxacin	0.000	0.000	0.194	100	25
Ciprofloxacin	0.000	0.000	0.192	100	25
Flumequine	0.000	0.000	0.031	50	12.5
Sulfonamides					
Sulfacetamide	0.000	0.000	0.020	100	25
Sulfadiazine	0.000	0.000	0.026	100	25
Sulfadimethoxine	0.005	0.013	0.199	100	25
Sulfamerazine	0.000	0.000	0.068	100	25
Sulfamethazine	0.000	0.000	0.136	100	25
Sulfamethoxypyridazine	0.000	0.000	0.080	100	25
Sulfapyridine	0.000	0.000	0.052	100	25
Sulfaquinoxaline	0.000	0.000	0.065	100	25
Sulfathiazole	0.000	0.000	0.022	100	25
Tetracyclines					
Chlortetracycline	0.000	0.000	0.246	100	25
4-epi-Chlortetracycline	0.000	0.000	0.367	100	25
Doxycycline	0.000	0.000	0.500	100	25
Oxytetracycline	0.000	0.000	0.763	100	25
4-epi-Oxytetracycline	0.000	0.000	0.155	100	25
Tetracycline	0.000	0.000	0.662	100	25
4-epi-Tetracycline	0.000	0.000	0.321	100	25
Macrolides[*]					
Erythromycin	0.000	0.000	28,486	40	20
Spiramycin	0.000	0.000	1,840,127	200	50
Neo Spiramycin	0.000	0.000	3,591,201	200	50
Tilmicosin	8,771	34,221	83,013	50	12.5
Tylosin[**]	-	-	-	-	-
Lincosamides[*]					
Lincomycin	0.000	0.000	10,943,649	150	37.5

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). [*]Calculations without isotopically labelled Internal Standard (IS) correction. **Tylosin eliminated due to its poor sensitivity in whey.

III.1.3.3.2. Precision and Trueness

Three matrix-matched calibration curves were carried out on different days for fresh cheese and whey, using five calibration points ranging from 0.25 to 4 and from 0.50 to 8 of MRL fixed in milk. Linearity was evaluated by means of coefficients of determination, whose values (R^2> *0.995*) were adequate for the two matrices.

The precision and trueness results for fresh cheese and whey (Table 12 and Table 13, respectively) were in accordance with the Commission Decision 2002/657/EC (EC, 2002b) for a quantitative screening method. Furthermore, relative standard deviation percentages in terms of repeatability (RSD_r) were lower than within-laboratory reproducibility values (RSD_R).

As an exception, for fresh cheese, cloxacillin and desfuroylceftiofur at high quality control (HQC) showed inadequate recovery percentages, with values above 110% and below 80%, respectively. In the case of whey, also for the HQC, nafcillin surpassed slightly the deviation range established for recovery parameter (EC, 2002b), with a percentage of 116%.

Regarding precision results, only in the case of fresh cheese, reproducibility (RSD_R) for desfuroylceftiofur at HQC was significantly higher than values recommended by Horwitz equation (EC, 2002b).

In contrast to the results obtained in the present study for cheese, previous studies by UHPLC-MS/MS found improper accuracy for macrolide and sulfonamide groups (Gómez Pérez *et al*., 2013). Also, Schwaiger *et al*. (2018) indicated unsatisfactory recovery (below 50%) and repeatability (above 30%) percentages in soft cheese for some macrolides; enrofloxacin and sulfathiazole included recovery percentages higher than 150% and a high RSD_r for sulfathiazole.

Overall, the results obtained in this validation procedure for liquid whey were in accordance with studies about the quantification of veterinary drugs in whey powders by UHPLC-MS/MS, reporting acceptable trueness and precision percentages. As an exception, Wittenberg *et al.* (2017) and Zhao *et al.* (2017) indicated that some antibiotics from β-lactam, macrolide, sulfonamide and tetracycline families were not adequately recovered with high repeatability percentages at some fortified levels. The recovery of nafcillin was not appropriate, with a value above 110% (Zhao *et al.*, 2017), a result that was also observed in the present study.

Table 12. Trueness (T), repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) percentages obtained in the validation process for fresh cheese

Antibiotics	LQC			MQC			HQC		
	T	RSD_r	RSD_R	T	RSD_r	RSD_R	T	RSD_r	RSD_R
β-lactams									
Ampicillin	110	8	10	77	12	13	106	15	20
Benzylpenicillin	110	12	18	85	9	18	98	7	24
Cloxacillin	106	13	21	92	3	24	132	1	13
Dicloxacillin	110	15	16	82	7	13	100	7	9
Nafcillin	100	7	7	88	4	5	96	9	11
Oxacillin	110	5	6	82	8	11	104	9	17
Cefalexin	100	15	15	104	14	18	94	10	18
Cefoperazone	101	7	23	106	11	18	104	3	17
Ceftiofur	100	18	23	99	3	15	105	12	16
Desfuroylceftiofur	86	9	16	103	9	12	65	3	39
Macrolides									
Erythromycin	94	9	16	98	5	8	94	10	12
Spiramycin	89	7	17	107	6	10	104	10	14
Neo Spiramycin	88	7	7	109	5	9	108	7	9
Tilmicosin	107	10	10	97	11	12	91	12	16
Tylosin	101	6	24	96	12	21	110	9	15
Lincosamides									
Lincomycin	95	5	7	110	3	5	94	9	12
Quinolones									
Danofloxacin	81	10	10	105	7	10	98	10	12
Enrofloxacin	93	18	21	105	7	10	91	11	17
Ciprofloxacin	95	7	16	104	8	15	102	7	12
Flumequine	104	13	31	100	16	20	81	5	6
Sulfonamides									
Sulfacetamide	94	8	9	96	9	10	101	10	16
Sulfadiazine	81	16	26	100	8	11	93	10	16
Sulfadimethoxine	82	11	16	107	11	12	101	8	11
Sulfamerazine	85	16	26	102	11	13	87	10	12
Sulfamethazine	88	16	20	104	10	11	85	10	14
Sulfamethoxypyridazine	85	18	19	99	10	11	89	9	11
Sulfapyridine	82	18	21	102	8	12	88	12	14
Sulfaquinoxaline	88	13	15	97	6	10	89	7	12
Sulfathiazole	98	13	16	98	7	7	104	8	10
Tetracyclines									
Chlortetracycline	106	4	5	90	11	18	91	4	17
4-epi-Chlortetracycline	90	10	14	103	7	9	91	7	17
Doxycycline	94	11	14	94	11	17	92	5	18
Oxytetracycline	93	7	9	93	10	13	92	7	18
4-epi-Oxytetracycline	101	14	17	94	8	18	101	3	13
Tetracycline	100	7	7	94	7	15	107	7	16
4-epi-Tetracycline	92	5	10	95	13	18	92	8	16

Trueness is expressed as recovery (%) (n= 18); Repeatability (n= 6) and Reproducibility (n= 18) are expressed as RSD (%); RSD: Relative Standard Deviation; LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control.

Table 13. Trueness (T), repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) percentages obtained in the validation process for whey

Antibiotics	LQC			MQC			HQC		
	T	RSD_r	RSD_R	T	RSD_r	RSD_R	T	RSD_r	RSD_R
β-lactams									
Ampicillin	93	24	35	108	19	36	93	19	29
Benzylpenicillin	97	17	21	98	8	10	95	10	18
Cloxacillin	88	18	33	107	17	23	110	13	15
Dicloxacillin	81	11	15	101	15	23	93	13	19
Nafcillin	107	18	22	107	16	24	116	13	19
Oxacillin	91	19	33	107	16	24	94	10	19
Cefalexin	108	15	27	103	10	20	96	11	16
Cefoperazone	103	18	24	91	15	21	90	11	17
Ceftiofur	108	14	27	103	14	23	91	10	18
Desfuroylceftiofur	106	16	23	98	13	18	109	10	16
Macrolides									
Erythromycin	96	16	29	93	14	21	107	11	17
Spiramycin	101	16	25	108	13	19	96	6	15
Neo Spiramycin	87	12	15	91	10	14	109	10	16
Tilmicosin	100	20	21	89	15	24	97	11	18
Lincosamides									
Lincomycin	90	9	12	93	6	12	101	5	13
Quinolones									
Danofloxacin	91	11	21	104	7	10	102	7	14
Enrofloxacin	86	13	27	102	9	16	94	7	17
Ciprofloxacin	89	9	12	92	8	18	102	5	16
Flumequine	94	15	29	92	14	21	108	8	13
Sulfonamides									
Sulfacetamide	110	16	23	104	15	22	98	11	16
Sulfadiazine	105	12	19	97	12	15	98	11	18
Sulfadimethoxine	95	12	19	98	9	10	96	8	9
Sulfamerazine	103	16	26	107	8	22	101	11	16
Sulfamethazine	82	14	14	100	13	20	87	9	18
Sulfamethoxypyridazine	99	15	18	91	10	22	92	5	16
Sulfapyridine	91	13	26	110	13	21	85	12	18
Sulfaquinoxaline	97	15	27	102	11	19	98	10	18
Sulfathiazole	108	14	27	91	14	19	106	10	18
Tetracyclines									
Chlortetracycline	100	12	21	93	12	18	98	10	16
4-epi-Chlortetracycline	98	9	17	92	8	13	100	7	11
Doxycycline	110	12	14	95	9	17	105	9	11
Oxytetracycline	95	11	18	95	12	23	99	9	18
4-epi-Oxytetracycline	104	14	28	100	11	19	96	10	16
Tetracycline	96	10	18	105	12	22	104	9	18
4-epi-Tetracycline	93	12	20	102	10	14	109	9	11

Trueness is expressed as recovery (%) (n= 18); Repeatability (n= 6) and Reproducibility (n= 18) are expressed as RSD (%); RSD: Relative Standard Deviation; LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control.

III.1.4. Conclusions

According to the validation parameters obtained, it can be concluded that the UHPLC-HRMS methodology, using the Orbitap Exactive™ analyser, is an efficient analytical tool for the quantitative screening of β-lactam, lincosamide, macrolide, quinolone, sulfonamide and tetracycline antibiotics in fresh cheese and whey, selecting as best sample treatment 0.2 M acetate buffer pH 5.2/acetonitrile (20/80, v/v) solvent combination for the extraction and C18 Dispersive Solid Phase Extraction (DSPE) as clean-up step.

III.2. TRANSFER OF ANTIBIOTICS FROM GOAT'S MILK TO RENNET CURD AND WHEY FRACTIONS DURING THE CHEESE-MAKING PROCESS

III.2.1. Introduction

During the cheese-making process, drugs present in milk can be transferred to rennet curd or released into the whey fraction to a greater or lesser extent. Antibiotic residues in dairy products could lead to hazards for the consumer, especially the development of multi-drug resistant bacteria (WHO, 2019). Additionally, contaminated whey, which is also destined to animal feed (Fresno *et al.*, 2015) or discharged into land or receiving waters (Prazeres *et al.*, 2012), would affect animal health and/or have negative environmental implications.

Scientific literature data about the transfer of drugs during the cheese-making process is limited, and in most cases focused on a reduced number of veterinary drugs. For the tetracyclines, studies on the distribution of antibiotics from sheep (Cabizza *et al.*, 2017) and cow milk (Gadja *et al.*, 2018) to cheese and whey reported concentration factors in cheese up to four and six times, respectively.

As well, high retention rates above 50% were obtained by Quintanilla *et al.* (2019b) in fresh cheese from goat's milk spiked with different β-lactams, aminoglycosides, macrolides and quinolones, with oxytetracycline showing the lowest retention rate in the cheese fraction (37.5%). However, in ripened cheeses (Quintanilla *et al.*, 2019a), lower retention rates were detected in all cases at the beginning of maturation, except for quinolones and especially for oxytetracycline, which was widely retained (68%) in the cheese.

On the other hand, some authors (Hakk *et al.*, 2016; Shappell *et al.*, 2017; Lupton *et al.*, 2018) have tried to develop theoretical models that contribute to predict the partitioning of antimicrobials into the different milk fractions and, hence, the risk for potential human exposure. These empirical equations related the obtained results to the physicochemical properties of the veterinary drugs considered such as lipophilicity (log P), ionization (log D), and the ability to bind proteins.

Therefore, the aim of this study was to evaluate the transfer of numerous antibiotics belonging to the β-lactam, macrolide and lincosamide, quinolone, sulfonamide and tetracycline groups from goat's milk to rennet curd and whey fractions using UHPLC-HRMS.

III.2.2. Material and Methods

III.2.2.1. Experimental Design

The transfer of 36 antibiotic substances from milk to curd and whey fractions during the cheese-making process was evaluated. The experimental cheeses were produced at laboratory scale, using antibiotic-free goat's milk spiked with five different concentrations of antibiotics ranging from 0.25 to 4 times the MRL established for such substances in milk. Cheeses from antibiotic-free milk were also included to be used as reference (control). Milk, rennet curd and whey samples were analysed in duplicate, using UHPLC-HRMS (Orbitrap Exactive^{TM}) to investigate the partitioning of antibiotics in the different dairy matrices. The effect of the antibiotics on the pH and the chemical composition of the whey fraction was also evaluated. The experimental design (Figure 22) was replicated on three different days, making three cheeses for each antibiotic concentration. This experimental study was carried out in the Public Health Laboratory of Valencia-FISABIO and in the Institute for Animal Science and Technology (ICTA) of the Universitat Politècnica de Valencia (UPV).

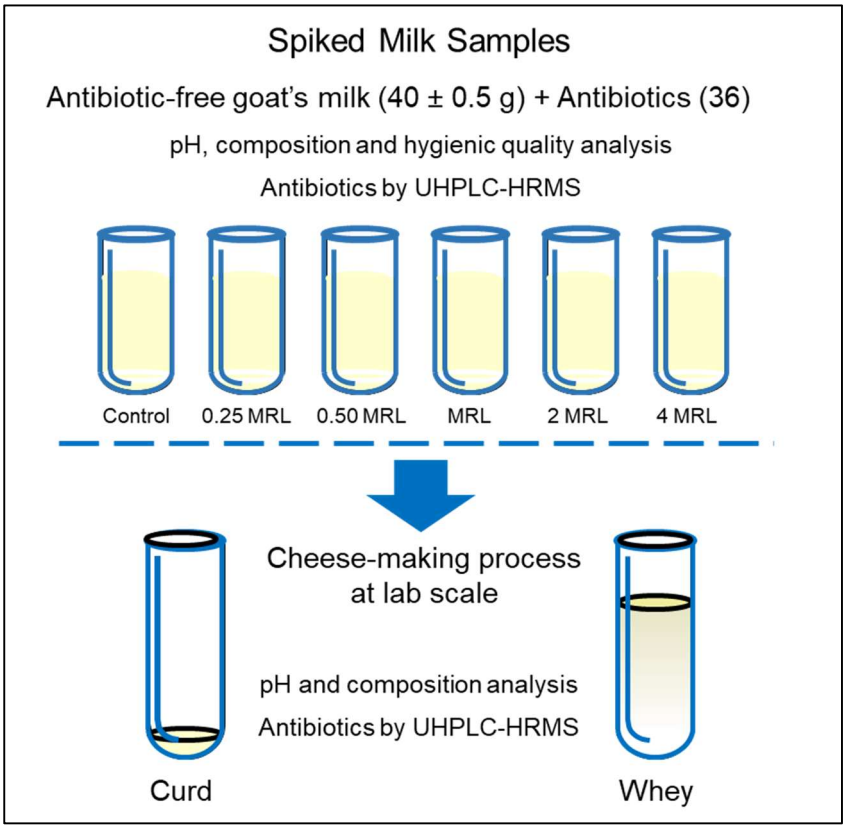


Figure 22. Experimental design to evaluate the transfer of antibiotics from milk to rennet curd and whey fractions

III.2.2.2. Antibiotics and Spiked Samples

Table 14 presents the commercial references and range of concentrations of the 36 antibiotic substances, as well as the 5 internal standards used in this study. For each of them, a stock solution was prepared in methanol at a concentration ranging from 250 to 1,500 µg/mL, which was stored at -20°C for further use.

Antibiotics present in milk, curd and whey samples were quantified by matrix-matched calibration curves using the following internal standards: penicillin G-D7 for β-lactams, norfloxacin-D5 for quinolones, sulfadimethoxine-D6 for sulfonamides, and demeclocycline for tetracyclines, at a final concentration of 100 µg/kg. Macrolides and lincosamides were quantified by external calibration (without internal standard).

Raw goat's milk for cheese production was spiked at five different drug concentrations (Table 14), following the recommendations of the International Dairy Federation (ISO/IDF, 2003a,b). For this, working solutions containing simultaneously all the antibiotic substances considered were daily prepared by diluting conveniently the standard stock solutions made previously.

III.2.2.3. Cheese-making Process

Raw milk for cheese production was obtained daily from the experimental heard of Murciano-Granadina goats of the Universitat Politècnica de València (Valencia, Spain). Animals were in mid-lactation (70-150 days after delivery), had good health status and did not receive any veterinary drugs, neither before nor during the experimental period.

Whey and curd samples were obtained from a laboratory scale cheese-making procedure according to Giraldo *et al.* (2017). Thus, raw milk (40 g) was heated at 33ºC in a water bath (Thomas Scientific, Madrid, Spain) and curdled using animal rennet (commercial solution 1:10000. Suministros Arroyo, Santander, Spain) in 50 mL conical centrifuge tubes (Merck®, KGaA, Darmstadt, Germany). After coagulation (30 min at 33ºC), the curd was cut and heated for 15 min at 35ºC, being mixed with a scraper at the beginning and 10 minutes after heating starting. Then, the tubes were centrifuged (3,000 rpm, 10 min. Allegra X-15 Centrifuge Performance. Beckman Coulter, Barcelona, Spain) and the whey separated using a metallic tea strainer (Figure 23).

Milk, curd, and whey fractions were accurately weighed to apply a mass balance in order to calculate the partitioning of antibiotics thorough cheese-making. Cheese yield (expressed as kg curd/100 kg milk) and the moisture percentage of curd were also defined, attributing its liquid content to interstitial whey amount contained into rennet curd.

Table 14. Antibiotics used to evaluate the partitioning of antibiotics during the cheese-making process

Antibiotics	Reference	Log P	EU-MRL (µg/kg)	Concentration ranges (µg/kg)
β-lactams				
Ampicillin	59349[1]	1.35	4	1, 2, 4, 8, 16
Benzylpenicillin	46609[1]	1.67	4	1, 2, 4, 8, 16
Cloxacillin	46140[1]	2.53	30	7.5, 15, 30, 60, 120
Dicloxacillin	46182[1]	3.02	30	7.5, 15, 30, 60, 120
Nafcillin	32071[1]	3.52	30	7.5, 15, 30, 60, 120
Oxacillin	46589[1]	2.05	30	7.5, 15, 30, 60, 120
Cefalexin	33989[1]	0.65	100	25, 50, 100, 200, 400
Cefoperazone	32426[1]	1.43	50	12.5, 25, 50, 100, 200
Ceftiofur	34001[1]	2.05	100	25, 50, 100, 200, 400
Desfuroylceftiofur	D289980[2]	-	100	25, 50, 100, 200, 400
Penicillin G-D7*	32985[1]			100
*Macrolides***				
Erythromycin	46256[1]	2.83	40	10, 20, 40, 80, 160
Spiramycin	46745[1]	3.06	200	50, 100, 200, 400, 800
Neo Spiramycin	N390040[2]	-	200	50, 100, 200, 400, 800
Tilmicosin	33864[1]	4.95	50	12.5, 25, 50, 100, 200
Tylosin	33847[1]	3.27	50	12.5, 25, 50, 100, 200
*Lincosamides***				
Lincomycin	15443869[3]	0.91	150	37.5, 75, 150, 300, 600
Quinolones				
Danofloxacin	33700[1]	1.20	30	7.5, 15, 30, 60, 120
Enrofloxacin	33699[1]	1.88	100	25, 50, 100, 200, 400
Ciprofloxacin	33434[1]	0.65	100	25, 50, 100, 200, 400
Flumequine	45735[1]	2.41	50	12.5, 25, 50, 100, 200
Norfloxacin-D5*	CH001[4]			100
Sulfonamides				
Sulfacetamide	46770[1]	0.07	100	25, 50, 100, 200, 400
Sulfadiazine	35033[1]	-0.12	100	25, 50, 100, 200, 400
Sulfadimethoxine	46794[1]	1.48	100	25, 50, 100, 200, 400
Sulfamerazine	46826[1]	0.34	100	25, 50, 100, 200, 400
Sulfamethazine	46802[1]	0.80	100	25, 50, 100, 200, 400
Sulfamethoxypyridazine	46858[1]	0.32	100	25, 50, 100, 200, 400
Sulfapyridine	31738[1]	0.03	100	25, 50, 100, 200, 400
Sulfaquinoxaline	45662[1]	1.30	100	25, 50, 100, 200, 400
Sulfathiazole	46902[1]	0.05	100	25, 50, 100, 200, 400
Sulfadimethoxine-D6*	SA001[4]			100
Tetracyclines				
Chlortetracycline	C4881[1]	-0.53	100	25, 50, 100, 200, 400
4-epi-Chlortetracycline	268231000[5]	-	100	25, 50, 100, 200, 400
Doxycycline	33429[1]	-0.54	100	25, 50, 100, 200, 400
Oxytetracycline	46598[1]	-1.50	100	25, 50, 100, 200, 400
4-epi-Oxytetracycline	257711000[5]	-	100	25, 50, 100, 200, 400
Tetracycline	31741[1]	-0.62	100	25, 50, 100, 200, 400
4-epi-Tetracycline	233121000[5]	-	100	25, 50, 100, 200, 400
Demeclocycline*	46161[1]			100

Log P: partitioning coefficient accessed from www.chemspider.com on December, 2019, using the ADC Lab-predicted values; data missing: not found in the literature. EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). *Isotopically labelled Internal Standard (IS). **External calibration (without IS). [1]Sigma-Aldrich Química, S.L. (Madrid, Spain); [2]Toronto Research Chemicals, Inc. (Toronto, Canada); [3]Honeywell Riedel-de-Haën, A.G. (Seelze, Germany); [4]WITEGA Laboratorien Berlin-Adlershof GmbH. (Berlin, Germany); [5]Acros Organics B.V.B.A. (Geel, Belgium).

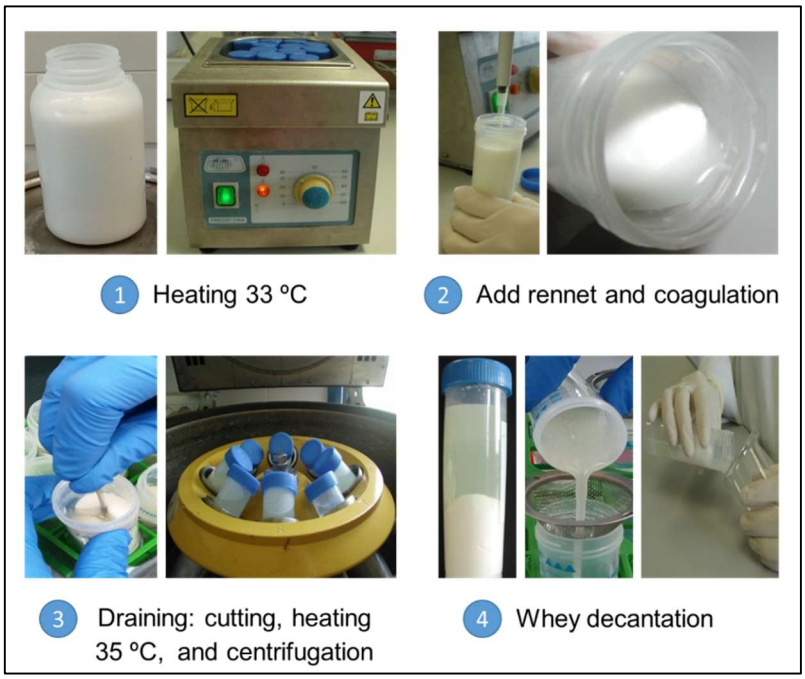


Figure 23. Cheese-making procedure at lab scale

III.2.2.4. Milk, Curd, and Whey Analysis

A conventional pH-meter Basic 20 (Crison, Barcelona, Spain) was applied to check the pH value in milk, curd, and whey samples. MilkoScan 6000 (Foss, Hillerød, Denmark) was used to determine the gross composition of milk and whey samples. Hygienic quality of the raw goat's milk used for cheese production was also evaluated using Fossomatic 5000 (Foss) to determine the Somatic Cell Count (SCC), and Bactoscan FC (Foss) for the Bacterial Count (BC). In the case of cheese, total solids content was determined by means oven-drying standard method (ISO/IDF, 2004). Milk and whey analysis were carried out in the Interprofessional Laboratory of the Valencian Community (LICOVAL, UPV).

Antibiotic concentrations in milk, curd and whey samples were analysed at the Public Health Laboratory of Valencia (LSPV) by UHPLC-HRMS, according to the previous validation of a screening quantitative test for the milk samples (unpublished data) and following the analytical procedure validated in accordance with Commission Decision 2002/657/EC (EC, 2002b), described in Study 1, in the case of fresh cheese and whey. Milk, curd, and whey samples were analysed in duplicate in three different experimental days (n= 6 per antibiotic concentration and matrix).

III.2.2.5. Statistical Analysis

Concentration ratios between curd and whey fractions (curd/whey) were calculated to evaluate the partitioning of antibiotics during cheese-making. Normalized drug

distribution rates, expressed as percentage, were also calculated by applying a mass balance.

To investigate factors affecting the drug distribution and the chemical composition of whey fraction, a multifactor ANOVA test was performed using Statgraphics Centurion software (StatPoint Technologies, Inc., Warrenton, VA). The antibiotic concentration in milk for cheese production (AC= 0.25 MRL, 0.50 MRL, MRL, 2 MRL, and 4 MRL) and experimental replicate (ER= 1, 2, or 3) were considered for this purpose. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P< 0.05$.

The relationship between drug lipophilicity (log P) and drug partitioning (log curd/whey) was evaluated using a lineal regression model. The Log P values for the antibiotic substances considered (Table 14) were obtained from ChemSpider (www.chemspider.com). As the percentage of moisture (or included whey) can vary between cheeses, antibiotic concentration in dry curd fraction (0% moisture) was used for the calculation of the logarithm of the concentration ratios. According to Shappell *et al.* (2017), antibiotic concentration in dry curd fraction was calculated by subtracting the whey-entrained drug amounts from the wet curd fraction, taking into account that the interstitial whey of the wet curd had the same drug concentration as the whey fraction.

III.2.3. Results and Discussion

III.2.3.1. Milk, Curd and Whey Composition

Raw milk used in this study as negative milk (antibiotic-free) showed a good hygienic quality and a characteristic gross composition for Murciano-Granadina goat's milk. The mean values for the quality parameters considered were (mean±standard deviation): 6.86±0.05 for the pH value, 5.32±0.37% for fat, 3.62±0.06% for protein, 4.63±0.04% for lactose, and 8.98±0.06% for total solids. Logarithm of the SCC was 5.86±0.10 and 4.34±0.29 to that of the logarithm of the BC.

Table 15 shows the chemical composition of the whey samples, the percentage of the total solids of curd and the cheese yield, as well as the pH values of both matrices, according to the two factors (AC and ER) considered. The ANOVA test results suggests that curd total solids were unaffected ($P> 0.05$) by the antibiotic concentration present in milk, showing similar characteristics to those reported by other authors making cheeses at laboratory-scale (Shappell *et al.*, 2017).

Table 15. Average values of parameters in rennet curd and whey made at different antibiotic concentrations and ANOVA F-ratio for Antibiotic Concentration (AC) and Experimental Replicate (ER)

Parameters	Antibiotic Concentration (µg/kg) (AC)							Experimental Replicate (ER)			
	Control	0.25 EU-MRL	0.50 EU-MRL	1 EU-MRL	2 EU-MRL	4 EU-MRL	SE	1	2	3	SE
Curd											
pH	6.74	6.73	6.74	6.73	6.74	6.71	0.006	6.75^{b}	6.79^{c}	6.66^{a}	0.005
Total solids (%)	30.36	30.74	30.06	31.54	30.99	31.55	0.181	30.53^{a}	31.30^{b}	30.75^{a}	0.128
CY (kg cheese/100kg milk)	3.21	3.38	3.29	3.48	3.40	3.49	0.081	3.29	3.49	3.36	0.057
Whey											
pH	6.65	6.66	6.66	6.66	6.64	6.65	0.007	6.66^{b}	6.70^{c}	6.61^{a}	0.005
Fat (%)	0.37	0.38	0.39	0.45	0.49	0.58	0.043	0.44	0.45	0.45	0.031
Protein (%)	1.08^{b}	1.05^{ab}	1.04^{ab}	1.02^{ab}	0.98^{ab}	0.90^{a}	0.034	1.09^{b}	0.96^{a}	0.98^{a}	0.023
Lactose (%)	5.14^{ab}	5.05^{a}	5.06^{a}	5.09^{ab}	5.16^{ab}	5.28^{b}	0.043	5.23^{b}	5.13^{ab}	5.03^{a}	0.031
Total solids (%)	7.18	7.08	7.12	7.20	7.26	7.42	0.029	7.34^{b}	7.16^{a}	7.13^{a}	0.021

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). SE: Standard Error. CY: cheese yield, expressed as kg of cheese per 100 kg of milk.

$^{a, b, c}$: Different letters in the same row indicate significant differences ($P< 0.05$).

Although significant differences were detected for protein (P< 0.05) and lactose (P< 0.05) in whey samples from milk spiked with the highest antibiotic concentration (4 MRL), they could be considered irrelevant.

Regarding experimental replicate factor, significant differences (P< 0.05) were also detected both in curd and whey fractions for most of the parameters considered, which could be related to the characteristics of the different milk samples used for cheese production. However, the cheese-making efficiency characterized by cheese yield (CY) was similar (P>0.05) in all cases.

III.2.3.2. Drug Distribution from Milk to Curd and Whey

Antibiotic concentration ratios between rennet curd and whey fractions were calculated according to the drug concentration present in milk for cheese production (Table 16). In general, the curd/whey ratios were drug-dose independent (P> 0.05) and lower than one (< 1) for most of the antibiotics considered, suggesting that such substances were mainly released into the whey, reaching higher concentrations than those calculated for the curd fraction. However, some antibiotics including most β-lactams, tilmicosin, danofloxacin, ciprofloxacin, sulfadimethoxine, sulfaquinoxaline and chlortetracycline showed higher susceptibility to be concentrated in the curd matrix, reaching concentrations up to 3.4 times higher than drug concentration initially present in milk.

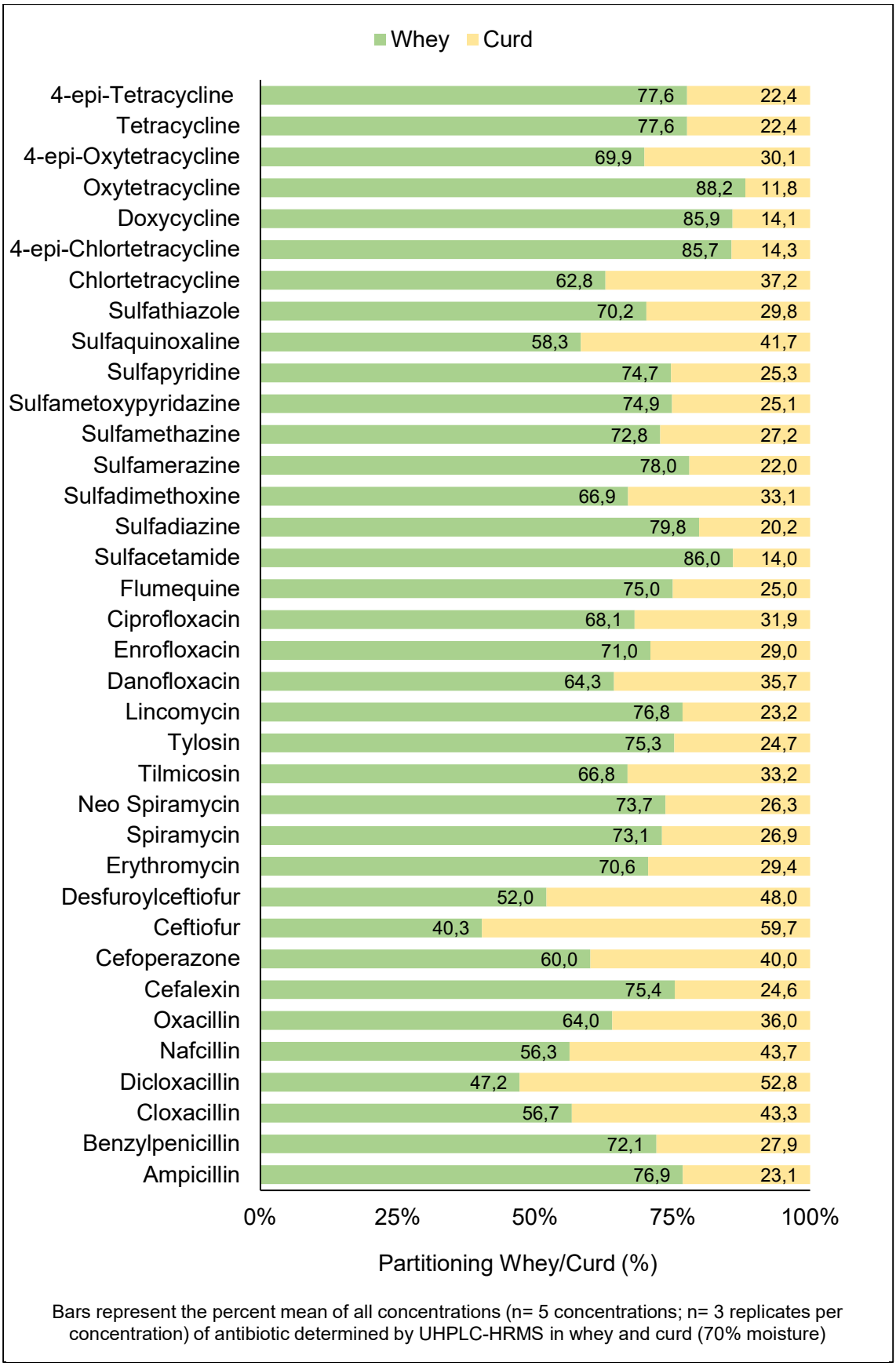
To evaluate the partitioning of antibiotics during cheese-making, normalised drug distribution rates were calculated considering the cheese yield and the antibiotic concentration in milk, curd, and whey fractions. As shown in Figure 24, antibiotics present in milk were mainly released into the whey fraction (up to 85.99%) during the drainage of the experimental cheeses. Thus, in general, the percentage of antibiotics retained in the curd fraction was lower than 50% in all cases, except for ceftiofur (59.7%) and dicloxacillin (52.8%), and highly variable between drugs.

Similar curd retention percentages to those obtained in this study were reported by Shappell *et al.* (2017) for oxytetracycline (15%), erythromycin (22%) and sulfadimethoxine (28%), when assessing the transfer of different veterinary drugs from skim milk to whey and curd fractions. Only in the case of benzylpenicillin (12%) was the result half of that shown in this experiment. In a similar study, Lupton *et al.* (2018) reported a higher retention rate close to 50% for ciprofloxacin.

Table 16. Antibiotic concentration ratios between rennet curd and whey fractions according to the drug levels in milk used for cheese production

Antibiotics	Equivalent drug concentration in raw milk (µg/kg)					SE
	0.25 EU-MRL	0.50 EU-MRL	1 EU-MRL	2 EU-MRL	4 EU-MRL	
β-lactams						
Ampicillin	0.725	0.741	0.669	0.838	0.864	0.0874
Benzylpenicillin	-	0.697^{a}	0.836^{ab}	1.059^{bc}	1.146^{c}	0.0597
Cloxacillin	3.216	1.686	1.848	1.778	1.685	0.3988
Dicloxacillin	-	2.548	2.980	2.735	2.967	0.3192
Nafcillin	-	1.633^{a}	2.234^{c}	1.873^{ab}	1.909^{ab}	0.0894
Oxacillin	1.478	1.224	1.482	1.374	1.389	0.1153
Cefalexin	0.869	1.053	0.737	0.857	0.722	0.1164
Cefoperazone	-	1.693	1.635	1.837	1.460	0.1234
Ceftiofur	4.042	3.292	3.341	3.669	3.562	0.4639
Desfuroylceftiofur	-	1.602	2.052	2.721	2.790	0.3448
Macrolides						
Erythromycin	-	0.810	1.136	1.233	0.124	0.0774
Spiramycin	0.842	0.815	0.901	0.915	0.948	0.0648
Neo Spiramycin	0.718	0.784	0.921	0.904	0.972	0.0649
Tilmicosin	1.127	1.183	1.252	1.355	1.180	0.1836
Tylosin	-	0.807	0.824	0.827	0.773	0.0113
Lincosamides						
Lincomycin	0.677	0.668	0.738	0.792	0.791	0.0582
Quinolones						
Danofloxacin	2.613^{b}	1.235^{a}	1.207^{a}	1.112^{a}	1.155^{a}	0.1917
Enrofloxacin	0.778	0.878	1.086	1.025	1.102	0.0904
Ciprofloxacin	1.310	1.075	1.129	1.015	1.069	0.0645
Flumequine	0.792	0.765^{ab}	1.066^{b}	0.855^{ab}	0.902^{ab}	0.1147
Sulfonamides						
Sulfacetamide	0.292^{a}	0.452	0.413	0.442	0.365	0.1026
Sulfadiazine	0.540	0.613	0.678	0.620	0.593	0.0645
Sulfadimethoxine	1.740	1.217	1.163	1.083	1.020	0.2012
Sulfamerazine	0.497	0.683	0.782	0.731	0.657	0.0863
Sulfamethazine	0.966	0.932	0.926	0.848	0.851	0.0870
Sulfamethoxypyridazine	0.693	0.851	0.924	0.809	0.794	0.0969
Sulfapyridine	0.848	0.789	0.862	0.850	0.782	0.1134
Sulfaquinoxaline	2.741	1.771	1.782	1.666	1.532	2.2315
Sulfathiazole	0.859	0.907	1.164	1.260	1.107	0.1914
Tetracyclines						
Chlortetracycline	-	1.650	1.698	1.360	1.079	0.1604
4-epi-Chlortetracycline	0.699^{c}	0.510^{b}	0.512^{b}	0.428^{ab}	0.343^{a}	0.0298
Doxycycline	0.411	0.426	0.448	0.417	0.315	0.0583
Oxytetracycline	0.354	0.373	0.363	0.315	0.231	0.0572
4-epi-Oxytetracycline	-	1.027	1.223	1.202	0.847	0.1467
Tetracycline	0.854	0.712	0.777	0.697	0.634	0.1050
4-epi-Tetracycline	-	0.774	0.778	0.704	0.558	0.1000

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). SE: Standard Error. Data missing: drugs with CCβ out of evaluated concentration range for some of the three matrices (milk, cheese, whey) considered. $^{a, b, c}$: different letters in the same row indicate significant differences (P< 0.05).



Bars represent the percent mean of all concentrations (n= 5 concentrations; n= 3 replicates per concentration) of antibiotic determined by UHPLC-HRMS in whey and curd (70% moisture)

Figure 24. Drug distribution rates (%) between rennet curd and whey fractions

Lipophilicity (log P) is usually considered to predict the partitioning of veterinary drugs into the different milk fractions (Hakk *et al.*, 2016; Shappell *et al.*, 2017; Lupton *et al.*, 2018) and related products. Thus, lipophilic drugs would be concentrating in cream, butter, and hard cheeses, while more hydrophilic drugs would be distributed in whey products.

To explain the partitioning of the 36 antibiotics used in this study, the logarithm of the curd (0% moisture)/whey ratio was calculated for plotting with the log P of such substance. As shown in Figure 25, antibiotics having increased log P (more lipophilic) tended to be more retained in curd and presented, therefore, higher curd/whey concentration ratios. However, this trend, which was not observed in the quinolone group, presented some relatively low adjustments (R^2 between 0.21 and 0.67) compared to those encountered by other authors in different veterinary drugs (R^2= 0.70), including antibiotics, anti-inflammatory and antiparasitic substances, with log P values ranging from 1.5 to 6.6 (Shappell *et al.*, 2017). Only in the case of macrolides and sulfonamides more adequate adjustments with R^2 values close to 0.7 were reached, similarly to data obtained by Shappell *et al.* (2017). The great range of variation of this parameter might explain the increased relationship between log P and the distribution of such substances during milk processing obtained by these authors, in contrast to what was observed in this study.

On the other hand, recently published studies suggest that the cheese-making process itself might exercise a larger influence on the distribution of veterinary drugs in the milk fractions than the solubility characteristics of such substances or their ability to binding proteins. In this sense, a large transfer of antibiotics from goat's milk to fresh cheeses (moisture content of 56%) was observed by Quintanilla *et al.* (2019b), who reported retention rates above 50% for different substances such as benzylpenicillin (66.8%), cloxacillin (75.2%), erythromycin (64.6%), enrofloxacin (51.1%) and ciprofloxacin (57.3%), with the only exception being oxytetracycline (37.5%), which was mainly released into the whey fraction. However, in ripened cheeses (moisture content of 40%) the same authors (Quintanilla *et al.*, 2019a) indicated retention rates lower than 20% for most of the antibiotics considered, including benzylpenicillin (16.4%), cloxacillin (15.6%), and erythromycin (7.4%). Only quinolones (enrofloxacin: 39.4% and ciprofloxacin: 56.4%), and especially oxytetracycline (68%), were highly retained in the rennet curd, reaching concentrations higher than those present in milk for cheese production. Similar results were reported by Cabizza *et al.* (2017) in ripened cheeses from sheep milk spiked with oxytetracycline at MRL equivalent antibiotic concentration.

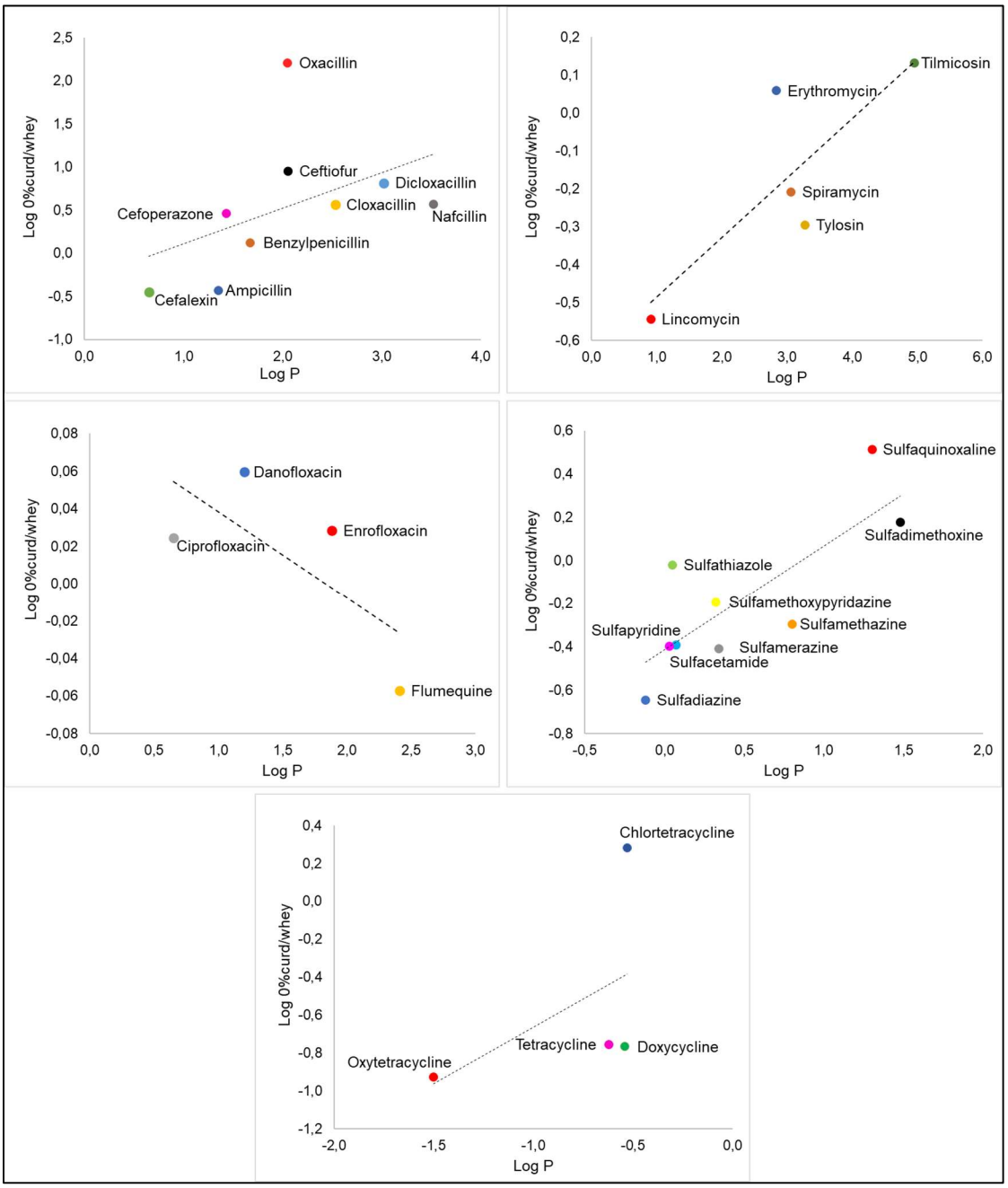


Figure 25. Relationship between the logarithm of concentration curd (0% moisture) to whey ratio and drug lipophilicity (Log P)

Moisture content in the cheeses, closely related to the cheese-making procedure, could be linked to the different drug distribution obtained between fresh and ripened cheeses at the beginning of maturation.

Thus, a low curd drainage as occurs in the fresh cheese production process would increase the concentration of water-soluble antibiotics in the rennet curd, reducing that of fat-soluble drugs such as quinolones or tetracyclines. Contrary, an intense drainage characteristic of the ripened cheeses processes would favour the major release of water-soluble drugs into the whey, while those able to interact with the main curd

components such as tetracyclines, would be retained to a greater extent. Other aspects concerning the cheese-making procedure such as heat treatments, calcium chloride addition, salting process, ripening, etc. could be related to possible antibiotic transformations and partial drug elimination during milk processing. Differences between cheese elaboration at lab scale and industrial processing transformation should also be considered.

On the other hand, Ozdemir *et al.* (2018) supported differences in the drug distribution into milk fractions between *in vitro* and *in vivo* studies, when assessing the transfer of amoxicillin and tylosin using HPLC-UV. Agreeing with the results obtained in this study, tylosin, independent of its lipophilic nature, was mostly released in water-soluble milk fraction (skimmed milk: 93.91%, cream: 6.42% and casein: 2.11%) during *in vitro* assay, attributing this behaviour to its binding degree to milk proteins, which were mostly accumulated in skimmed milk, and its zwitterionic property. Similarly, amoxicillin also interacted to a greater extent with skimmed milk fraction (skimmed milk: 92.36%, cream: 7.42% and casein: 0.82%) due to its characteristic hydrophilicity. However, for the *in vivo* experiment, the behaviour of both antibiotics was different, with significantly decreasing percentages of the two drugs in the water-soluble fractions, although a larger proportion of amoxicillin remained in skimmed milk than in cream and casein, and the amount of tylosin in cream increased considerably (73.13% for amoxicillin in skimmed milk, and 63.34% for tylosin in cream). Also, Quintanilla *et al*. (2018), when assessing the transfer of macrolides from goat's milk to ripened cheeses in an *in vivo* study, indicated a low retention rate (5-6) of such substances in the cheeses. Therefore, it is essential to study the transfer of antibiotics from milk to dairy products in *in vivo* conditions to consider the biochemical transformations that have occurred in the animal organism affecting the drug partitioning.

III.2.4. Conclusions

Results herein indicate that antibiotics present in milk for cheese production are mainly transferred from milk to whey during cheese-making, which could have potential implications for consumers, livestock, and environmental conditions. In general, drug distribution was not affected by the antibiotic concentration initially present in milk, which ranged from 0.25 to 4 times the MRL legally established for raw milk, and it was poorly related to the drug lipophilicity. Therefore, it would be of interest to include other aspects related to the characteristics of the veterinary drugs as well as the different cheese-making processes to reach a more thorough assessment of their distribution in the different milk matrices.

Chapter IV. Assessment of Screening Tests for Antibiotics Detection in Whey

IV.1. EVALUATION OF THE COMMERCIAL TESTS TO SCREEN ANTIBIOTICS IN WHEY

IV.1.1. Introduction

The presence of antibiotics in dairy products like cheese and whey is an important food safety and health hazard mainly due to the generation of antimicrobial resistance, among others, and also promotes negative effects on the dairy industry. Antibiotics can be transferred from milk to cheese and whey during the cheese-making process. Therefore, it is also necessary to have appropriate methodologies for the detection of antibiotics in milk derivates. Drugs in dairy products have not been regulated and their potential effect is unknown.

Several commercially available tests have been developed to monitor antibiotic residues in milk. Many of the screening tests are microbial methods based on the inhibition of *Geobacillus stearothermophilus var. calidolactis*. The majority of microbial screening methods have been validated for their use in cow milk (Le Bréton *et al.*, 2007; Stead *et al.*, 2008; Perme *et al.*, 2010), with information on performance of these tests in sheep and goat's milk being rather limited (Beltrán *et al.*, 2015).

Furthermore, a simplest and least time-consuming method has been developed for the use in farms, dairies and milk quality laboratories. Mata *et al.* (2016) combined the microbial inhibitor test Eclipse Farm 3G with an e-Reader device that integrates incubation at 65ºC and a continuous monitoring of the colour change allowing the interpretation of the results in an objective and rapid manner.

However, in farms and dairies receptor-binding assays are more commonly applied than microbial tests due to their simple and fast response (< 10 min). These tests have been developed for the simultaneous detection of the most used antimicrobial families such as β-lactams and tetracyclines and for also other groups (aminoglycosides, macrolides, quinolones, and sulfonamides). Receptor-binding assays were initially developed for cow (Perme *et al.*, 2010; Reybroeck *et al.*, 2010; Salter *et al.*, 2011). In recent years the suitability of these tests has also been studied for sheep (Beltrán *et al.*, 2014a) and goat's milk (Beltrán *et al.*, 2014b).

Nevertheless, information about the suitability of milk screening methods for the analysis of antibiotics in whey is practically non-existent. Therefore, the objective of this study is the evaluation of the tests commercially available for milk applied to the detection of antibiotics in whey.

IV.1.2. Material and Methods

IV.1.2.1. Experimental Design

The Eclipse Farm 3G microbial test coupled to an e-Reader device (Zeulab, Zaragoza, Spain), hereinafter referred to as Eclipse Farm-eReader test, and the Twinsensor, 3Aminosensor, Tylosensor and Quinosensor receptor-binding assays (Unisensor, Liège, Belgium) were chosen to evaluate their performance in whey.

All tests were evaluated in accordance with Commission Decision 2002/657/EC (EC, 2002b) in the Institute for Animal Science and Technology (ICTA, UPV) laboratories. Antibiotic-free whey samples from individual goats were analysed in triplicate to calculate the test specificity (false-positive rate). The detection profile of the screening tests was evaluated according to the IDF recommendations (ISO/IDF, 2003a,b), and the Detection Capability (CCβ) was calculated following the CRLs guidelines (CRLs, 2010). The effect of the pH value of the whey samples was used to evaluate the ruggedness of the microbial screening test.

IV.1.2.2. Whey, Antibiotics and Spiked Samples

Whey was obtained from a laboratory-scale cheese-making procedure using antibiotic-free milk from the experimental heard of Murciano-Granadina goats of the Universitat Politècnica de València (UPV, Valencia, Spain). Animals had a good health status and did not receive any veterinary drugs neither before nor during the experimental period.

The cheese manufacturing was described in the previously experiment “Transfer of antibiotics from goat’s milk to rennet curd and whey fractions during the cheese-making process” in Chapter III.

The IDF recommendations (IDF, 2014b) established that the pH of the whey samples must be between 6.5 to 7.0 to be tested by screening methods commercially available for milk. Therefore, pH was checked before use (pH-meter Basic 20, Crison, Barcelona, Spain), and adjusted when necessary. The gross composition of whey samples was also determined using an infrared spectrophotometer (MilkoScan 6000, Foss, Hillerød, Denmark), at the Interprofessional Laboratory of the Valencian Community (LICOVAL, UPV).

Since the original validation was carried out in cow milk, an abridged validation was developed for the whey samples (CRLs, 2010), and representative antibiotics from the most widely used veterinary drug residues in the treatment of infectious diseases in dairy goats (Berruga *et al.*, 2008b) were selected in this experiment.

Table 17 summarizes the commercial references of the antibiotics used in this study, the solvent employed for the preparation of antibiotic stock solutions, and the range of concentrations evaluated to calculate the detection limits of the different screening methods assessed. Drugs were stored and handled according to the manufacturer's instructions. For use, antibiotics were dissolved (1 mg/mL) in water or in an appropriate solvent in a 25 mL volumetric flask at the time when analyses were carried out to avoid problems related to instability. The intermediate and working solutions were prepared by diluting stock solutions in distilled water.

Spiked whey samples were prepared following IDF recommendations (ISO/IDF, 2003a,b) and tested immediately thereafter.

Table 17. Antibiotics used to assess the performance of commercial screening tests in whey

Antibiotics	Reference[1]	Solvent	Concentration ranges (µg/kg)	
			Eclipse Farm-eReader	Receptor-binding assays
β-lactams				
Amoxicillin	A8523	H_2O	2-6	1-5
Benzylpenicillin	P3032	H_2O	1-5	1-5
Cefalexin	C4895	H_2O	40-80	250-1,250
Aminoglycosides				
Gentamicin	G3632	H_2O	50-150	120-200
Macrolides				
Tylosin	T627	H_2O	10-30	10-50
Quinolones				
Enrofloxacin	17849	AcOH 5%/H_2O	500-2,500	20-100
Tetracyclines				
Oxytetracycline	O4636	HCl 0.1N/H_2O	50-150	10-50

[1]Sigma-Aldrich Química, S.A. (Madrid, Spain).

IV.1.2.3. Commercial Screening Tests

IV.1.2.3.1. Microbial Screening Test

The Eclipse Farm-eReader test (Zeulab) is a microbial inhibition tube test that uses *Geobacillus stearothermophilus var. calidolactis* for the detection of antimicrobials in milk. Tubes contain a nutrient agar medium spread with the target bacteria and bromocresol purple as pH indicator. When the test is incubated at 65°C, the microbial growth lowers the pH and causes the colour change of the medium from blue to yellow. The presence of antibiotics in milk samples above the detection limits of the test inhibits the growth of the bacteria, and no colour changes are observed. The e-Reader device (Zeulab) combines the incubation at the selected temperature and the monitoring of the

colour changes along the assay. An internal software automatically detects the end point of the assay using a negative (antibiotic-free) control sample as a reference, and then reads the colour in each tube with values expressed in arbitrary units (AU).

Whey samples were analysed following the test procedure recommended by the manufacturers for milk samples (Figure 26). Thus, a volume of 100 µL of sample was added into a test tube and incubated in the e-Reader device at 65°C until the end point of the assay was reached at 40 AU in approximately 120 min.

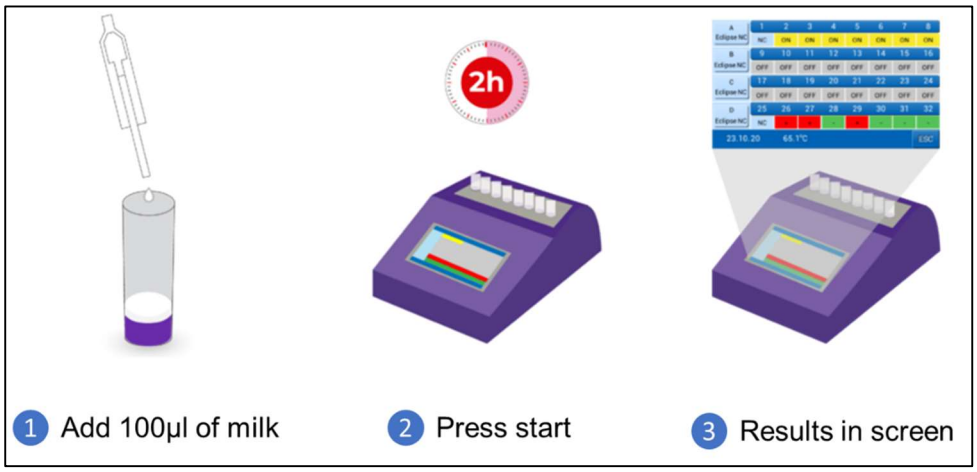


Figure 26. Analytical procedure of Eclipse Farm-eReader test

Source: Zeulab (2020)

After the incubation of whey samples, the formation of colour gradients ranging from blue to yellow in the culture medium was observed, which could influence on the interpretation of the results by the e-Reader device. To avoid this problem, a pre-experiment was carried out, checking different treatments of whey samples prior to their analysis. Hence, 10 blank (antibiotic-free) whey samples were analysed applying four different procedures: centrifugation (3,000 rpm, 10 minutes. Allegra X-15 Centrifuge Performance. Beckman Coulter, Barcelona, Spain), heat treatment (85ºC, 10 minutes. Thermostirred water bath. Fisher Scientific S.L., Madrid, Spain), centrifugation and heating (under the same conditions) and diffusion for one hour at room temperature, followed by rinsing with distilled water.

Before starting validation, the cut-off level of the Eclipse Farm-eReader test using whey was calculated following the indications by Mata *et al.* (2016). For this, 75 whey samples from individual goats were tested and then the mean value and standard deviation from the e-Reader results were calculated. The cut-off level was determined as the mean value plus three times the standard deviation, considering those samples with values higher than the cut-off level as positive.

IV.1.2.3.2. Receptor-Binding Tests

The evaluated receptor-binding assays are competitive tests involving specific binding reagents with high affinity to the drugs for which they have been developed. The test requires the use of two components: a microwell containing a predetermined amount of antibody linked to gold particles and dipstick made up of a set of membranes with two capture lines: the control line and the test line. The Twinsensor test allows simultaneous detection of both β-lactam and tetracycline antibiotics in milk samples. The 3Aminosensor test is applied for the detection of gentamicin, neomycin and streptomycin, the Tylosensor test is specific for tylosin, and the Quinosensor test is used for the detection of quinolones (ciprofloxacin and enrofloxacin).

Test procedures (Figure 27) in general include two stages: 1) preliminary incubation of the binding reagents with the milk sample resulting in the interaction of the antibiotics, if present, and 2) the milk solution is transferred onto an immunochromatographic medium by which a coloured signal development takes place when passing the various binding positions. Specific binding reagents that do not interact with antibiotic residues during preliminary incubation are bound at the corresponding binding positions and coloured lines appear.

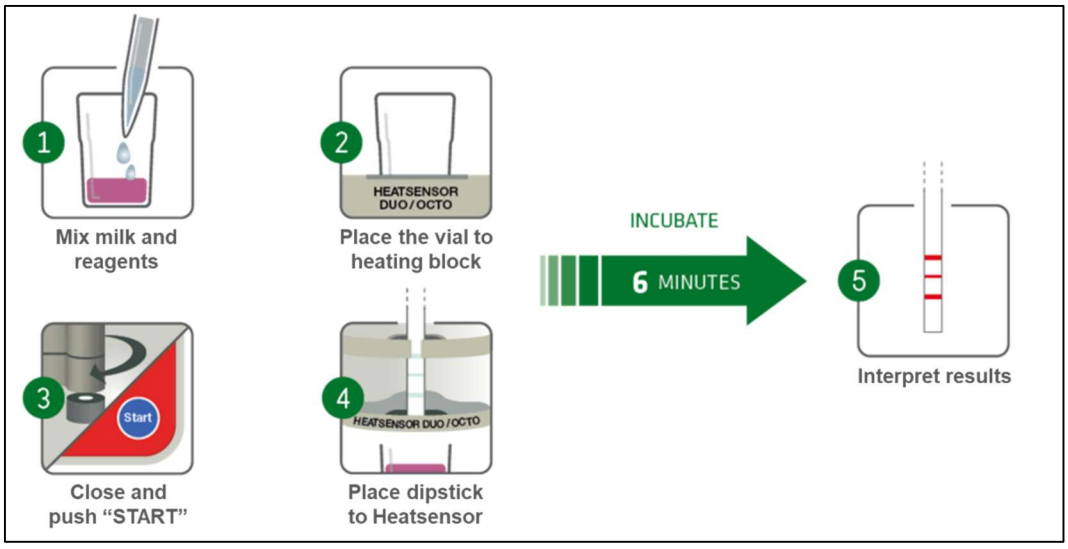


Figure 27. Procedure of Unisensor receptor-binding tests

Source: Unisensor (2020)

Whey samples were analysed following the test procedure indicated by the manufacturer for this matrix, which includes the adjustment of the pH with 0.1 N sodium hydroxide when its value is below than 6.5. The total incubation time was six minutes, although 3 additional minutes of incubation were suggested by the manufacturer if the control line on the test strip was not defined.

Results were classified as positive or negative both visually, by three trained technicians, and instrumentally. For visual interpretation of the results (Figure 28), a sample was classified as positive when the intensity of the antibiotic test line was as distinct as or lighter in colour than the control line. For a valid test, it is necessary that the control line appears after the incubation time.

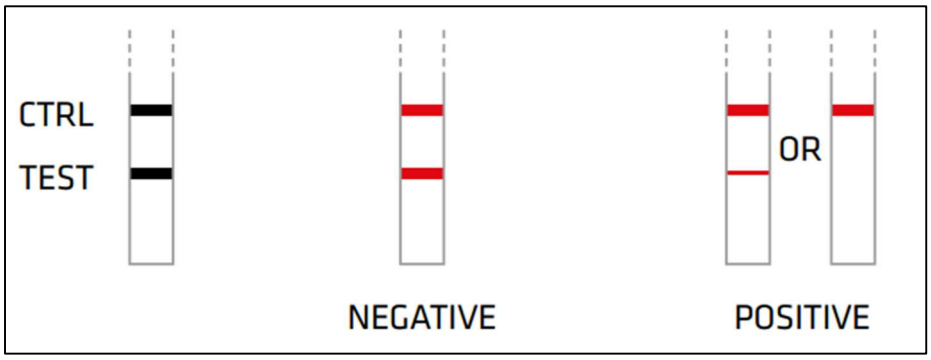


Figure 28. Interpretation of the Unisensor tests results

Source: Unisensor (2020)

For instrumental classification of the test results, dipsticks were inserted into Readsensor (Unisensor, Liege, Belgium, EC) reader system immediately after the required incubation time, and numerical data were recorded to categorize the test results. Negative results above 1.10 and positive results below 0.90, and intermediate values (0.90-1.10) were considered low positive results. The performance of the reader system was checked on a daily basis by testing negative and positive (benzylpenicillin: 4 µg/kg and oxytetracycline: 100 µg/kg, gentamicin: 100 µg/kg, tylosin: 50 µg/kg, enrofloxacin: 100 µg/kg) cow milk controls just before the whey analysis.

IV.1.2.4. Performance of Commercial Screening Tests

IV.1.2.4.1. Microbial Screening Test

The specificity of the Eclipse Farm-eReader test was evaluated using 100 antibiotic-free whey samples from individual goats, that were analysed in triplicate. Samples giving only one positive result were reanalysed and only those showing positive outcomes in at least two replicates were eventually classified as positive. Specificity was calculated as the number of negative results with respect to the total of whey samples, expressed as percentage.

The CCβ values of the commercial screening tests were investigated according to the CRLs guidelines (CRLs, 2010). Given the limitations of the e-Reader device for testing simultaneously a large number of samples (only six or eight tubes per run), the CCβ of this bioassay was calculated in three steps according to Mata *et al.* (2016).

Activity profiles for the seven antibiotics considered were initially established using whey samples from bulk raw goat's milk. One replicate of five concentrations around the expected Detection Limit (DL) for every compound was analysed in this step. Then, the DLs of the Eclipse Farm-eReader for these substances were calculated (second step) following a procedure adapted from ISO/IDF (2003a). To optimize the number of assays to be performed, only the lowest antibiotic concentration giving a positive result in the previous step was tested in five replicates. Finally, in the third step, the CCβ values were determined according to the CRLs (CRLs, 2010), the number of replicates analysed are summarized in Table 7 in the Introduction section. Thus, each analyte had to be tested 20, 40 or 60 times on different days, depending on the closeness of the detection limit to the regulatory MRL and no more than 5% of false-compliant results were considered. When the resulting detection capability for any substance was higher than its corresponding MRL fixed in milk, only 20 replicates were tested at CCβ concentration to verify the suitability of the test in the whey matrix.

The ruggedness of the Eclipse Farm-eReader test was evaluated by assessing the effect of the whey samples pH on the test performance. For this purpose, two types of whey samples were considered: acid whey samples when pH was lower than 6.5, and standard whey samples when pH was at or above 6.5 (IDF, 2014b). According to the CRLs guidelines (CRLs, 2010), for each type of whey considered, ten different samples free of antibiotics, and ten negative samples spiked individually with antibiotics at their corresponding CCβ concentration were analysed.

An ANOVA test using Statgraphics Centurion XVI.II software (StatPoint Technologies, Inc., Warrenton, VA) was applied to assess differences between both considered pH conditions. Tukey's multiple-comparison test was used for paired comparison of the treatment means, and the level of significance was determined at $P < 0.05$.

IV.1.2.4.2. Receptor-Binding Tests

The performance of the receptor-binding tests was assessed using the protocol mentioned previously for the Eclipse Farm-eReader test. Regarding CCβ values, the antibiotic concentration ranges used to determine the DLs of the receptor-binding assays are presented in Table 17. Their suitability to detect antibiotic residues at or below MRL was also evaluated according to the CRLs guidelines (CRLs, 2010). However, the ruggedness study was considered not necessary, as a previous adaptation of the tests for screening antibiotics in whey samples had been performed by the manufacturer.

IV.1.3. Results and Discussion

IV.1.3.1. Microbial Screening Test

In the pre-experiment carried out to check different treatments of whey samples prior the analysis, the diffusion of the whey samples at room temperature for one hour showed the best results (Figure 29). Thus, this step was included in the Eclipse Farm-eReader procedure. Giraldo *et al.* (2019) using the same test for the screening of antibiotics in sheep and goat's milk samples also applied a diffusion step before the analysis of milk samples.

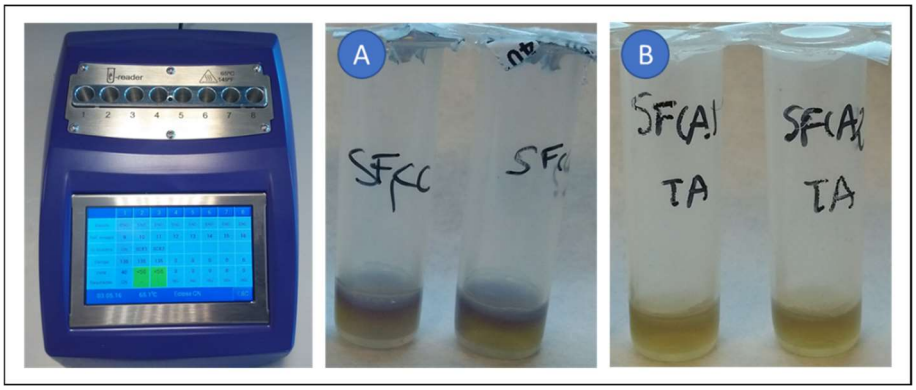


Figure 29. Eclipse Farm- eReader test results of whey analyses (A: without diffusion, B: with diffusion)

The cut-off level of the Eclipse Farm-eReader test was calculated according to Mata *et al.* (2016). The average reading values from the analysis of 75 antibiotic-free whey samples was 41.17±6.99 AU (Figure 30) and the calculated cut-off level (mean value of the negative samples plus three times the standard deviation) was 62.14 AU.

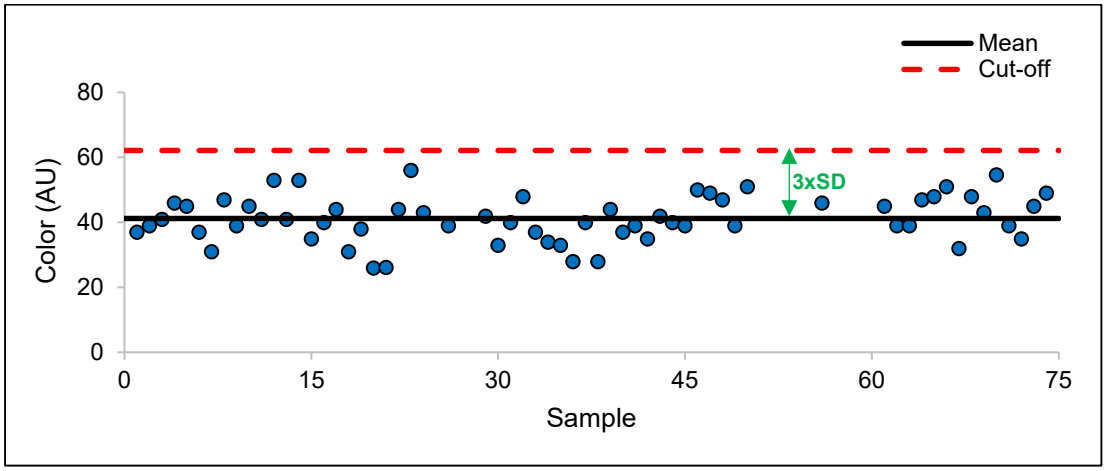


Figure 30. Cut-off value of the Eclipse Farm-eReader test for screening antibiotics in whey samples

The cut-off value obtained for whey was close to that obtained by Giraldo *et al.* (2019) using sheep and goat's milk samples (65 AU), and slightly higher than that calculated previously by Mata *et al.* (2016) in cow milk (55 AU).

Table 18 shows the chemical composition of the 100 whey samples from individual goats used to calculate the false-positive rate of the Eclipse Farm-eReader test. Whey samples having around 6% of total solids and pH values ranging from 6.5 to 7.0 were in accordance with the IDF recommendations (IDF, 2014b) for the analysis of whey using antibiotic screening tests commercially available for milk.

Table 18. Chemical composition of whey samples (n= 100) used in the specificity study of Eclipse Farm-eReader test

Parameters	Mean	SD	Min	Max
pH	6.56	0.04	6.50	6.69
Fat (%)	0.71	0.16	0.33	1.21
Lactose (%)	4.90	0.10	4.64	5.10
Protein (%)	1.08	0.14	0.83	1.31
Total solids (%)	6.70	0.17	6.36	7.12

SD: Standard Deviation; Min: Minimum; Max: Maximum.

The specificity calculated for the Eclipse Farm-eReader test was 100% (no false-positive results). High specificity values ranging from 97% to 100% were also obtained by other authors using the Eclipse Farm-eReader test in milk from different species. Mata *et al.* (2016) indicated a false-positive rate of 2.7%, when 300 antibiotic-free cow milk samples were tested. Similarly, Giraldo *et al.* (2019) obtained a specificity of 99.5% and 100% for sheep and goat's milk, analysing a total of 250 and 150 individual milk samples, respectively.

The DLs and the CCβ values of the Eclipse Farm-eReader test are presented in Table 19. In general, the CCβ values were higher than their respective DLs, highlighting gentamicin with a CCβ three times greater than the DL calculated for this substance.

Considering the MRL fixed in the EU for raw milk, DLs for whey resulted in values at or below the MRLs for all substances, with the exception of enrofloxacin since *Geobacillus stearothermophilus var. calidolactis* presents a limited sensibility for the detection of quinolones (Beltrán *et al*., 2015). These results are in accordance to those reported by Mata *et al.* (2016) for the β-lactam group, using the Eclipse Farm-eReader test in cow milk. In the case of non β-lactam drugs, specifically for gentamicin, tylosin and oxytetracycline substances, the detection limits were higher, to a greater or lesser extent, than those obtained in whey.

Table 19. Detection Limit (DL) and Detection Capability (CCβ) of Eclipse Farm e-Reader test for antibiotics in whey

Antibiotics	EU-MRL (µg/kg)	DL		CCβ		
		Concentration (µg/kg)	e-Reader value Mean±SD	Concentration (µg/kg)	Positives/ Total	e-Reader value Mean±SD
β-lactams						
Amoxicillin	4	4	71±5	5	20/20	107±12
Benzylpenicillin	4	3	107±15	3	40/40	117±24
Cefalexin	100	50	63±18	60	38/40	108±19
Aminoglycosides						
Gentamicin	100	30	72±4	100	59/60	94±13
Macrolides						
Tylosin	50	15	66±5	20	19/20	86±14
Quinolones						
Enrofloxacin	100	2,000	71±7	2,000	20/20	75±8
Tetracyclines						
Oxytetracycline	100	60	68±12	100	60/60	95±12

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). DL: ISO/IDF (2003a). CCβ: CRLs (2010). Positive results: e-Reader value> 62 AU. SD: Standard Deviation.

For sheep and goat's milk, Giraldo *et al.* (2019) also indicated equal detection limits for penicillins using the Eclipse Farm-eReader test. However, the DLs of non β-lactam antibiotics were higher than in whey samples, even above safety limits established in Regulation Nº 37/2010 (EU, 2010) for aminoglycosides (gentamicin: DL≥ 250 µg/kg) and tetracyclines (oxytetracycline: DL= 150 µg/kg).

The sensitivity results herein differ from those obtained by Mata *et al.* (2016) in cow milk, who found lower CCβ values for amoxicillin (4 µg/kg), cefalexin (50 µg/kg) and gentamicin (50 µg/kg), showing the suitability of the method for the detection of these substances at or below safety levels. However, higher CCβs were obtained by Giraldo *et al.* (2019) in sheep and goat's milk, except for amoxicillin that was detected at 4 µg/kg, being consistent to the MRL established in milk for this penicillin. The differences in the sensitivity results obtained by the Eclipse Farm-eReader test in whey and milk from goats could be related to their different chemical composition, with significantly higher percentages of fat and protein in the case of milk, or the lack of somatic cells in whey.

Similar CCβ values were obtained by Giraldo *et al.* (2017) in whey samples using another version of the microbial test in microtiter format (Eclipse 100 test, Zeulab). Moreover, Beltrán *et al*. (2015), assessing the performance of commercially available microbial screening tests based on *Geobacillus stearothermophilus var. calidolactis* (BRT MRL, Delvotest DA, Delvotest SP-N and Eclipse 100) in goat's milk, reported lower or similar detection capabilities for β-lactams than those obtained for whey in this study, excluding the BRT MRL test with a CCβ above MRL in milk for cefalexin. As regards non β-lactam antibiotics, CCβ values were higher for aminoglycosides, macrolides and tetracyclines, being consistent to the obtained whey results for only gentamicin with the BRT test, and tylosin using the Delvotest SP-NT test. The sensitivity of method for enrofloxacin in goat's milk was in accordance with the results obtained in whey, due to the limited sensitivity of this microorganism for quinolones detection.

Regarding the ruggedness study (Table 20), the false-positive rate was not affected by the pH of the whey samples, as all the antibiotic-free whey samples showed results below the cut-off value (62 AU). Nevertheless, concerning test sensitivity, whey samples with pH values ranging from 5.46 to 6.22 showed significantly lower readings ($P< 0.05$) than those obtained for whey samples at or above 6.5 pH, both fortified at the CCβ concentration; an exception was cefalexin, with similar values in the two cases.

The detection capabilities are affected by the whey pH, increasing significantly the percentage of false-negative results, which in this study reached values between 10%

for benzylpenicillin up to 100% in the case of tylosin and enrofloxacin. Hence, in agreement to the IDF recommendations (IDF, 2014b), for screening antibiotics in whey using the microbial Eclipse Farm-eReader test, the pH of the whey samples must be adjusted before analysis when values are below 6.50.

Table 20. Effect of pH on the false-negative rate of Eclipse Farm-eReader test in whey (n= 10)

Antibiotics	eReader value Mean±SD		Positives/ Total	
	pH≥ 6.5	pH< 6.5	pH≥ 6.5	pH< 6.5
β-lactams				
Amoxicillin	108±12[b]	94±16[a]	10/10	10/10
Benzylpenicillin	117±24[b]	81±22[a]	10/10	9/10
Cefalexin	102±21	100±16	10/10	10/10
Aminoglycosides				
Gentamicin	92±11[b]	49±13[a]	10/10	2/10
Macrolides				
Tylosin	86±14[b]	40±9[a]	10/10	0/10
Quinolones				
Enrofloxacin	86±12[b]	35±9[a]	10/10	0/10
Tetracyclines				
Oxytetracycline	95±12[b]	72±10[a]	10/10	8/10

SD: Standard Deviation; [a, b]: Different letters in the same row indicate significant differences (P< 0.05).

IV.1.3.2. Receptor-Binding Tests

The mean quality parameters of the 100 antibiotic-free whey samples used for the specificity experiment are presented in Table 21 and are consistent, in terms of pH and composition, with the characteristics required in whey samples to be analysed through milk microbial inhibitor tests (IDF, 2014b).

The specificity of the Twinsensor, 3Aminosensor and Quinosensor tests was 100% (no false-positive results), and no significant differences were found between the visual and instrumental interpretation of the test results. However, in the case of Tylosensor, 52 out of 100 whey samples gave positive screening results when interpretation of the results was carried out instrumentally, while visual classification reported a higher specificity percentage (91%). Therefore, it might be necessary to modify Readsensor (Unisensor) operating parameters for achieving appropriate instrumental readings of the Tylosensor test results.

Table 21. Chemical composition of whey samples (n= 100) used in the specificity study of receptor-binding tests

Parameters	Mean	SD	Min	Max
pH	6.57	0.05	6.50	6.67
Fat (%)	0.68	0.16	0.33	1.07
Protein (%)	1.13	0.13	0.83	1.31
Lactose (%)	4.88	0.11	4.51	5.10
Total solids (%)	6.76	0.17	6.44	7.12

SD: Standard Deviation; Min: Minimum; Max: Maximum.

Although there are no published studies on the specificity of receptor-binding tests to detect antibiotics in whey, the results obtained in this study could be compared with those reported previously for milk from different species. In this sense, Reybroeck and Ooghe (2007) and Perme *et al.* (2010) also found 100% specificity using the Twinsensor test for the detection of antibiotics in bulk raw cow milk. For aminoglycoside, macrolide and quinolone groups, studies using the same Unisensor receptor-binding tests (Reybroeck and Ooghe, 2012) obtained 100% of specificity in cow milk. In sheep and goat's milk, Beltrán *et al.* (2014 a,b) indicated specificity results in accordance with that obtained in this study for whey. For goat's milk, the Twinsensor test showed 1% positive results, while no false-positive outcomes were reported using the SNAP β-lactam, SNAP Tetracycline and Betastar Combo tests.

Detection Limits (DLs) of the receptor-binding tests were calculated in whey according to the visual and instrumental interpretation of the results (Table 22). The DL values calculated were lower than the MRLs indicated for milk, except for cefalexin and gentamicin. In general, the results obtained for Twinsensor and Tylosensor tests agreed with those indicated by manufacturer in cow milk. However, DLs calculated using 3Aminosensor and Quinosensor tests in whey samples were about twice as high as those obtained for cow milk by the Unisensor company.

As for the Detection Capabilities (CCβs), the results were agreed with the DL values obtained previously (Table 22). The CCβ values reported by Beltrán *et al.* (2014b) for penicillins when assessing the performance of the Betastar Combo, SNAP β-lactam and Twinsensor receptor-binding assays in goat's milk were lower or equal to those obtained for whey in this study. The detection capability of benzylpenicillin (≤ 2 μg/kg) was lower than in whey using the SNAP β-lactam and Twinsensor tests; the SNAP β-lactam also showed a high sensitivity for the detection of cefalexin (CCβ= 75 μg/kg). For oxytetracycline, CCβ values (≤ 50 μg/kg) calculated in goat's milk with Betastar Combo, SNAP Tetracycline and Twinsensor were higher than that obtained by the Twinsensor test using whey samples.

Table 22. Detection Limit (DL) and Detection Capability (CCβ) of receptor-binding tests for antibiotics in whey

Antibiotics	EU-MRL (µg/kg)	DL		CCβ		
		Concentration (µg/kg)	Readsensor value Mean±SD	Concentration (µg/kg)	Positives/ Total	Readsensor value Mean±SD
β-lactams						
Amoxicillin	4	3	0.60±0.07	3	40/40	0.58±0.10
Benzylpenicillin	4	3	0.34±0.11	3	40/40	0.34±0.14
Cefalexin	100	500	0.62±0.18	500	20/20	0.71±0.13
Aminoglycosides						
Gentamicin	100	160	0.88±0.19	160	20/20	0.85±0.16
Macrolides						
Tylosin	50	10	0.96±0.15	10	19/20	0.93±0.11
Quinolones						
Enrofloxacin	100	40	0.87±0.05	40	20/20	0.78±0.17
Tetracyclines						
Oxytetracycline	100	10	0.34±0.13	10	20/20	0.48±0.21

EU-MRL: European Union Maximum Residue Limit fixed in milk (EU, 2010). DL: ISO/IDF (2003b). CCβ: CRLs (2010). SD: Standard Deviation.

IV.1.4. Conclusions

From the results obtained in this study, it can be concluded that the use of Eclipse Farm-eReader microbial test and Twinsensor, 3Aminosensor, Tylosensor and Quinosensor receptor-binding assays for the detection of antibiotics in whey shows, in general, detection capabilities below or at MRL fixed in milk with suitable specificity percentages. However, it might be convenient to make some modifications to achieve lower detection profiles. Considering the lack of regulation for whey, this study could be a first step towards the adequacy of screening tests to monitor whey samples for antimicrobials in a future control strategy.

IV.2. PERFORMANCE OF MICROBIAL BIOASSAYS IN MICROTITER PLATES TO DETECT ANTIBIOTICS IN WHEY

IV.2.1. Introduction

The microbial tests based on the inhibition of *Geobacillus stearothermophilus var. calidolactis* have been used worldwide in monitoring programmes for the screening of antibiotics in milk. Although this bacterium is very sensitive to β-lactam antibiotics, its detection profiles in the case of non β-lactam antibiotics are rather limited, especially for aminoglycosides, macrolides, quinolones and tetracyclines (Althaus *et al.*, 2003; Montero *et al.*, 2005; Le Bréton *et al.*, 2007; Linage *et al.*, 2007; Stead *et al.*,2008; Sierra *et al.*, 2009a,b; Beltrán *et al.*, 2015), to be used as an analytical quality control strategy.

Hence, some studies proposed the combination of different bacteria test as a complementary option to detect non β-lactam substances in accordance with the MRL levels, using microbial bioassays in microtiter plates with a dichotomous response. In this sense, Nagel *et al.* (2013a) improved the antibiotic coverage achieved by commercial inhibitor tests normally using *Geobacillus stearothermophilus* in cow milk combining this microorganism with *Bacillus subtilis*, which presents a greater sensitivity for the detection of macrolides (erythromycin and spiramycin) and quinolones (ciprofloxacin, enrofloxacin and marbofloxacin), and with *Bacillus cereus* in the case of tetracyclines. In sheep milk, detection limits close to MRL were also obtained for macrolide and quinolone groups with the implementation of a microbial bioassay consisting in *Geobacillus stearothermophilus* and *Bacillus subtilis* microorganims (Nagel *et al.*, 2012).

The microorganism alternatives most used are *Bacillus subtilis* for macrolides and quinolones, *Escherichia coli* for quinolones, and *Bacillus cereus* for tetracyclines. In addition, Nagel *et al.* (2013b, 2014) assessed thermophilic bacteria to reduce incubation time of bioassays using *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*, respectively, for the detection of β-lactams in milk in less than 2.5 hours.

Concerning whey, qualitative screening methods have not still been developed and studies about the suitability of milk screening tests for the detection of antibiotics in matrices with similar composition such as whey are practically non-existant (IDF, 2001; Berruga *et al.*, 2005). The aim of this study is to evaluate alternative bioassays to *Geobacillus stearothermophilus* to improve the detection of non β-lactams residues in whey.

IV.2.2. Material and Methods

IV.2.2.1. Experimental Design

A microbial system composed of four different bioassays with dichotomous response (positive or negative) in microtiter plate format was evaluated for the detection of antibiotics in whey. Therefore, Eclipse 100 test (Zeulab, Zaragoza, Spain) using *Geobacillus stearothermophilus var. calidolactis* and three in-house bioassays that use *Bacillus subtilis*, *Geobacillus thermocatenulatus* and *Geobacillus thermoleovorans*, respectively, in accordance with the Commission Decision 2002/657/EC (EC, 2002b). Experimental procedures were performed in the ICTA (UPV) laboratories using raw milk from its experimental herd of Murciano-Granadina goats.

The assessment of the performance of the microtiter plate bioassays included a specificity study through the analysis per triplicate of 100 antibiotic-free whey samples from individual goats to calculate the false-positive rate (ISO/IDF, 2003a).

According to the methodology described by the International Dairy Federation (ISO/IDF, 2003a), to study the sensitivity of the different bioassays, Detection Limits (DLs) for 23 different antibiotics, belonging to the β-lactam, aminoglycoside, macrolide, lincosamide, quinolone, and tetracycline families, were calculated from the logistic regression equations constructed making 12 replicates per 8 different antibiotic concentrations.

IV.2.2.2. Whey, Antibiotics and Spiked Samples

Antimicrobial-free goat's whey samples were obtained according to cheese-making protocol at lab-scale described previously in the study "Transfer of antibiotics from goat's milk to rennet curd and whey fractions during the cheese-making process" in Chapter III. It was verified that the pH of whey samples was adequate, ranging from 6.5 to 7, and if necessary, this parameter was adjusted with 0.1 N sodium hydroxide. Chemical parameters (fat, protein and total solids) from whey samples were also analysed using MilkoScan 6000 (Foss, Hillerød, Denmark) (LICOVAL, UPV).

A total of 23 antibiotic substances provided by Sigma-Aldrich Química, S. A. (Madrid, Spain) were assessed in the whey matrix applying different concentration ranges (Table 23). Antibiotic stock solutions (1 mg/mL) were prepared on a daily basis before use, dissolving solid patterns in water and/or other suitable solvent. Working solutions were obtained diluting previous stock patterns in distilled water, while spiked whey samples were fortified following the recommendations of the International Dairy Federation (ISO/IDF, 2003a).

Table 23. Antibiotics used to assess the performance of microtiter plate bioassays in whey

Antibiotics	Reference[1]	Solvent	Concentration ranges (µg/kg)			
			G. stearothermophilus	*B. subtilis*	*G. thermocatenulatus*	*G. thermoleovorans*
β-lactams						
Amoxicillin	A8523	H_2O	0.25-3.75	0.50-6.50	0.50-6.50	0.50-6.50
Ampicillin	A9393	H_2O	0.50-6.50	4-22	0.50-9.50	0.50-6.50
Benzylpenicillin	PENNA	H_2O	0.25-3.25	1-7	0.50-6.50	0.50-6.50
Cloxacillin	C9393	H_2O	5-65	50-350	5-65	40-130
Oxacillin	46589	MeOH/H_2O	5-35	50-350	1-7	20-140
Cefalexin	C4895	H_2O	10-70	100-700	10-70	50-650
Cefapirin	43989	H_2O	1-7	10-70	2-14	5-95
Cefazolin	C5020	H_2O	2.50-17.50	50-350	2.50-17.50	50-350
Cefoperazone	C4292	NaOH 0.1 N/H_2O	25-175	200-1,400	25-175	100-950
Cefquinome	32472	H_2O	25-175	25-175	25-175	50-350
Aminoglycosides						
Gentamicin	G3632	H_2O	50-350	200-1,400	50-350	50-350
Neomycin	N1876	H_2O	200-1,400	1,000-7,000	500-3,500	100-700
Streptomycin	S6501	H_2O	500-3,500	1,000-7,000	1,000-7,000	500-3,500
Macrolides						
Erythromycin	E6376	EtOH/H_2O	50-350	20-80	50-350	20-140
Spiramycin	59132		500-3,500	50-350	25-175	100-1,300
Tylosin	T6271	H_2O	20-140	50-350	20-140	20-140
Lincosamides						
Lincomycin	31727	H_2O	200-1,400	200-1,400	200-1,400	50-350
Quinolones						
Enrofloxacin	33699	AcOH 5%/H_2O	500-5,900	50-350	500-6,500	2,000-8,000
Ciprofloxacin	17850	HCl 0.1N	250-5,050	50-350	250-5,050	2,000-8,000
Tetracyclines						
Chlortetracycline	C4884	NaOH 0.1N/H_2O	50-350	50-350	50-350	400-1,000
Oxytetracycline	O4636	HCl 0.1N/H_2O	25-175	50-350	25-265	50-650
Tetracycline	T3258	HCl 0.1N/H_2O	25-175	100-700	25-265	50-650

[1]Sigma-Aldrich Química, S.A. (Madrid, Spain).

IV.2.2.3. Preparation of Microtiter Plate Bioassays

The Eclipse 100 test (Zeulab) is a microtiter plate bioassay containing agar medium with *Geobacillus stearothermophilus var. calidolactis* (Gst) spores, and bromocresol purple as pH indicator. Positive or negative results are obtained after the incubation period (65ºC, 120-150 min), checking the purple or yellow well colour, respectively. The composition and operational conditions of the three in-house bioassays used in combination with the Eclipse 100 test are shown in Table 24.

The general procedure to prepare the microtiter plate bioassays was as follows: the different culture media were prepared according to the manufacturers' instructions and sterilized (121ºC for 15 minutes). Then, the media were cooled down to approximately 60 ºC and their pH adjusted with 0.1 N HCl or 0.1 N NaOH, according to each bioassay specification. Culture media were seeded with the corresponding bacteria test, and the different reagents described in Table 24 added, and 100 µl of the inoculated culture medium were placed into each microtiter wells plate. The plates were sealed and used after being kept for 16 hours at 4ºC.

For use, an appropriate volume of the whey sample (100 µl for *B. subtilis* plate, and 50 µl for *G. thermocatenulatus* and *G. thermoleovorans* plates) was added into each microwell. After incubation (Figure 31), the visual interpretation of the test results was carried out by 3 trained technicians, assessing colour changes of the culture media to be classified as negative (*B. subtilis*: pink; *G. thermocatenulatus* and *G. thermoleovorans*: light-yellow) or positive (*B. subtilis*: light-blue, *G. thermocatenulatus*: purple and *G. thermoleovorans*: black). Two coincident results were considered for statistical calculations.

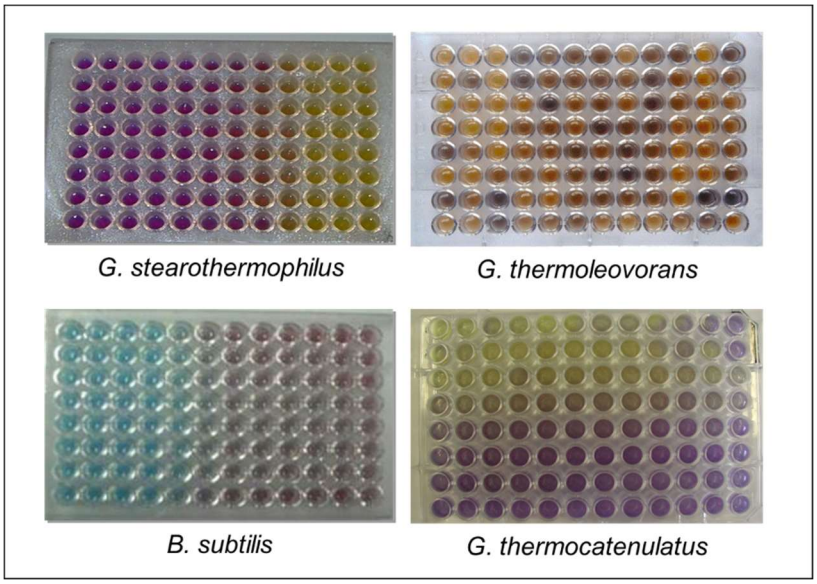


Figure 31. Microtiter plate bioassays after incubation

Table 24. Composition and operational conditions of microtiter plate bioassays

Bacteria test	Culture medium	pH	Supplements	Indicator	Incubation T[a]/t
Bacillus subtilis (Bs) $1.75x10^8$ spores/mL Ref. 1.10649 (Merck)	Müeller Hinton: 38 g/L Ref. BK048HA (Bioser)	7.2	Glucose: 10 g/L Ref. 1.08342 (Merck)	2,3,5-TTC: 150 mg/L Ref. T8877 (Merck) Toluidine blue: 20 mg/L Ref. T3260 (Merck)	40ºC/ 5 h
Geobacillus thermocatenulatus (Gtc) $8.0x10^{10}$ spores/mL Ref. LMG-19007 (DSMZ)	Plate Count Agar (PCA) Agar Nutritive: 15 g/L Ref. 413792 (Panreac) Casein peptone: 5 g/L Ref. 403898 (Panreac) NaCl: 3 g/L Yeast extract: 2.5 g/L Ref. 403687 (Panreac)	7.4	Glucose: 20 g/L Ref. 1.08342 (Merck)	Bromocresol Purple: 75 mg/L Ref. 2090 (Mallinckrodt)	65ºC/ 1 h 30 min
Geobacillus thermoleovorans (Gtl) $2.4x10^9$ spores/mL Ref. LMG-9823 (DSMZ)	Müeller Hinton: 38 g/L Ref. BK048HA (Bioser)	8.2	Glucose: 10 g/L Ref. 1.08342 (Merck) Clavulanic acid: 6 mg/L Ref. L0720000 (Merck)	Brilliant black: 200 mg/L Ref. 211842 (Merck) Toluidine blue: 10 mg/L Ref. T3260 (Merck)	65ºC/ 1 h 30 min

Merck, Darmstadt, Germany; DSMZ Leibniz Institute, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany; Bioser SA, Barcelona, Spain; Panreac, Barcelona, Spain; Mallinckrodt, Dublin, Ireland. 2,3,5-TTC: 2,3,5-triphenyltetrazolium chloride.

IV.2.2.4. Performance of Microtiter Plate Bioassays in Whey

The false-positive rate of the four microtiter plate bioassays was calculated testing 100 antibiotic-free whey samples from individual goats. Samples were collected and analysed in triplicate, on different days. Moreover, such samples were also checked after a heat treatment at 85ºC for 10 min to inactivate the natural inhibitors present in whey. In both cases, samples giving positive results were retested (three replicates) and only those with two positive results were eventually classified as positive.

The detection profiles of the four microtiter bioassays were evaluated using seven antibiotic concentrations (Table 23), which were analysed in twelve replicates to build a dose-response curve from the positive frequencies obtained for each one. Data were analysed using Statgraphics Centurion XVI.II software (StatPoint Technologies, Inc., Warrenton, VA). The following logistic regression model was applied to calculate the detection limits of the tests, with the antibiotic concentration giving 95% of positives.

$$L_{ij} = \text{logit}\,[P_i] = \beta_0 + \beta_1\,[A]_i + \varepsilon_{ij}$$

where: L_{ij}= lineal logistic model; $[P_i]$= logit [Pp/(1-Pp)]: the probability of a "positive" response / probability of a "negative" response; β_0, β_1= coefficients estimated for the logistic regression model, indicating "β_1" parameter the slope of the dose-response curve; $[A]_i$= antibiotic concentration; ε_{ij}= residual error. The adjusted coefficients of determination (R^2) were applied as a rank correlation between the observed responses and the predicted probabilities.

IV.2.3. Results and Discussion

IV.2.3.1. Specificity of Microtiter Plate Bioassays

As shown in Table 25, whey samples used in this study were in accordance with IDF recommendations (IDF, 2014b).

Table 25. Chemical composition of whey samples (n= 100) used in the specificity study of microtiter plate bioassays

Parameters	Mean	SD	Min	Max
pH	6.69	0.08	6.52	6.82
Fat (%)	0.56	0.21	0.20	0.95
Protein (%)	2.33	0.07	2.22	2.45
Lactose (%)	3.49	0.10	3.33	3.67
Total solids (%)	6.35	0.19	6.06	6.68

SD: Standard Deviation; Min: Minimum; Max: Maximum.

The specificity study (Table 26) of the bioassays containing *G. stearothermophilus* and *B. subtilis* showed a high percentage of non-compliant results for untreated whey samples (up to 10%), while for the plates with *G. thermocatenulatus* and *G. thermoleovorans* specificity was above than 98% regardless of the heat treatment performed (Table 26). When the heat treatment (85ºC, 10 min) was applied prior to the whey analysis, the specificity increased significantly in both cases: 97.9% for *G. stearothermophilus* and 100% for *B. subtilis*. Therefore, this pre-treatment should be considered for replicating analysis when a non-compliant result is obtained for screening antibiotics in whey using the microtiter plate bioassays.

Table 26. Specificity of microtiter plate bioassays in whey

Bioassays	No pre-treatment samples		Heating 85ºC 10 min	
	Positives/ Total	Specificity (%)	Positives/ Total	Specificity (%)
G.stearothermophilus	10/100	90	2/100	98
B. subtilis	11/100	89	0/100	100
G. thermocatenulatus	0/100	100	0/100	100
G. thermoleovorans	1/100	99	0/100	100

Studies performed with the Eclipse 100 commercial test in goat's milk showed better specificity percentages (99.4%) analysing 350 individual antibiotic-free milk samples (Beltrán *et al.*, 2015). The chemical composition of whey matrix, richer in soluble protein such as β-lactoglobulin, β-lactoalbumin, seroalbumin, lactoferrin and inmunoglobulins (Bravo, 2012), could have a negative influence on the increase of false-positive rate interfering with the antimicrobial activity of the bacteria test to a greater extent than in the case of milk.

Also, Molina *et al.* (2003) showed that after the application of a heat treatment (85°C for 10 min) to inactivate the natural inhibitors present in sheep milk, an increase of test specificity was achieved in agreement with the results obtained in this study for whey.

IV.2.3.2. Detection Limits of Microtiter Plate Bioassays

The coefficients of the logistic regression equations obtained for the four microtiter plate bioassays are presented in Table 27. High β_1 values, suggesting a suitable sensitivity, were obtained in all cases for the detection of β-lactam antibiotics, although they were particularly higher for bioassays containing *G. stearothermophilus* and *G. thermocatenulatus*. For the other substances, results were highly variable.

Table 27. Parameters of logistic regression model applied to the analysis of antibiotics using microtiter plate bioassays in whey

Antibiotics	*G. stearothermophilus*			*B.subtilis*			*G. thermocatelunatus*			*G. thermoleovorans*		
	β_0	β_1	R^2	β_0	β_1	R^2	β_0	β_1	R^2	β_0	β_1	R^2
β-lactams												
Amoxicillin	-37.009	48.135	96.85	-34.007	16.835	84.63	-6.702	3.341	77.80	-4.582	2.818	72.07
Ampicillin	-37.009	24.068	96.85	-89.335	7.579	88.13	-4.634	2.111	67.01	-3.688	2.036	63.06
Benzylpenicillin	-33.551	33.551	84.32	-48.555	16.185	84.32	-20.418	19.320	85.84	-5.421	3.361	76.87
Cloxacillin	-20.418	1.932	85.84	-48.518	0.328	85.22	-20.418	1.932	85.84	-15.595	0.213	68.20
Oxacillin	-33.041	4.386	96.29	-59.524	0.473	96.85	-64.740	16.185	84.32	-59.524	1.182	96.85
Cefacetrile	-70.378	8.021	96.99	-21.402	0.428	82.69	-5.251	0.283	73.56	-29.702	0.594	82.70
Cefalexin	-10.049	0.375	80.88	-49.934	0.164	85.44	-11.179	0.312	78.24	-4.018	0.023	67.00
Cefapirin	-85.621	24.463	96.99	-33.041	2.193	96.29	-16.086	7.238	85.67	-19.265	1.857	84.23
Cefazoline	-29.702	5.940	82.70	-33.041	0.439	96.29	-59.524	9.455	96.85	-29.469	0.298	82.74
Cefoperazone	-59.524	0.945	96.85	-59.524	0.118	96.85	-48.378	0.650	84.51	-34.925	0.171	85.44
Cefquinome	-48.555	0.647	84.32	-33.041	0.877	96.29	-85.621	0.979	96.99	-65.289	0.325	84.51
Aminoglycosides												
Gentamicin	-49.066	0.325	84.63	-59.524	0.118	96.85	-48.518	0.328	85.22	-48.518	0.328	85.22
Neomycin	-53.036	0.086	88.86	-33.041	0.022	96.29	-59.524	0.047	96.85	-30.160	0.149	83.19
Streptomycin	-33.041	0.044	96.29	-29.702	0.015	82.70	-51.205	0.017	86.82	-29.702	0.030	82.70
Macrolides												
Erythromycin	-33.041	0.439	96.29	-11.012	0.264	74.40	-33.041	0.439	96.29	-5.723	0.132	72.27
Spiramycin	-14.093	0.008	83.60	-29.702	0.297	82.70	-85.621	0.122	96.99	-9.564	0.026	77.06
Tylosin	-30.679	0.827	90.12	-51.187	0.357	91.50	-105.936	1.182	96.85	-9.213	0.129	74.00
Lincosamides												
Lincomycin	-59.524	0.118	96.85	-5.230	0.012	69.39	-6.951	0.111	74.70	-29.610	0.307	84.66
Quinolones												
Enrofloxacin	-37.009	0.024	96.85	-7.204	0.067	77.09	-33.551	0.017	84.32	-64.371	0.016	88.51
Ciprofloxacin	-29.399	0.039	96.85	-30.160	0.298	83.19	-23.466	0.023	84.63	-12.507	0.003	76.55
Tetracyclines												
Chlortetracycline	-59.524	0.473	96.85	-29.702	0.297	82.70	-49.066	0.325	84.63	-20.558	0.033	78.86
Oxytetracycline	-33.041	0.877	96.29	-31.859	0.308	85.84	-9.167	0.104	76.17	-33.285	0.170	85.22
Tetracycline	-59.524	0.945	96.85	-105.936	0.236	96.85	-5.305	0.088	68.82	-50.990	0.172	85.44

β_0, β_1: coefficients estimated for the logistic regression models; R^2: adjusted coefficients of determination.

The Detection Limits (DLs) calculated from the logistic regression equations are summarized in Table 28. Although no MRLs have been established in whey, the obtained results are comparable to safety levels fixed in milk.

In general, DLs obtained in whey were below MRLs for the most β-lactams considered using the bioassays containing *G. stearothermophilus* and *G. thermocatenulatus*, with oxacillin, cefacetrile, cefapirin and cefazoline, being detected at concentrations between 7.5 and 20 times lower than their respective MRLs. The detection limits were also lower than those obtained by other authors in milk from different species for penicillins and cefalosporins, assessing different microbial tests using *G. stearothermophilus* (Nagel *et al.*, 2012, 2013a; Beltrán *et al.*, 2015). High DLs for β-lactams were also indicated by Gasparotti *et al.* (2018) using *G. thermocatenulatus* microtiter plate bioassay in cow milk.

Of the three aminoglycosides analysed, gentamicin and neomycin were detected close to the MRL using *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* microtiter plate bioassays, while *B. subtilis* reported a detection limit slightly above MRL fixed in milk for neomycin. Neither bioassay showed suitable sensitivity to detect streptomycin.

For aminoglycosides, DLs significantly higher than those obtained for whey have been reported by other authors, using in-house microtiter plate bioassays based on the inhibition of *G. stearothermophilus* and *B. subtilis*, in milk from sheep (Nagel *et al.*, 2012) and cow (Nagel *et al.*, 2013a). According to the results obtained in this study, a suitable DL for neomycin in cow milk was reported by Itatí (2016) and Gasparotti *et al.* (2018), using *G. thermoleovorans* and *G. thermocatenulatus* as test microorganism, respectively. Similar detection levels for aminoglycosides were obtained by Beltrán *et al.* (2015) using commercial screening methods in sheep and goat's milk, neither microbial test showed a suitable sensitivity for the detection of streptomycin in milk at safety level.

Regarding macrolides, Table 28 shows that erythromycin was detected around the MRL in milk using *B. subtilis* and *G. thermoleovorans*, while spiramycin was only detected below MRL by *B. subtilis*, tylosin by *G. stearothermophilus* and lincomycin by *G. thermocatenulatus* and *G. thermoleovorans*. Results herein for macrolides are similar to those reported for sheep and cow milk using *B. subtillis* and *G. stearothermophilus* (Nagel *et al.*, 2012; Nagel *et al.*, 2013a). Itatí (2016), using *G. thermoleovorans* in cow milk, observed lower sensitivity to detect erythromycin, while the DL for lincomycin agreed with that obtained in this study for whey.

Table 28. Detection Limits (DLs) of microtiter plate bioassays in whey

Antibiotics	EU-MRL (µg/kg)	DL (µg/kg)			
		G. stearothermophilus	*B.subtilis*	*G. thermocatelunatus*	*G. thermoleovorans*
β-lactams					
Amoxicillin	4	1	2	3	3
Ampicillin	4	2	12	4	3
Benzylpenicillin	4	1	3	1	2
Cloxacillin	30	12	157	12	87
Oxacillin	30	8	132	4	53
Cefacetrile	125	9	57	29	55
Cefalexin	100	35	322	45	298
Cefapirin	60	4	16	3	12
Cefazoline	50	5	82	7	109
Cefoperazone	50	66	529	79	221
Cefquinome	20	80	41	91	210
Aminoglycosides					
Gentamicin	100	160	529	157	157
Neomycin	1,500	653	1,641	1,321	222
Streptomycin	200	820	2,198	3,242	1,099
Macrolides					
Erythromycin	40	82	53	82	66
Spiramycin	200	2,056	110	724	485
Tylosin	50	41	152	92	95
Lincosamides					
Lincomycin	150	529	703	89	106
Quinolones					
Enrofloxacin	100	1,660	152	2,176	4,081
Ciprofloxacin	100	830	111	1,142	5,348
Tetracyclines					
Chlortetracycline	100	132	110	160	704
Oxytetracycline	100	41	113	116	213
Tetracycline	100	66	461	94	313

EU-MRL: European Union Maximum Residue Limits fixed in milk (EU, 2010). DL: ISO/IDF (2003a).

The microbial bioassay using *B. subtilis* was the only test showing a suitable detection profile for quinolones in whey samples, with DL values close to their respective MRL fixed in milk. The high sensitivity of this microorganism to detect quinolones was emphasized by other authors using this bioassay to detect such substances in sheep (Nagel *et al.*, 2012) and cow milk (Nagel *et al.*, 2013a).

For tetracyclines, suitable DLs were observed for the bioassays containing *G. stearothermophilus*, *B. subtilis* (chlortetracycline and oxytetracycline) and *G. thermocatenulatus* (oxytetracycline and tetracycline) in order to ensure compliance with EU regulations in milk. However, both *G. stearothermophilus* and *B. subtilis* resulted in considerably higher DL values when they were used for screening antibiotics in sheep and cow milk samples (Nagel *et al.*, 2012; Nagel *et al.*, 2013a). Detection capabilities above MRLs were obtained by Beltrán *et al.* (2015) for tetracyclines using different commercial tests based on *G. stearothermophilus* in milk from small ruminants.

In Figure 32, the detection pattern obtained from every assessed bioassay is graphically expressed in relation to the MRL established in milk. A logarithmic transformation to DL/MRL for each antibiotic was applied. Thus, the different polygons, from the inner to the outer, correspond to concentrations ranging from 100 x (DL/MRL) to 0.01 x (DL/MRL), respectively.

Those quotients at the MRL line indicate an adequate DL for the corresponding antibiotic substance. However, antibiotics situated closer to the centre of the graphic (10 and 100 lines) should be present at a higher concentration than MRL to be detected by the bioassay in question. Finally, when DL/MRL ratios are more distanced from the interior polygon, achieving 0.1 and 0.01 values, the rate of false non-compliant results is increased significantly, possibly leading to an unjustified penalization of farmers.

In the present study, *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* showed a good sensitivity for the β-lactams family, and the application of different alternatives to commercial inhibitor test would be more related to the improvement of the incubation time, significantly shorter with the last two microorganisms.

As shown in Figure 32, only in the case of macrolides and quinolones, *B. subtilis* reported significantly better detection limits, close to MRL established in milk, than the rest of *Geobacillus* microorganisms. The combination of *G. stearothermophilus* and *B. subtilis* plates was previously indicated by Nagel *et al.* (2012) as an improvement of the antibiotic detection level in sheep milk with respect to the use of a single commercial test using *G. stearothermophilus*.

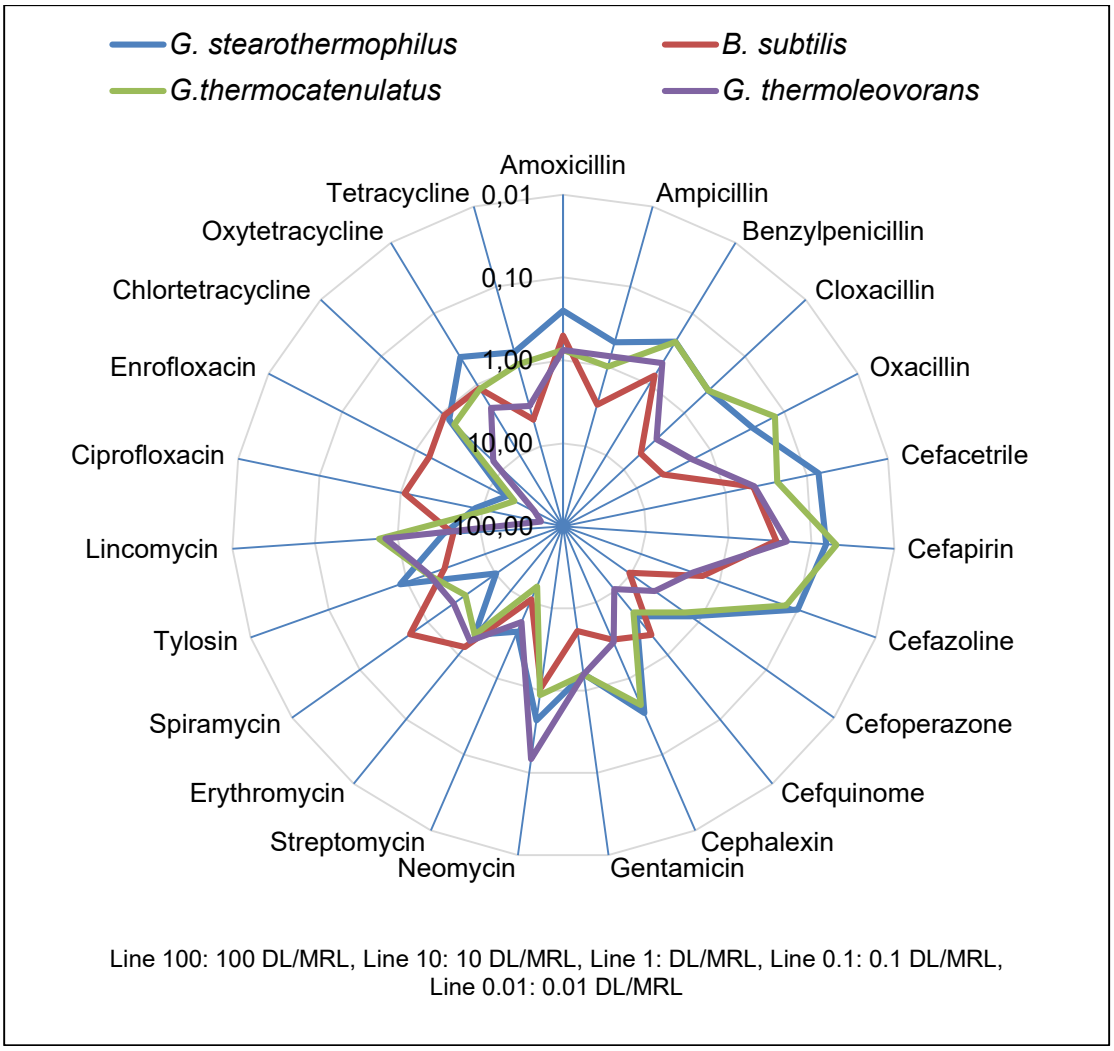


Figure 32. Detection pattern of the four microtiter plate bioassays

IV.2.4. Conclusions

To conclude, this study provides an adequate tool for the detection of a wide range of antibiotics in whey in two different ways. As first, complementing the lack of sensitivity of *G. stearothermophilus* for non β-lactam antibiotics such as macrolides and quinolones by means of its simultaneous use with *B. subtilis*. And the second alternative is related to the replacement of *G.stearothermophilus* by other thermophilic bacteria (*G. thermocatenulatus* or *G. thermoleovorans*) that, in addition to presenting a good sensitivity to β-lactams, allow shortening the incubation time.

IV.3. EVALUATION OF SCREENING TEST FOR ANTIBIOTIC RESIDUES (STAR) IN WHEY

IV.3.1. Introduction

Microbial inhibitor tests are extensively used in routine screening of milk samples due to their broad-spectrum of detection and capacity to screen a large number of samples, in addition to a characteristic low cost and relatively easy use. Among the widely used microorganisms, *Geobacillus stearothermophilus var. calidolactis* is the most commonly used by commercial inhibitor tests as it presents the highest sensitivity to detect β-lactam antibiotics.

However, none of the commercially available tests is satisfactory for the detection close to MRL of other non β-lactam antibiotics, especially aminoglycosides, macrolides, or quinolones. In addition, the high false-positive rate of these methods makes post-screening verification of the type of drug in suspected milk samples necessary. Therefore, some authors have developed multiplate microbiological systems using different microorganisms and specific methodological conditions (pH, temperature, and incubation time) for the semi-quantitative analysis (post-screening) of different antimicrobials by means of measuring the microbial inhibition zone.

The Community Reference Laboratories for residues (CRLs) of Fougères (France) developed the Screening Test for Antibiotic Residues (STAR), consisting in five different plates seeded with *Geobacillus stearothermophilus* for β-lactams and sulfonamides, *Bacillus subtilis* for aminoglycosides*, Kocuria varians* for macrolides*, Escherichia coli* for quinolones and *Bacillus cereus* for tetracyclines, that was validated in accordance to the Commission Decision 2002/657/EC criteria for antibiotic detection in cow milk (Gaudin *et al.*, 2004) and meat (Gaudin *et al.*, 2010).

The STAR protocol has become the method of choice for screening antibiotics in meat and milk in many European countries to limit the range of antibiotics to be tested by confirmation and, thus, reduce the cost of quantitative analysis.

Post-screening methods for the identification of antibiotics in dairy products like whey have still not been studied. Therefore, the objective of this study is the evaluation of the STAR multiplate microbiological system as a post-screening test for the assessment and semi-quantification of antibiotic residues in whey.

IV.3.2. Material and Methods

IV.3.2.1. Experimental Design

The Screening Test for Antibiotic Residues (STAR), a multiplate bioassay using five test microorganisms sensitive towards different antibiotic substances inoculated in a plate: *Bacillus stearothermophilus* for β-lactams and sulfonamides (currently *Geobacillus*, Nazina *et al.,* 2001), *Bacillus subtilis* for aminoglycosides, *Kocuria varians* for macrolides, *Escherichia coli* for quinolones, and *Bacillus cereus* for tetracyclines, was validated in whey following the International Dairy Federation criteria (ISO/IDF, 2003a).

The test specificity (false-positive rate) was calculated using antibiotic-free whey samples which were analysed in duplicate in two different days. For the calculation of the Detection Limits (DLs), antibiotics belonging to β-lactam, aminoglycoside, macrolide and lincosamide, quinolone, and tetracycline groups were selected and five different concentrations of them were tested in twelve replicates.

A linear regression model was established for each antibiotic to relate the antibiotic concentration and the zone of microbial growth inhibition. The lowest antibiotic concentration assessed in the regression model corresponded to the DL calculated.

IV.3.2.2. Whey, Antibiotics and Spiked Samples

Goat's whey samples were obtained from the experimental herd of Murciano-Granadina goats of the ICTA (UPV), and evaluated similarly to the methodology described previously in Chapter III.

The 17 antibiotic substances (commercial references), the solvent employed for the preparation of antibiotic stock solutions, and the range of concentrations to calculate the detection limits of each plate are shown in Table 29. Commercial drugs were stored and handled according by the manufacturers' instructions. For use, they were dissolved (1 mg/mL) at the time when analyses were carried out to avoid problems related to instability.

Spiked milk samples were prepared following the recommendations of the International Dairy Federation (ISO/IDF, 2003a), and tested simultaneously by the different plates immediately after spiking.

IV.3.2.3. Preparation of STAR plates and Analytical Procedure

The STAR protocol includes five plates using different test microorganisms, each one sensitive to specific veterinary drugs. Table 30 details the bacteria test, spore's

concentration in agar, composition and pH of the culture media, incubation conditions, antibiotic control discs, and the residue detection objectives of every plate bioassay.

Table 29. Antibiotics used to assess the performance of the STAR protocol in whey

Plate	Antibiotics	Reference[1]	Solvent	Concentration ranges (µg/kg)
Bacillus stearothermophilus (Bst)	*β-lactams*			
	Amoxicillin	A8523	H_2O	2-32
	Ampicillin	A9393	H_2O	2.5-40
	Benzylpenicillin	PENNA	H_2O	2-32
	Cloxacillin	C9393	H_2O	15-240
	Cefazolin	C5020	H_2O	5-80
Bacillus subtilis pH 7.2 (Bs7.2)	*Aminoglycosides*			
	Gentamicin	G3632	H_2O	125-2,000
	Neomycin	N1876	H_2O	1,500-24,000
	Streptomycin	S6501	H_2O	500-8,000
Kocuria varians pH 8 (Kv8)	*Macrolides*			
	Erythromycin	E6376	EtOH/ H_2O	15-240
	Spiramycin	59132	H_2O	100-1,600
	Tylosin	T6271	H_2O	150-2,400
	Lincosamides			
	Lincomycin	31727	H_2O	250-4,000
Escherichia coli pH 8 (Ec8)	*Quinolones*			
	Enrofloxacin	33699	AcOH 5%/H_2O	20-320
	Ciprofloxacin	17850	HCl 0.1N/H_2O	5-80
Bacillus cereus pH 6 (Bc6)	*Tetracyclines*			
	Chlortetracycline	C4881	NaOH 0.1N/H_2O	250-4,000
	Oxytetracycline	O4636	HCl 0.1N/H_2O	112.5-1,800
	Tetracycline	T3258	HCl 0.1N/H_2O	112.5-1,800

[1]Sigma-Aldrich Química, S.A. (Madrid, Spain).

Once prepared, 11.4 mL of inoculated culture medium were added on each square plate dish (plate size 120 x 120 mm). After solidification, 9 paper discs (diameter 9 mm) were equidistantly positioned on the surface of the test media (Figure 33), and immediately 30 µl of whey sample was added on the corresponding discs to be analysed. Plates were left at room temperature for 30 minutes and then, incubated accordingly (Table 30). After diffusion and incubation, diameters of the inhibitory zones were measured, including the diameter of the paper disc (diameter 9 mm), in duplicate, using a calliper (range 0-150 mm, accuracy ±0.02 mm, Ceosa, Huelva, Spain). Samples were classified as positive when inhibitory zones showed a diameter at or above 11 mm (Gaudin *et al.*, 2004).

Table 30. Composition and operational conditions of the STAR protocol for screening antibiotics in whey

Plate	Bacteria test	Concentration	Culture medium	pH	Incubation T^a/t	Control discs	Residue detection
Bst	*Bacillus stearothermophilus* Ref. ATCC 10149 (Merck)	5x10^5 spores/mL	Diagnostic Sensitive Test (DST): 40 g/L Ref. CM0261 (Oxoid) Trimethoprim (TMP): 0.5 µg/mL (Sigma)	7.4	55ºC/ 12-15 h	Sulfamethazine 1,000 µg/L	β-lactams and sulfonamides
Bs7.2	*Bacillus subtilis* Ref. BGA (Merck)	5x10^4 spores/mL	Test agar: 27.5 g/L Ref. 110664 (Merck)	7.2	30ºC/ 18 h	Streptomycin 2,000 µg/L	Aminoglycosides
Kv8	*Kocuria varians* Ref. ATCC 9341 (Pasteur Institute)	5x10^4 spores/mL	Test agar: 27.5 g/L Ref. 110664 (Merck)	8.0	37ºC/ 24 h	Tylosin 1,000 µg/L	Macrolides and Lincosamides
Ec8	*Escherichia coli* Ref. ATCC 11303 (Pasteur Institute)	1x10^5 spores/mL	Test agar: 27.5 g/L Ref. 110664 (Merck)	8.0	37ºC/ 18 h	Ciprofloxacin 100 µg/L	Quinolones
Bc6	*Bacillus cereus* Ref. ATCC 11778 (Pasteur Institute)	3x10^4 spores/mL	Test agar: 25 g/L Ref. 110664 (Merck)	6.0	30ºC/ 18 h	Chlortetracycline 500 µg/L	Tetracyclines

Merck, Darmstadt, Germany. Pasteur Institute, Paris, France. Oxoid, Unipath Ltd., Basingtoke, UK. Sigma-Aldrich Química, S.A., Madrid, Spain.

For each batch of culture media prepared, one negative (antibiotic-free) and one positive cow milk controls were included to verify operational conditions of the test plates and, additionally, one negative and one positive whey controls were used. The antibiotic concentrations assessed as positive controls were established according to Gaudin *et al.* (2004). Exceptionally, the chlortetracycline concentration was increased 2.5 times with respect to that indicated for cow since the sensitivity of *B. cereus* microorganism was lower in the case of whey.

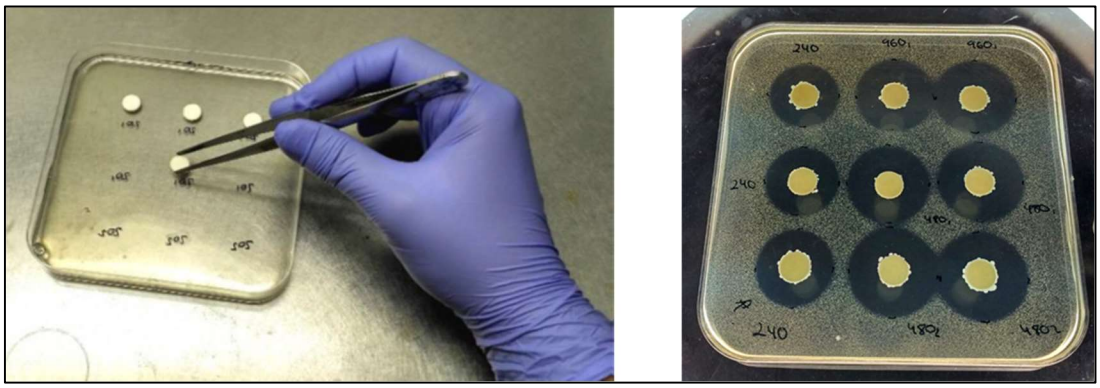


Figure 33. STAR protocol plate for screening antibiotics in whey

IV.3.2.4. Performance of STAR Protocol in Whey

The specificity (false-positive rate) of the STAR protocol for the screening of antibiotics in whey was evaluated testing 100 whey samples free of antibiotics, obtained from a cheese-making procedure at lab-scale using individual milk from goats (Chapter III). Two batches of culture media were prepared, including for each one both negative (antibiotic-free) and positive controls from whey and cow milk, analysed in duplicate. Considering a maximum of 9 paper disks per plate, a total of 24 plates were employed for each microorganism.

A second experiment was conducted to evaluate the Detection Limits (DLs) of the STAR protocol using 17 antibiotics belonging to 6 different families (β-lactams, aminoglycosides, macrolides and lincosamides, quinolones and tetracyclines). Five different concentrations in twelve replicates were analysed with several culture medium batches. For each one, positive and negative controls from cow milk were used, analysing in duplicate, to check the suitability of the bioassays, in addition to one negative and one positive whey controls (Figure 34), making a total of 8 plates per antibiotic.

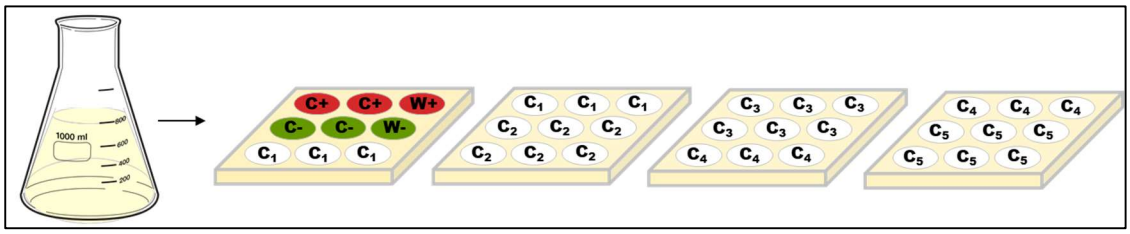


Figure 34. Experimental design of STAR protocol: Detection Limit (DL) study in whey

In agreement to IDF (ISO/IDF, 2003a), the detection limits were calculated as the lowest antibiotic concentration that produces 95% of positives (inhibition zone ≥ 11 mm diameter).

Finally, to establish the relationship between the inhibition zone diameters and the antibiotic concentration, five different increasing antibiotic concentrations (each double that of the previous), from the initial concentration corresponding to DL previously obtained, and twelve repetitions for each one were assessed. With these results, a linear regression model was made.

IV.3.2.5. Statistical Analysis

To establish a relationship between the inhibition zone diameters and the concentrations of antibiotics present in whey, the following linear regression model was applied:

$$D_{ij} = \beta_0 + \beta_1 \log C_i + \varepsilon_{ij}$$

where D_{ij}: diameter of inhibitory zone (mm), β_0 and β_1: parameters calculated by the linear regression model, log C_i: logarithm of antibiotic concentration (µg/kg), and ε_{ij}: residual error.

Statistical analysis was performed using Statgraphics Centurion XVI.II software (StatPoint Technologies, Inc., Warrenton, VA).

IV.3.3. Results and Discussion

IV.3.3.1. STAR Specificity

As shown in Table 31, the pH and the chemical composition of 100 antibiotic-free whey samples used to calculate the specificity of the STAR protocol were in agreement with the IDF recommendations (IDF, 2014b) since the mean pH was between 6.5 and 7.0 and the total solids percentage reached the value of 6.3.

Table 31. Chemical composition of whey samples (n= 100) used in the specificity study (false-positive rate) of the STAR protocol

Parameters	Mean	SD	Min	Max
pH	6.61	0.03	6.58	6.67
Fat (%)	0.56	0.20	0.35	0.97
Protein (%)	1.09	0.12	0.86	1.25
Lactose (%)	4.80	0.11	4.55	4.95
Total solids (%)	6.33	0.20	5.85	6.56

SD: Standard Deviation; Min: Minimum; Max: Maximum.

The positive control discs used for the five test plates showed inhibition zone diameters above 11 mm as follows: 16.07±2.33 for sulfamethazine (1,000 µg/L), 13.46±2.01 for streptomycin (2,000 µg/L), 18.98±3.37 for tylosin (1,000 µg/L), 20.13±0.86 for ciprofloxacin (100 µg/L) and 18.82±3.99 for chlortetracycline (500 µg/L). No significative variability was observed between the different days of experiment since Relative Standard Deviation (RSD) did not exceed 0.2%. Overall, the mean of inhibitory zone diameters obtained for control discs in whey was larger than the reported by Gaudin *et al.* (2004) in cow milk, as an exception of chlortetracycline that was not detected at antibiotic concentration level assessed in milk.

Specificity was calculated as the percentage of negative samples from the total of whey samples analysed. According to Table 32, high specificity values ranging from 98 to 100% were obtained for the *B. stearothermophilus*, *B. subtilis*, *E. coli*, and *B. cereus* bioassays. However, plates using *Kocuria varians* showed lower specificity percentages, increasing false-positive results for macrolides. Similar results were obtained by Gaudin *et al.* (2004) with *K. varians* bioassay in cow milk, considering in this case inhibition zones equal to 0 mm.

Table 32. Specificity of the STAR protocol in whey

Plate	Results and diameters of the inhibition zone		
	Negative (< 11 mm)	Positive (≥ 11 mm)	Specificity (%)
Bst	98	2	98
Bs7.2	100	0	100
Kv8	80	20	80
Ec8	100	0	100
Bc6	100	0	100

IV.3.3.2. Detection Limits of STAR Protocol

Table 33 shows Detection Limits (DLs) below or at MRL in milk obtained for cefazolin and the quinolone group. Overall, detection limits were around double the MRL in milk for benzylpenicillin, cloxacillin, erythromycin, spiramycin, oxytetracycline and tetracycline; values of four times the MRL fixed in milk were also found in the case of amoxicillin, ampicillin, gentamicin, neomycin and chlortetracycline, and even above for certain substances such as streptomycin, tylosin and lincomycin.

Table 33. Detection Limits (DLs) and inhibition diameters of STAR protocol in whey

Plate	Antibiotics	EU-MRL (µg/kg)	DL (µg/kg)	Mean (mm)	SD (mm)	Min (mm)	Max (mm)	RSD (%)
Bst	*β-lactams*							
	Amoxicillin	4	16	14.6	1.7	12.4	19.0	0.12
	Ampicillin	4	20	16.3	0.9	15.1	17.7	0.06
	Benzylpenicillin	4	8	14.7	1.3	13.2	17.3	0.08
	Cloxacillin	30	60	14.3	2.2	11.1	18.8	0.16
	Cefazolin	50	20	14.2	1.1	12.2	15.6	0.08
Bs7.2	*Aminoglycosides*							
	Gentamicin	100	500	12.9	0.6	12.0	13.8	0.05
	Neomycin	1,500	6,000	13.7	1.6	11.7	16.2	0.11
	Streptomycin	200	2,000	12.3	0.8	11.4	14.5	0.07
Kv8	*Macrolides*							
	Erythromycin	40	60	14.2	1.4	12.1	16.5	0.10
	Spiramycin	200	400	13.1	0.4	12.2	13.5	0.03
	Tylosin	50	300	12.4	0.5	11.4	12.9	0.04
	Lincosamides							
	Lincomycin	150	1,000	14.8	0.9	13.6	16.1	0.06
Ec8	*Quinolones*							
	Enrofloxacin	100	40	14.2	0.9	12.4	15.3	0.06
	Ciprofloxacin	100	20	13.4	0.7	12.3	14.9	0.05
Bc6	*Tetracyclines*							
	Chlortetracycline	100	500	19.9	4.0	14.4	26.7	0.20
	Oxytetracycline	100	225	15.1	0.7	14.0	16.3	0.05
	Tetracycline	100	225	16.5	0.7	15.2	17.6	0.04

EU-MRL: European Union Maximum Residue Limits fixed in milk (EU, 2010). DL: ISO/IDF (2003a). SD: Standard Deviation; Min: Minimum; Max: Maximum. RSD: Relative Standard Deviation.

Considering the Detection Limits results jointly obtained for each bioassay, for penicillins and cefalosporins, inhibitory zone diameters ranging from 14 to 16 mm could evidence the presence of such antibiotics in whey with a probability of 95%. In the case of aminoglycosides, macrolides and quinolones, the sensitivity criteria of the three families was similar to each other, with mean diameter between 12 and 15 mm, slightly lower than those indicated for β-lactams. The strongest variability among drugs of the same antibiotic group was observed for tetracyclines. Thus, while oxytetracycline and

tetracycline showed mean diameters of inhibition zones around 15-16 mm, for chlortetracycline, the mean diameter at DL concentration reached 20 mm. Therefore, to detect chlortetracycline residues with a probability of 95% an increase of the inhibitory zone is required.

The sensitivity results obtained for whey were similar to those indicated in cow milk (Gaudin *et al.*, 2004) with STAR protocol for β-lactams, some macrolides, quinolones and tetracyclines, highlighting the case of cefazolin and chlortetracycline, whose detection limits were ten times higher and lower, respectively, than those obtained in this study with whey matrix.

Nouws *et al.* (1999), using a seven plate multiresidue bioassay (NAT-screening) in cow milk, estimated detection levels significantly lower, between 2 and 10 times, than those obtained in this study for whey. Likewise, the DLs resulting from the present study for penicillins were also 4 times higher than those reported by Shitandi and Gathoni (2005) in cow milk. In sheep milk, Althaus *et al.* (2009), using the NAT-screening method proposed by Nouws *et al.* (1999), reported DLs higher, for some antibiotics up to 5 times, than those calculated in whey. Only in the case of aminoglycosides, the detection limits in sheep milk were significantly lower than in whey.

IV.3.3.3. Semi-Quantification of Antibiotics by STAR Protocol in Whey

A linear regression model between logarithm of the antibiotic concentration and their corresponding inhibition zone diameter was performed to establish a semi-quantification range for every antibiotic studied in whey using the STAR protocol. The parameters estimated from the regression equations (β_0 and β_1), lineal correlation coefficients (R^2) and the diameters of the inhibitory zones corresponding to the range of antibiotic concentrations considered are presented in Table 34.

Regarding parameters from the linear regression model, high lineal correlation coefficients (R^2), between 0.90 and 0.96, were found for the five β-lactams evaluated, and for the most of non β-lactams antibiotics R^2 were above 0.90, except for erythromycin (R^2= 0.82) and chlortetracycline (R^2= 0.72). Additionally, β_1 coefficient signalled the relation between antibiotic concentration and the microbial inhibition zone, indicating a higher value of this statistical parameter a greater increase in diameter when the antibiotic concentration is increased. While lower β_1 values were observed in Bs7.2, Kv8 and Bc6 plates, for β-lactams and quinolones, the values of β_1 coefficients were the highest reported with the STAR protocol in whey and, consequently, a greater sensitivity was obtained using *B. stearothermophilus* and *E. coli* microorganisms in this dairy matrix.

Table 34. Regression parameters, antibiotic concentrations, and inhibition diameters of STAR protocol in whey

Plate	Antibiotics	β_0	β_1	R^2	Concentration ranges (µg/kg)	Diameter ranges (mm)
Bst	*β-lactams*					
	Amoxicillin	-2.214	13.747	0.91	16-256	14.6-32.1
	Ampicillin	-1.492	13.555	0.96	20-320	16.3-33.1
	Benzylpenicillin	0.019	15.736	0.90	8-128	14.7-32.8
	Cloxacillin	-16.192	17.165	0.95	60-960	14.3-34.6
	Cefazolin	-4.5251	14.046	0.91	20-320	14.2-31.1
Bs7.2	*Aminoglycosides*					
	Gentamicin	-8.263	8.049	0.95	500-8,000	12.9-23.3
	Neomycin	-14.604	7.543	0.90	6,000-96,000	13.7-22.5
	Streptomycin	-18.294	9.215	0.94	2,000-32,000	12.3-23.1
Kv8	*Macrolides*					
	Erythromycin	-8.1064	12.249	0.82	60-960	14.2-29.8
	Spiramycin	-23.721	13.753	0.97	400-6,400	13.1-29.1
	Tylosin	-18.246	12.080	0.97	300-4,800	12.4-26.8
	Lincosamides					
	Lincomycin	-42.399	19.102	0.98	1,000-16,000	14.8-38.2
Ec8	*Quinolones*					
	Ciprofloxacin	3.619	7.989	0.94	20-320	13.4-23.7
	Enrofloxacin	1.156	8.211	0.96	40-640	14.2-24.3
Bc6	*Tetracyclines*					
	Chlortetracycline	-13.261	11.618	0.72	500-8,000	19.9-33.2
	Oxytetracycline	-17.554	14.196	0.94	225-3,600	15.1-32.0
	Tetracycline	-18.393	15.112	0.97	225-3,600	16.5-34.9

β_0, β_1: coefficients estimated for the linear regression model; R^2: lineal correlation coefficients.

The ranges of semi-quantification for each bioassay with a 95% of confidence (error β≤ 5%) were established considering the DL that produced the largest microbial inhibition zone and the maximum antibiotic concentration assessed for any substance within the same plate equivalent to the smallest diameter measured. Thus, in the case of Bst plate, diameters ranging between 16 and 31 mm could be evidence of the presence of β-lactam antibiotic at or above the Maximum Residue Limit fixed in milk, since in the case of cefazolin the MRL (50 µg/kg) was included within the range of concentrations evaluated. Similarly, for bioassay using *E.coli*, microbial growth inhibition corresponding to diameters between 14 and 24 mm indicates a possible non-compliant result above regulation limits established in milk. For the rest of plates evaluated, although in their semi-quantification ranges Maximum Residue Limits fixed in milk were excluded, the obtained diameter ranges provide information about the estimated drug concentration that could contain a whey sample for a specific antibiotic family.

From the obtained regression equations (Table 34), the antibiotic concentrations equivalent to diameters of inhibitory zone of 11 mm, 13 mm, 15 mm, 17 mm, and 19 mm were calculated. The resulting concentrations for the assessed diameters are presented in Table 35. For the most of antibiotics studied, the antibiotic concentrations corresponding to inhibition zone diameters were found to be above the MRL established in milk, as an exception of some β-lactams, erythromycin, quinolones and tetracyclines. Thus, it seems that sensitivity of Bs7.2 and Kv8 plates for whey is significantly lower than the reported previously for cow milk (Gaudin *et al.*, 2004).

Some antibiotic concentration ranges, corresponding to inhibition zone diameters between 11 mm and 19 mm, included the MRL concentration fixed in milk: cefazolin close to 19 mm, erythromycin from 11 mm to 13 mm, enrofloxacin between 17-19 mm, ciprofloxacin slightly above evaluated diameters (> 19 mm), and oxytetracycline and tetracycline at smallest evaluated diameters (11-13 mm). Also, for some substances such as benzylpenicillin, cloxacillin, and chlortetracycline, the antibiotic concentration obtained for the minimum diameter assessed (11 mm) approached significantly to their Maximum Residue Limit legally fixed in milk. Drugs could be classified attending on the relationship existing between their sensitivity, which is defined as the antibiotic concentration equivalent to inhibition zone diameter ≥ 11 mm, obtained from lineal regression equations and the MRL in milk.

Thus, in agreement with the study conducted by Gaudin *et al.* (2004) in cow milk with the STAR protocol, antibiotic concentrations below MRL were reported for erythromycin and quinolones, lower than four times MRL for penicillins, gentamicin and

spiramycin, and sensitivities greater than four times regulation established in milk in the case of streptomycin. In the case of oxytetracycline and tetracycline, sensitivities reached in whey have been closer to MRL fixed in milk than the reported for cow milk.

Table 35. Antibiotic concentrations at different inhibition diameters (11, 13, 15, 17, 19 mm) of STAR protocol in whey

Plate	Antibiotics	EU-MRL (µg/kg)	Concentrations (µg/kg)				
			C_{11mm}	C_{13mm}	C_{15mm}	C_{17mm}	C_{19mm}
Bst	*β-lactams*						
	Amoxicillin	4	9	13	18	25	35
	Ampicillin	4	8	12	16	23	32
	Benzylpenicillin	4	5	7	9	12	16
	Cloxacillin	30	38	50	66	86	112
	Cefazolin	50	13	18	25	34	47
Bs7.2	*Aminoglycosides*						
	Gentamicin	100	247	438	777	1377	2440
	Neomycin	1,500	2,479	4,566	8,407	15,481	28,506
	Streptomycin	200	1,510	2,489	4,103	6,762	11,147
Kv8	*Macrolides*						
	Erythromycin	40	36	53	77	112	163
	Spiramycin	200	335	468	654	914	1,277
	Tylosin	50	264	386	565	827	1,211
	Lincosamides						
	Lincomycin	150	624	795	1,011	1,287	1,638
Ec8	*Quinolones*						
	Enrofloxacin	100	16	28	49	85	149
	Ciprofloxacin	100	8	15	27	47	84
Bc6	*Tetracyclines*						
	Chlortetracycline	100	123	182	271	402	598
	Oxytetracycline	100	103	142	196	272	376
	Tetracycline	100	88	119	162	220	298

EU-MRL: European Union Maximum Residue Limits fixed in milk (EU, 2010).

Other screening methods, including different number and/or type of microorganisms, indicated higher inhibition zone diameters for cefazolin, tylosin, oxytetracycline and tetracycline in cow milk (Nouws *et al.*, 1999) and for macrolides, quinolones and tetracyclines in the case of sheep (Althaus *et al.*, 2009).

In spite of the discrepancies pointed out in the sensitivity results in whey and those highlighted for milk of the different species analysed by means of various multiplate systems, the STAR protocol may be used for whey analysis in the future. Maximum Residue Limits have not been established in whey and higher values than those established for milk could be estimated taking into account its high volume produced

per year, as well as the products derived from whey treatments are consumed in a limited way.

Then, with the purpose to express in a graphic manner the relationship between the antibiotic concentrations that produce diameters of inhibitory zone (mm) ranging from 11 mm to 19 mm and the MRL fixed in milk, Figure 35, Figure 36, Figure 37 and Figure 38 corresponding to β-lactam, aminoglycoside, macrolide and lincosamide, and tetracycline families were constructed.

In the aforementioned figures, the relationship (ratio) between the antibiotic concentrations corresponding to a certain diameter with respect to the equivalent concentration at MRL fixed in milk, is shown. This ratio is expressed logarithmically in basis 2. Ratios close to Line 1 indicate a closer proximity to the MRL. Yet, ratios close to the center of the figure indicate that the antibiotic concentration is far higher than that fixed as MRL.

For β-lactams (Figure 35), no penicillin reached Line 1 in the polygonal area, since MRL could not be detected with Bst plate in whey. Only for cloxacillin at a concentration equivalent to 11 mm of diameter and for cefazolin in the case of 19 mm, are ratios are close to the polygonal Line 1, indicating a fit relationship between the antibiotic concentration equivalent to these diameters and the concentration equal to MRL fixed in milk.

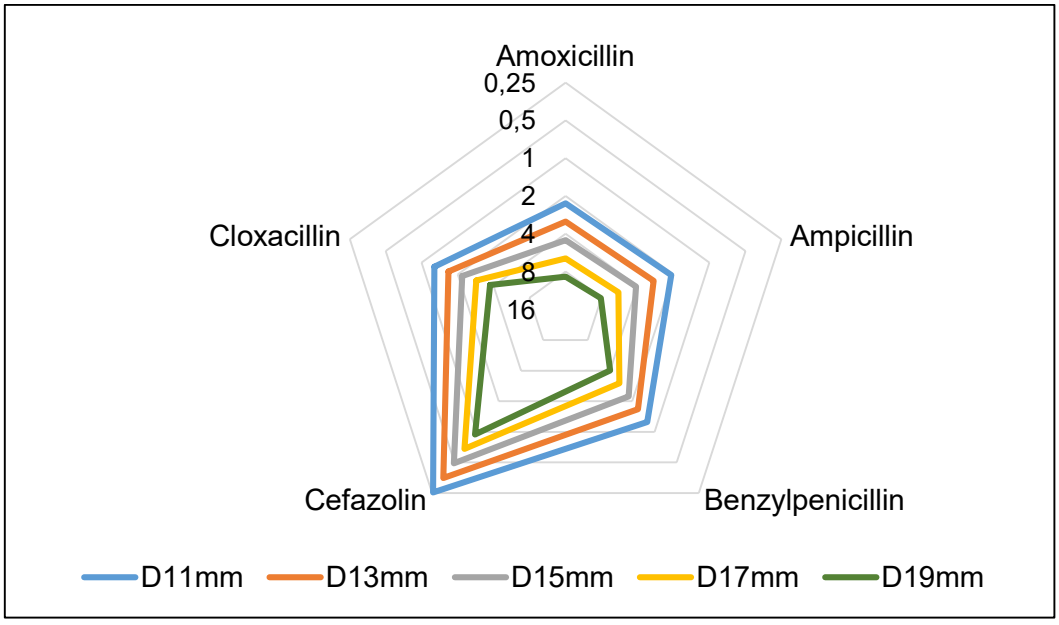


Figure 35. Relationship between β-lactam concentrations in whey at specific inhibitory zone diameters and MRL in milk

In a similar way, *B. subtilis* microorganism was not sufficiently sensitive to identify aminoglycosides below or at MRL concentration in milk since any ratio is close to Line 1. Only in the case of neomycin, concentration equivalent to 11 mm diameter is situated between Line 2 and Line1 (Figure 36).

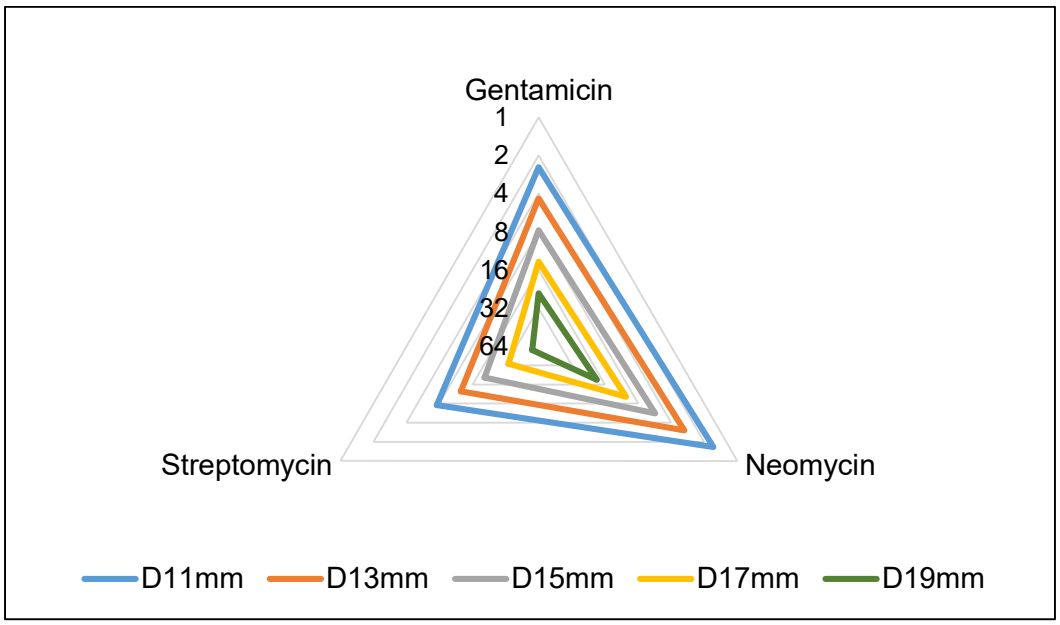


Figure 36. Relationship between aminoglycoside concentrations in whey at specific inhibitory zone diameters and MRL in milk

In the case of macrolides, for erythromycin, antibiotic concentration equivalent to MRL in milk was reached at 11 mm inhibitory zone, being the closest diameter to the polygonal line one (Figure 37).

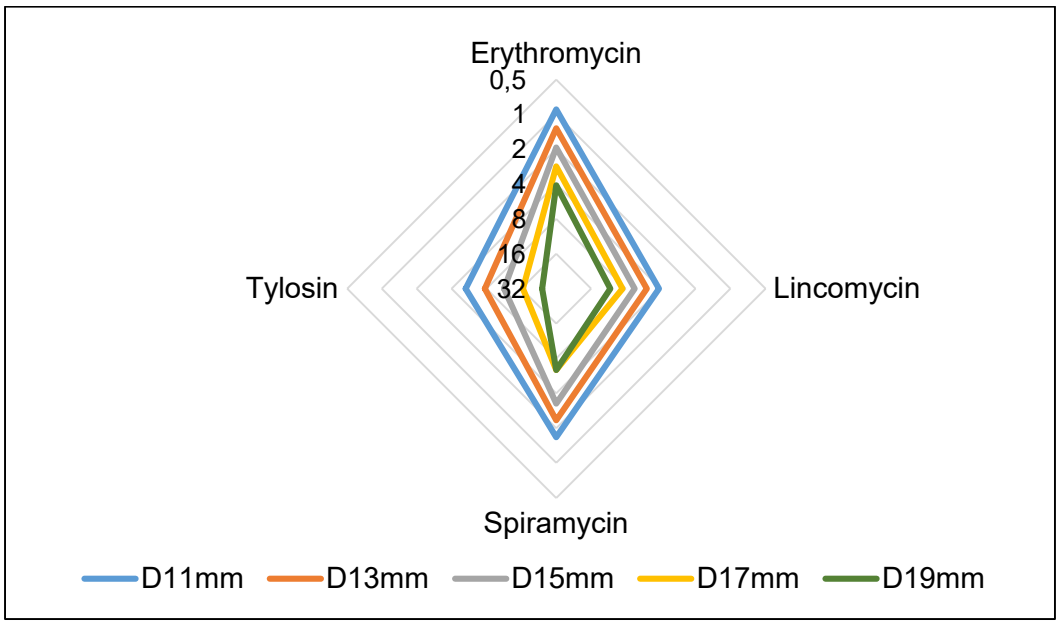


Figure 37. Relationship between macrolide concentrations in whey at specific inhibitory zone diameters and MRL in milk

For the three tetracyclines studied (Figure 38), inhibitory zones of 11 mm are situated on the polygonal line 1, representing a concentration equal to the MRL established for this group in milk.

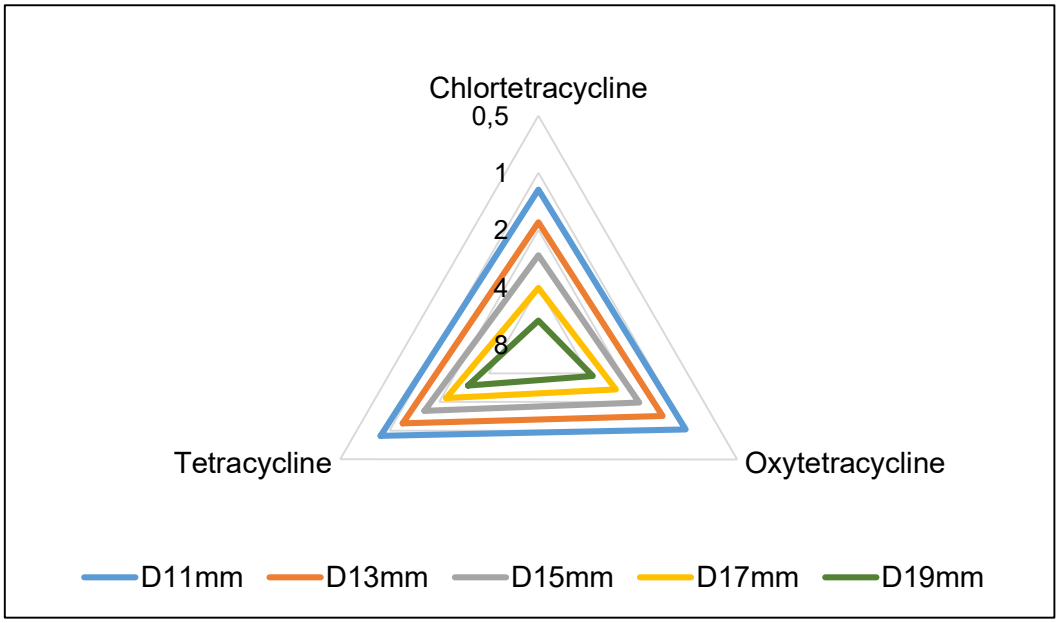


Figure 38. Relationship between tetracycline concentrations in whey at specific inhibitory zone diameters and MRL in milk

Finally, for quinolones group, radial figure could not be constructed since only two series (enrofloxacin and ciprofloxacin) are not able to represent any polygonal structure. However, considering the results obtained for quinolones family, it can be deduced that the range of diameters selected in the study is adequate to obtain information about the closeness between the results obtained with *E.coli* bioassay and MRL. Enrofloxacin would be detected at MRL concentration in a range between 17 mm and 19 mm, while ciprofloxacin at diameters somewhat above 19 mm.

IV.3.4. Conclusion

Although the obtained detection limits with the STAR protocol exceed the MRL established in the milk for the most of substances studied in whey, this multiplate microbiological system could become an adequate tool to identify antibiotic residues in whey and to estimate their concentration as post-screening methodology. An adequate microbial post-screening strategy could largely reduce the number of non-compliant samples that should be confirmed by chromatographic technology, with a considerable saving of time and resources.

Chapter V. General Discussion

Antibiotic therapy is an effective practice to treat bacterial infections in dairy livestock. However, the inappropriate use of veterinary products can lead to drug residues in milk, involving harmful consequences for consumer health, principally the development of antimicrobial resistances (Sharma *et al*., 2018), in addition to negative repercussions on the technological process (Cabizza *et al*., 2017; Quintanilla *et al*., 2019a).

The distribution of antibiotics during cheese-making into the different milk fractions, mainly cheese and whey, depends on the physicochemical properties of drugs and the characteristics of manufacture process. Studies about the lesser or greater affinity of antibiotics to interact with the fat and/or protein fraction of the milk and, therefore, to be retained in curd, are focused on a limited number of analytes: amphenicoles (Sniegocki *et al.*, 2015), tetracyclines (Cabizza *et al.*, 2017; Gajda *et al.*, 2018) or representative substances of some veterinary drugs such as antibiotics, antihelmintics and antiparasitics (Hakk *et al.*, 2016; Shappell *et al.*, 2017).

Therefore, this thesis focuses on the evaluation of the transfer of an extent number of antibiotics from milk to fresh cheese and whey by means the validation of a quantitative screening approach using UHPLC-HRMS method, Orbitrap Exactive™ analyser, and the establishment of the most appropriate quality control strategy to screen antibiotics in whey.

Liquid Chromatography tandem MS spectrometry (LC-MS/MS), mainly triple quadrupole (QQq), is the analytical quantitative approach used by excellence for the analysis of veterinary residues in milk, cheese, and whey (Gómez Pérez *et al*., 2013; Rossi *et al*., 2017; Zhao *et al*., 2017). However, the number of substances that can be determined in one injection is limited by working at unit-resolution scanning.

As alternative, for multiresidue analysis, the use of High and Ultra High Performance Liquid Chromatography (HPLC and UHPLC) coupled to High Resolution Mass Spectrometry (HRMS) provides optimal separation and narrow peak widths with a better specificity. Furthermore, full scan approach using High Resolution Mass Spectrometry (HRMS) and accurate mass measurements, for example by means Orbitrap Exactive™ analyzer, makes possible the simultaneous analysis of larger number of antibiotics with different physicochemical properties with high specificity since allows to distinguish isotope peaks and isomeric ions.

For multiresidue analysis by LC-HRMS, the use of generic sample treatments is a practice widely generalized with the aim to recover the maximum number of physiochemically different substances. Aqueous and organic solvent combinations are

the most used extractants to detect a wide range of polar and non-polar substances (Kaufmann *et al*., 2014; Cepurnieks *et al*., 2015). Acetonitrile is a water-miscible organic solvent that allows protein precipitation with a minimum coelution of endogenous substances typically present in other solvents as methanol. Aqueous solvents commonly used are water to favour the extraction of β-lactams (Han *et al*., 2015; Wittenberg *et al*., 2017), EDTA to avoid chelates formation between tetracyclines and metals (Aguilera-Luiz *et al*., 2008; Zhu *et al*., 2016), and also buffers at different pH that allow to keep stable acidity of medium (Li and Wu, 2017).

In this thesis, four combinations of solvents (ACN and three combinations of ACN with aqueous solutions in 20/80 proportion: water/ACN, McIlvaine EDTA-buffer/ACN and acetate buffer pH 5.2/ACN) were evaluated with results similar than those provided prior by literature. The use of acetonitrile (ACN) was the least recommended extraction alternative, since 17% of antibiotics were not detected in cheese and more than 50% in whey. In the case of McIlvaine EDTA-buffer/ACN (20/80, v/v), the extraction of tetracyclines was increased significantly, but some analytes were not detected in cheese and whey. On the other hand, although water in combination to acetonitrile (20/80, v/v) allowed the recovery of the most of antibiotics evaluated, benzylpenicillin was not detected in whey with this solvent combination. Thus, acetate buffer pH 5.2/ACN (20/80, v/v) was selected as extraction method since it provided the best response for β-lactam family, which is the most used antibiotic family in dairy livestock.

However, one of the main challenges of mass spectrometry comes from simple sample preparation that involves the presence of endogenous compounds from the sample that coelute at the same time that the analytes of interest provoking changes in the intensity of the detected signal, negative or positive matrix effect corresponding to ion suppression and signal enhancement phenomena (Freitas *et al*., 2015).

Based on the results obtained herein, the use of internal standard could decrease signal changes derived from the effect of matrix (cheese or whey) and extraction procedure, especially when these differences were > +50% or < -60%. Other studies previously carried out in milk (Wang *et al*., 2015) also observed an improvement on accuracy for a quantitative multiresidue analysis by UHPLC-qOrbitrap applying matrix-matched calibration curves along with isotopically labelled internal standards.

While a high ion suppression effect was observed considering analytes peak areas (ME: < -60%), from 27% in the case of fresh cheese up to 63% in whey, after correction with internal standard (ME/IS), the percentage of substances without matrix effect was increased considerably, especially for quinolones, sulfonamides and tetracyclines (up

to 100% for tetracyclines in whey). Contrarily, studies in milk and cheese indicated an enhancement effect for sulfonamides and tetracyclines (Gómez Pérez *et al.*, 2013; Wang *et al.*, 2015), and no signal changes for quinolones (Moretti *et al.*, 2016), without internal standard correction.

β-lactams was the antibiotic group mostly influenced by matrix effect, decreasing their signal above 60%, even after correction with IS. From the literature, studies about matrix effect in milk also indicated ion suppression effect for β-lactams ranging from 22% to 82% (Moretti *et al*., 2016).

Finally, macrolides showed an unusual behavior when correction with roxithromycin internal standard was considered. As it was also indicated in a study conducted by Wang *et al*. (2015) in milk, roxithromycin suffered a significant ion suppression phenomenon (ME: < -60%), while macrolides to which it corrected included a moderate matrix effect (ME: -60% - < -20%) in most studied cases. Consequently, a high enhancement effect (ME/IS: > +50%) was obtained for macrolides when using IS correction, and external matrix-matched calibration was preferably applied.

In the same way than for ME, absolute recovery percentages (RE) were increased when isotopically labelled Internal Standards were used (RE/IS), being recovery percentages mainly improved at low quality control (LQC) assessed. The lowest recovery values (RE) were obtained for sulfonamides and tetracyclines at LQC concentration (25 or 50 µg/kg) with percentages between 60-77%, in agreement to previous studies reported for milk (Moretti *et al.*, 2016) and cheese (Gómez Pérez *et al.*, 2013).

Regarding validation parameters obtained herein for fresh cheese and whey, resulting Detection Capabilities (CCβs) values at 0.25 or 0.5 of MRL fixed in milk were according with Commission Decision 2002/657/EC (EC, 2002b). Furthermore, the precision percentages in terms of repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) were also considered appropriate (EC, 2002b), in addition to repeatability results were lower than within-laboratory reproducibility, indicating the acceptable precision of method; and trueness (recovery) results were also satisfactory.

For cheese and liquid whey, multiresidue methods by UHPLC-HRMS have not been developed. Some studies using triple quadrupole mass spectrometer (QQq) indicated lower LODs and LOQs than the obtained in this study, but with important losses of some of the most employed drugs in lactating animals such as ampicillin, benzylpenicillin, cloxacillin, ceftiofur and erythromycin for cheese (Schwaiger *et al*.,

2018) and β-lactams, tylosin and tetracyclines in the case of whey powder derived (Wittenberg *et al*., 2017).

The multiresidue quantitative screening method (UHPLC-Orbitrap Exactive^{TM}), previously validated for dairy matrices was applied to study the transfer of antibiotics from milk to cheese and whey at lab scale.

In general, antibiotic concentration ratios between curd and whey indicated that the most of drugs were released in aqueous milk fraction, in accordance with others experiments (Hakk *et al.*, 2016; Shappell *et al*., 2017). Thus, the percentage of antibiotics retained in curd was lower than 50% in all cases, except for ceftiofur and dicloxacillin that remained in curd to a somewhat greater extent than in whey, with values of 59.7% and 52.8%, respectively.

Although most of the antibiotics evaluated tended to remain in whey fraction, a positive relation between drug distribution ratios and drug lipophilicity (Log P) was observed, especially for macrolides and sulfonamides (R^2> 0.6) in accordance with Shappell *et al.* (2017).

Other studies reported a higher transfer of antibiotics from milk to cheese compared with the results obtained in this thesis. Quintanilla *et al*. (2019b), making a study in fresh cheese from goat's milk containing antibiotics at MRL concentration, observed that the most of the substances evaluated (amoxicillin, benzylpenicillin, cloxacillin, neomycin, erythromycin, enrofloxacin and ciprofloxacin) were retained in cheese with percentages above 50%. This trend is attributed to the high whey content of this type of cheese. However, in ripened cheese, Quintanilla *et al*. (2019a) indicated that β-lactams (amoxicillin, benzylpenicillin and cloxacillin) and erythromycin were preferably released into the whey, while those groups with higher lipophilicity such as quinolones and oxytetracycline showed a concentration factor in cheese up to 2.7 and 4.3 times higher than the initial antibiotic concentration in milk.

Therefore, in addition to physicochemical properties of antibiotics, technological process could also influence on the behaviour of drugs into the different milk fractions. Thus, a major draining during cheese-making process could entail an increase of the concentration of antibiotics more lipophilic, while a lower elimination of whey could lead to higher retention of water-soluble antibiotics in the curd.

Although whey by-product has been traditionally transformed in foodstuffs as whey cheeses or used in animal feed, currently, the application of biotechnological processes has allowed the obtention of added-value products with important nutraceutical and pharmaceutical applications (Kareb and Aïder, 2018; Lappa *et al.*, 2019). For that, the

presence of veterinary drug residues in whey could compromise the suitability of these related products, being necessary to have appropriate methods for screening antibiotics in whey samples, avoiding negative implications on humans, animals, and environment.

Considering the greater tendency of antibiotics to be released in whey during cheese-making process, in addition to that the main uses of whey are related to the food industry, it would be convenient to establish an appropriate quality control strategy for the screening of antibiotics in whey. Moreover, Maximum Residue Limits have not still established in whey, being unknown the potential risk that the presence of drug residues in this dairy by-product could involve for human and animal health.

Microbial inhibitor tests are commonly used for the screening of antibiotics in milk by quality control laboratories since they are relatively inexpensive, user-friendly, and able to detect a great variety of antimicrobials in a large number of milk samples. At present, these methods, which were initially developed for cow milk (Le Bréton *et al*., 2007; Perme *et al*., 2010), are widely used in goat and sheep milk (Beltrán *et al*., 2015).

However, one of the main limitations of microbial inhibitor tests is their long incubation time, above 2.5 hours. Thus, recently, Eclipse Farm 3G microbial screening method was coupled to e-Reader automatic device (Eclipse Farm-eReader) with the objective to favour analysis in situ by farmers and dairy industry, making easier the interpretation of results in a more objective and rapid manner. Some studies indicated the suitability of Eclipse Farm-eReader for the detection of antibiotics in cow (Mata *et al*., 2016), with a specificity percentage above 95% and CCβ at or below Maximum Residue Limits. Furthermore, in a study conducted by Giraldo *et al*. (2019) in sheep and goat's milk, the false-positive rate of this analytical method did not exceed 5%, but the CCβ values were not appropriate in relation to the MRL for gentamicin and oxytetracycline.

The adoption of Eclipse Farm-eReader for the screening of antibiotics in whey was investigated in this thesis. In the case of whey samples, a diffusion at room temperature for one hour prior to the incubation step was required. The specificity of inhibitor test resulted in 100% and CCβ values were similar to MRL fixed in milk, as an exception of enrofloxacin with a detection capability 20 times regulation limit legally established in milk. The inadequate results obtained for quinolone groups are related with the lack of sensitivity of *Geobacillus stearothermophilus var. calidolactis* bacteria test for non β-lactams (Beltrán *et al*., 2015). Detection capabilities could be significantly affected ($P < 0.05$) when analysing whey samples with pH< 6.5, even reaching false negative rates

of 100% in the case of tylosin and enrofloxacin. These robustness results underline the need to adjust the pH before the analysis of whey samples with values between 6.5 and 7, as recommended by the International Dairy Federation (IDF, 2014).

Regarding biochemical qualitative screening methods, known as rapid tests, specific receptor-binding assays including lateral flow chromatography in reactive dipsticks are frequently used in farms and dairies as they are user-friendly and allows fast responses in less than 10 minutes.

Receptor-binding tests were initially validated in cow milk (Perme *et al*., 2010; Reybroeck *et al*., 2010; Salter *et al*., 2011). However, in recent years, studies conducted by Beltrán *et al*. (2014 a,b) indicated the suitability of some of these commercially available tests (Betastar Combo, Charm MRL, SNAP and Twinsensor) in sheep and goats with optimal specificity percentages, without no cross reactions and detection capabilities equal or lower to the MRLs for most β-lactams and tetracyclines. Information about the performance characteristics of receptor-protein assays in whey has not been reported.

Some of the rapid tests evaluated in this thesis (Twinsensor, 3Aminosensor and Quinosensor) for the detection of antibiotics in whey indicated a specificity of 100%. In the case of Tylosensor, although deficient false-positive rate of 52% was obtained using Readsensor (Unisensor) device, specificity percentage improved significantly (91%) when the results were interpreted visually. These commercial receptor-binding assays presented CCβ below or at Maximum Residues Limits fixed in milk, as an exception of cefalexin that was detected at 5 times MRL and gentamicin with values around 1.5 MRL established for milk. Overall, β-lactams showed detection capabilities higher than informed for milk, while oxytetracycline was detected in whey at a concentration level considerably lower than the results obtained by Beltrán *et al*. (2014b) in goat's milk.

Despite of the advantages of receptor-binding assays for their use especially on farms and dairies where a fast response (< 10 minutes) is required, their higher specificity towards particular substances or antibiotic families and the limited number of samples that can be analysed simultaneously in one reading, as well as their cost-expensive in relation to microbial screening methods, it forces to inhibitor tests are the analytical screening methodology preferably used by quality control laboratories.

As aforementioned, most current microbial screening tests are based on the inhibition of *Geobacillus stearothermophilus var. calidolactis*, which includes a high sensitivity for the detection of β-lactams, although with an inadequate detection

spectrum for some non β-lactam groups, mainly aminoglycosides, macrolides and quinolones.

To improve the detection profile of antibiotic residues in milk, some authors proposed microbial bioassays combining different bacteria test in microtiter plates with dichotomous response manufactured in house in an economical manner. Thus, sensitivity of macrolides, quinolones, sulfonamides and tetracyclines in milk was increased combining *Geobacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus cereus* bioassays (Nagel *et al*., 2013a). In sheep milk, a detection system based on two microplates containing *Geobacillus stearothermophilus* and *Bacillus subtilis* (Nagel *et al*., 2012) reached good detection limits for residues of macrolides and quinolones in comparison with commercially available inhibitor tests using *G. stearothermophilus*. Recently, some authors shown a trend towards the development of more rapid microbial methods using thermophilic bacteria such as *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus* (Nagel *et al*., 2013b, 2014), to reduce the incubation time below 2.5 hours.

For these reasons, a microbial bioassay constituted by four different microorganisms in microtiter plate format with dichotomous response (positive or negative) was evaluated, including Eclipse 100 commercial screening method, which uses *Geobacillus stearothermophilus var. calidolactis* as bacteria test, in comparison to three in-house bioassays that use *Bacillus subtilis*, *Geobacillus thermocatenulatus* and *Geobacillus thermoleovorans*. The obtained results indicated an adequate specificity, around 100%, for *G. thermocatenulatus* and *G. thermoleovorans* microorganisms, while for the analysis of whey samples with *G. stearothermophilus* and *B. subtilis* the false-positive rate was 10%. However, a previous heat treatment (85ºC, 10 min) on whey samples significantly improved specificity percentages for both microorganisms (98% and 100%, respectively). Therefore, it is recommended that in the case of obtaining positive results with *G. stearothermophilus* and *B. subtilis*, whey samples to be reanalysed with a previous heat treatment at 85ºC for 10 minutes approximately, as previously some studies also indicated on inhibitor tests in milk (Molina *et al*., 2003).

In general, Detection Limits (DLs) obtained in whey by means the use of thermophilic bioassays containing *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* were lower than MRLs fixed in milk, mainly in the case of β-lactams, while *B. subtilis* improved macrolide and quinolone detection ranges with DLs close to the safety levels established in milk. Consequently, the lack of sensitivity of *G.stearothermophilus* towards non β-lactams antibiotics as macrolides and quinolones

could improve by its simultaneous use with *B. subtilis* bioassay, while the paper of *G. thermocatenulatus* and *G. thermoleovorans* is related to the replacement of *G.stearothermophilus* to reach shorter incubation times, around 1.5 hours.

Currently, microbiological semi-quantitative methods including different microorganism combinations have been evaluated in order to increase the detection profile of antibiotic residues in milk, providing information about the identity of substances. Furthermore, the use of "Multiplate Microbiological Systems" as post-screening strategy limits the number of substances which need to be confirmed by physicochemical methodologies, reducing the cost of quantitative analysis.

Some of the existing microbiological post-screening methods are used by Official Control Laboratories and European Union Reference Laboratories (EURL), among which the Screening Test for Antibiotic Residues (STAR) stands out, developed by the EU Reference Laboratory for Veterinary Drug Residues (ANSES Fougères Laboratory, Fougères, France) and validated in cow milk by Gaudin *et al*. (2004). This STAR protocol is constituted by five different plates using *Bacillus stearothermophilus* for β-lactams and sulfonamides, *Bacillus subtilis* for aminoglycosides, *Kocuria varians* for macrolides, *Escherichia coli* for quinolones, and *Bacillus cereus* for tetracyclines. The lower sensitivity of the STAR protocol for β-lactams in relation to microbial commercial inhibitor tests is compensated by a higher range of detection as regards non β-lactam drugs (macrolides, lincosamides, quinolones, sulfonamides, tetracyclines…).

The results herein indicate that STAR protocol could be used as semi-quantitative tool for the analysis of antibiotics in whey matrix, since although for the most of substances evaluated detection limits were further from the MRL value fixed in milk, regulation of antibiotics in whey has not still established. The comparison of the DLs with respect to the MRL fixed in milk provides illustrative information about the suitability of this analytical method in whey. Additionally, high percentages of specificity, as an exception of *K.varians* plate, were reported in the antibiotic analysis of whey samples by STAR protocol.

Overall, only detection limits below MRL fixed in milk were obtained by STAR protocol for cefazolin (20 µg/kg), and quinolone family (enrofloxacin= 40 µg/kg and ciprofloxacin= 20 µg/kg), with DLs around the double of MRL in milk for benzylpenicillin, cloxacillin, erythromycin, spiramycin, oxytetracycline and tetracycline; also values of four times the MRL fixed in milk or even more, in agreement with the results obtained previously by Gaudin *et al*. (2004) in cow milk. It must be highlighted that Maximum Residue Limits have not been regulated in whey, and for their establishment, both the

huge whey volume produced annually and the small amount of this by-product commonly consumed in the form of value-added products must be considered. Therefore, it is expected that the MRLs in whey would equal higher antibiotic concentrations than those fixed in milk and higher diameters would be indicating a non-compliant result in the case of whey.

In summary, the most of veterinary residues studied shows a tendency to be transfer mostly from milk to whey. Qualitative and quantitative screening approaches evaluated in this thesis provide an adequate analytical control strategy to the analysis of antibiotics in whey, being the implementation of each one of these screening tests less or more recommended for the different key steps of the food chain. Thus, commercial microbial inhibitor tests based on *Geobacillus stearothermophilus* and microbial bioassays complementing the lack of sensitivity of this microorganism towards non β-lactam groups (mainly *Bacillus subtilis*) could be applied for a first bulk screening, obtaining information about the presence or absence of antibiotics in whey; in the case of receptor-binding assays, they provide qualitative specific data concerning the type of antibiotic family. The STAR protocol is capable to estimate semi-quantitatively the amount of one substance that is present as a post-screening analysis; and, in the same way, physicochemical quantitative screening methods, mainly liquid chromatography with mass spectrometry, can be used as post-screening step with more sensitivity and specificity; UHPLC-HRMS comprises an improvement in terms of resolution and accuracy for the development of multiclass-multiresidue methods.

The results obtained in this thesis indicate the suitability of qualitative as well as quantitative screening methods for the detection of antibiotics in whey and, thus, prevent the hazards related to the presence of veterinary drugs residues in food, especially with respect to human and animal health.

Chapter VI. Conclusions

Antibiotic residues in raw milk for cheese production could be widely transferred from milk to curd and whey fractions, posing a potential public health concern. To prevent antibiotics from reaching the food chain, it is, therefore, necessary to have control methods available for screening drug residues in such dairy products.

A suitable multiresidue UHPLC-HRMS method using Orbitrap Exactive^{TM} analyser has been validated according to Commission Decision 657/2002/CE recommendations, showing adequate performance for the quantitative screening of different antibiotic families in fresh cheese and whey samples.

The partitioning of antibiotics during the cheese-making process was established by the UHPLC-HRMS method. Antibiotics were mainly transferred from milk to whey, reaching concentrations higher than those present in the rennet curd fraction. In most cases, drug partitioning was dose-independent, and poorly related to drug lipophilicity.

The commercially available methods for screening antibiotics in milk, both microbial inhibitor tests and receptor-binding assays, were suitable for the detection of drug residues in whey samples, having pH values ranging from 6.5 to 7.0, although slight modifications in the test procedure were made in certain cases to improve their performance characteristics.

The simultaneous application of bioassays containing *Geobacillus stearothermophilus* and *Bacillus subtilis,* respectively, improves the detection profile of commercially available microbiological methods in whey. Thus, representing a relatively inexpensive and user-friendly alternative for the screening of a large number of samples in control laboratories.

The multiplate system *Screening Test for Antibiotics Residues* (STAR) may be used in post-screening to confirm the presence of antibiotics in whey and their preliminary identification and, hence, reduce the number of samples destined for quantitative analysis by LC-MS/MS, which is a more complex and expensive method.

Whey, traditionally used to produce whey cheeses and to feed animals, currently, has different food, pharmaceutical, biotechnological, and agricultural applications that could be compromised by the presence of drug residues. Given the suitability of the methods available, the screening of whey for antibiotics before use could be an interesting and beneficial strategy to avoid problems related to the presence of antibiotics reaching humans, animals, and/or the environment.

Chapter VII. References

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Annex

Table 1. Quality parameters of fresh cheese, and whey samples (n= 20)

Parameters	Cow				Goat				Sheep			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
Fresh cheese												
pH	6.58	0.08	6.49	6.68	6.64	0.14	6.44	6.82	6.48	0.02	6.46	6.49
Fat (%)	16.70	0.66	16	17.3	23.9	1.65	22.00	25.00	16.65	0.49	16.30	17.00
Protein (%)	12.87	2.5	10.30	15.30	14.57	0.84	13.60	15.10	13.35	0.49	13.00	13.70
Salt (%)	10.10	0.17	0.90	1.20	1.04	0.7	0.80	1.46	1.05	0.07	1.00	1.10
Total solids (%)	34.26	2.27	31.83	36.33	42.53	1.45	41.16	44.05	34.92	2.43	33.20	36.63
Whey												
pH	6.55	0.11	6.45	6.73	6.23	0.30	5.65	6.51	6.15	0.37	5.51	6.43
Fat (%)	0.54	0.19	0.37	0.83	0.90	0.27	0.42	1.13	0.51	0.75	0.05	1.84
Protein (%)	0.97	0.05	0.92	1.04	1.12	0.12	0.92	1.22	1.33	0.06	1.25	1.42
Lactose (%)	4.17	0.73	3.02	4.78	4.62	0.40	3.92	5.00	5.02	0.19	4.82	5.28
Total solids (%)	6.37	0.47	5.60	6.70	7.17	0.60	6.24	7.61	7.86	0.86	7.15	9.29

SD: Standard Deviation; Min: Minimum; Max: Maximum.

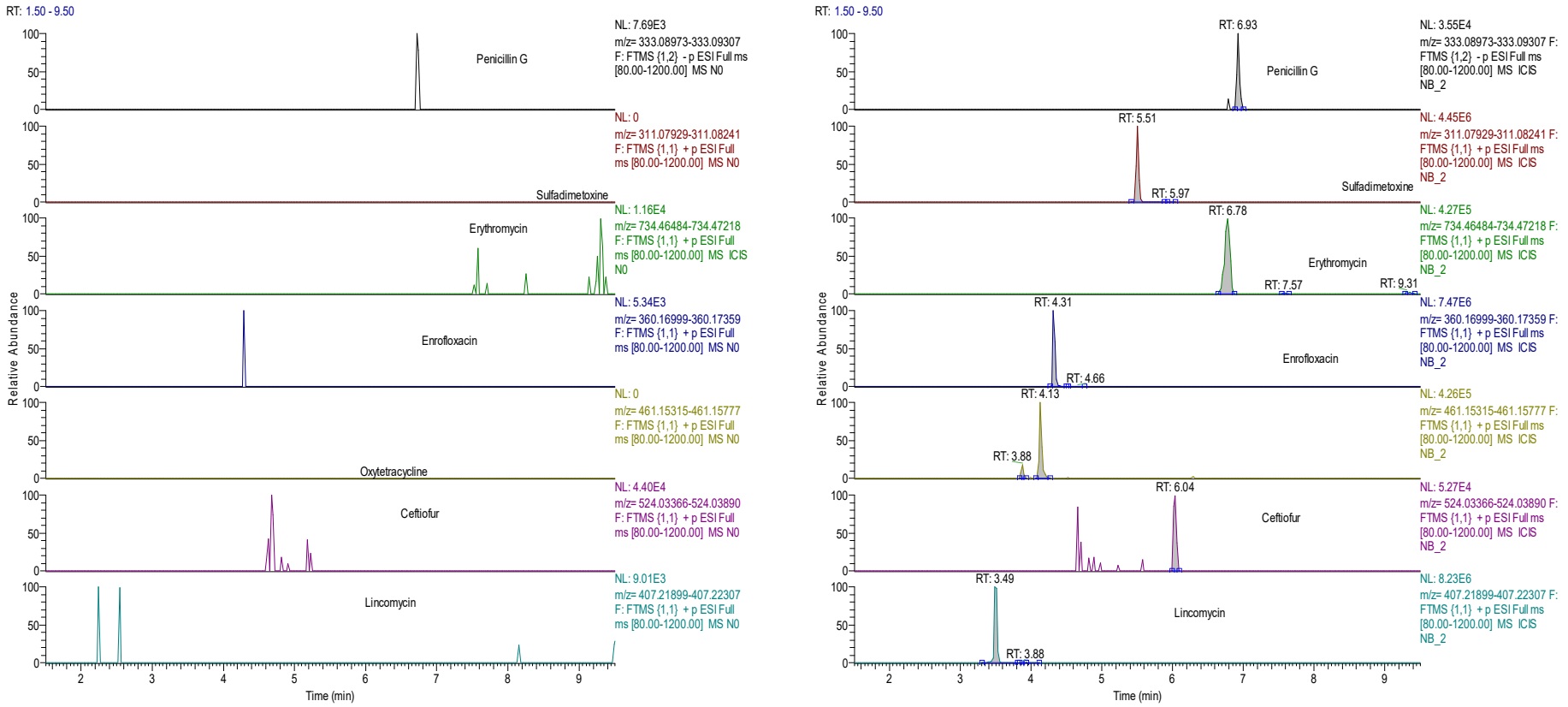

Figure 1. UHPLC–HRMS extracted ion chromatograms of representative compounds from each antibiotic

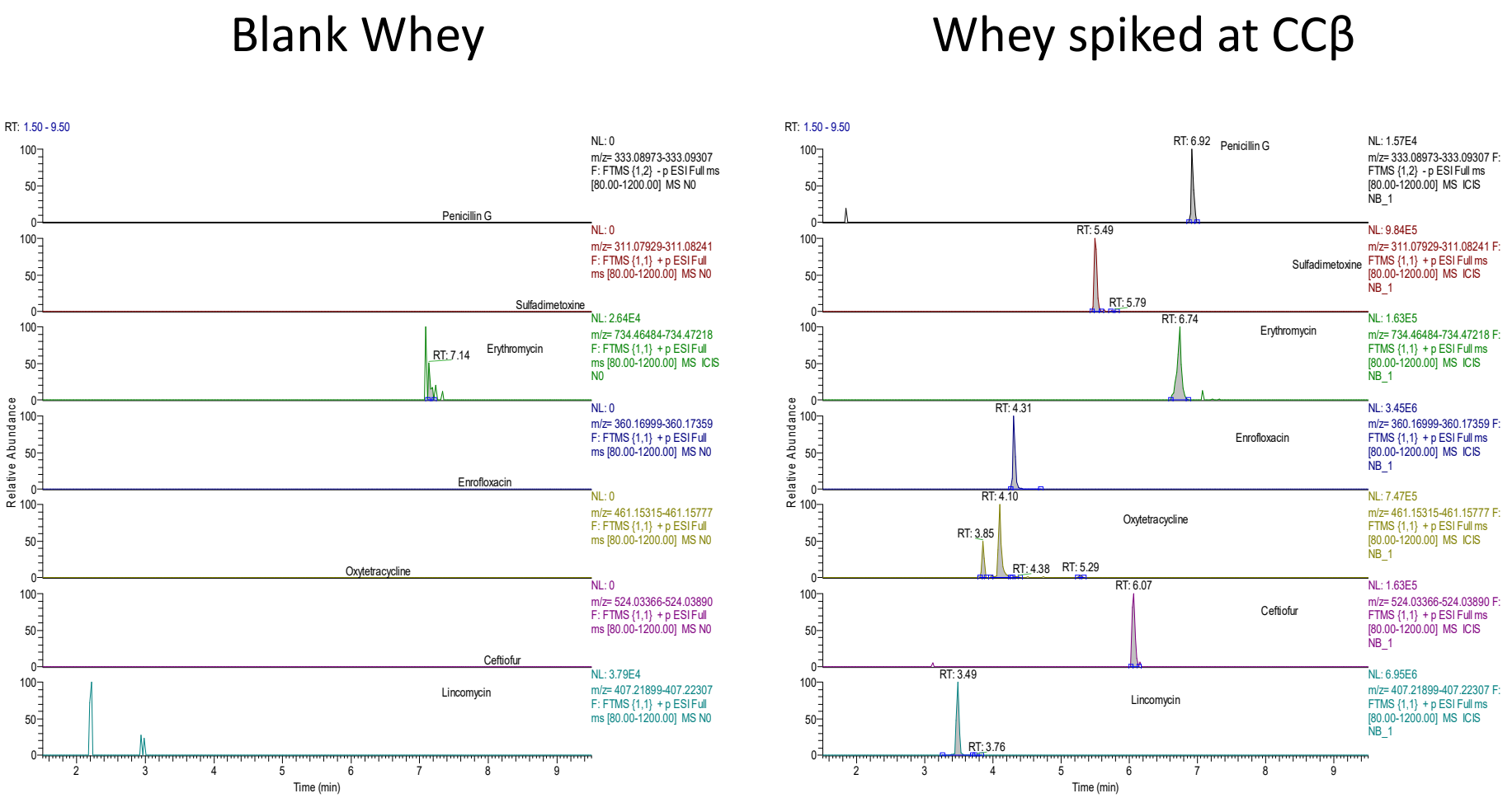

Figure 2. UHPLC–HRMS extracted ion chromatograms of representative compounds from each antibiotic