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

Paloma Quintanilla, Mª Carmen Beltrán, Bernardo Peris, Martín Rodríguez, Mª Pilar Molina*

Institute of Animal Science and Technology. Universitat Politècnica de València. Camino de Vera, s/n. 46022, Valencia, Spain

* Corresponding author: Mª Pilar Molina Pons
Institute of Animal Science and Technology
Universitat Politècnica de València
Camino de Vera, s/n
46022 Valencia, Spain
Phone: +34 96 387 7431
Fax: +34 96 387 7439
Email address: pmolina@dca.upv.es

Abstract

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

Keywords: antibiotics; macrolides; goat's milk; goat cheese

1. Introduction

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

Respect at the use of veterinary drug Spain is the second country after United Kingdom, which has used the most antimicrobial agents for goats and sheep species. In addition, among the sales of antimicrobial agents for food-producing species, the macrolides constitute the fourth most important group of antimicrobials applied, behind

66 the tetracyclines, penicillins and sulfonamides (EMA, 2017). Macrolides are antibacterial
67 compounds usually applied in veterinary medicine showing *in vitro* activity against a
68 wide range of pathogenic microorganisms including mycoplasma, Gram positive
69 bacteria, and some Gram negative bacteria like *Pasteurella* spp. (Clothier et al., 2012).
70 The antibacterial activity of such drugs is based on the inhibition of bacterial protein
71 synthesis by binding to bacterial 50S ribosomal subunits (Papich and Riviere, 2001).
72 Macrolides may also have an immune-modulating effect on cell-mediated immunity (Cao
73 et al., 2006).

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

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

83 Studies have been performed to evaluate the pharmacokinetics of macrolides in tissues
84 and plasma of some animal species including goats (Taha et al., 1999; Cárceles et al.,
85 2005). However, very little information is available on residual patterns of macrolides in
86 goat's milk with excretion times ranging from a few hours to several days (Ambros et al.,
87 2007; Amer et al., 2012). The aim of this work was: 1) to verify if the exceptional use of
88 macrolide antibiotics in dairy goats leads to residues in milk and cheese, thus posing a
89 risk for consumer health, and 2) to evaluate the effect of these treatments on cheese
90 manufacturing and the characteristics of matured cheeses.

2. Material and Methods

Experimental procedure

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

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

Three macrolide antibiotics (erythromycin, tylosin and spiramycin) registered for the use in cattle and pigs, were selected for this study. All the treatments were administrated after morning milking by the intramuscular route. The veterinary drugs used were: Pantoyet® (Laboratorios Syva, S.A. León, Spain), 200 mg/mL of erythromycin, dose: 0.5 mL/10 kg body weight on three consecutive days; Trelacón® (Laboratorios Elanco Valquímica, S.A. Madrid, Spain), 200 mg/mL of tylosin, dose: 0.5 mL/10 kg body weight on three consecutive days; and Mycogal® (Laboratorios Ovejero, S.A. León, Spain), 276.3 mg (1.05 MUI)/mL of spiramycin, dose: 1 mL/10 kg body weight in a single dose. The withdrawal period considered was seven days after the last drug administration, as stipulated by European legislation for the exceptional use of antibiotics, except for Mycogal® (spiramycin) for which two withdrawal times (seven and 14 days) were considered as the manufacturer's specification sheet indicates a withdrawal period (11 days) for dairy cows. During the experimental period, bulk milk samples (50 mL) were taken on a daily basis to detect the presence of drug residues.

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

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

Milk Analysis

Milk samples were analysed by MilkoScan 6000 (Foss, Hiller∅d, Denmark) to determine the chemical composition (fat, protein and total solids); somatic cell count (SCC) and total bacterial count (TBC) were obtained using Fossomatic 5000 (Foss) and Bactoscan FC (Foss), respectively. The milk pH value was measured by a conventional pH-meter (model Basic 20, Crison, Barcelona, Spain).

Cheese Analysis

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

Cheese samples were characterized twice whilst ripening (1 and 60 days) by assessing quality variables such as acidity, water activity (a_w), free fatty acids (FFA) and free amino acids (FAA) contents.

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

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

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

Antibiotic residues analysis

165 Antibiotic residues in goat's milk and cheeses were analysed at the Instituto
166 Lactológico de Lekunberri (Pamplona, Spain) using a liquid chromatography tandem
167 mass spectrometry (LC-MS/MS) technique. For chromatographic analysis, an Alliance
168 2695 high-performance liquid chromatograph with a diode-array detector from Waters
169 (Waters Chromatography Division, Milford, MA, USA) was employed. Separation of
170 compounds was accomplished using an XBridgeTM C18 column (Waters
171 Chromatography Division). Mass spectral analyses were performed on a Micromass
172 Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters Chromatography
173 Division). The calibration curves had previously been established for each macrolide
174 considered; the quantification limit (LOQ) being equal to 10 µg/kg for tylosin and
175 erythromycin, and 30 µg/kg for spiramycin. MassLynx 4.0 software (Waters) was used
176 to calculate the macrolide concentrations in goat's milk and cheeses.

177 *Statistical analysis*

178 Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. The Plains, Virginia,
179 USA) was used for the statistical analysis. The milk quality variables were analyzed using
180 a multifactorial ANOVA including the effects of the Milk sampling (PT: pre-treatment,
181 AT: 24 hours after treatment, and AW: after the withdrawal period) and the animal group
182 (1 to 2).

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

185 $$Y_{ijkl} = \mu + C_i + R_j + G_k + (C_i \times R_j) + (C_i \times G_k) + (R_j \times G_k) + e_{ijkl}$$

186 where: Y_{ijkl}= dependent variable; µ= mean; C_i= cheese-making (PT: pre-treatment, AT:
187 24 hours after treatment, and AW: after the withdrawal period); R_j= ripening time (1 or
188 60 days); G_k= animal group (1 or 2). $C_i \times R_j$ = Effect of interaction cheese-making and

ripening time; $C_i \times G_k$ = effect of interaction cheese-making and animal group; $R_j \times G_k$= effect of interaction ripening time and animal group; e_{ijkl}= residual error.

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

3. Results

The off-label use of erythromycin, tylosin, and spiramycin in dairy goats under conditions described in this study had no significant effect ($p>0.05$) on the milk quality parameters such as gross composition, pH, SCC, and TBC. In all cases, similar characteristics were observed in bulk milk obtained before drug administration as well as in the milk collected 24 hours after treatment, and at the end of the withdrawal period considered for each antibiotic. The mean values for gross composition (g/100 g) of raw milk used for cheese manufacture were as follows: 14.40±0.40 for total solids, 5.30±0.29 for fat, and 3.74±0.18 for protein. The pH-value was 6.72±0.05, SCC 707,800 cells/mL, and TBC 21,900 cfu/mL. However, residues of erythromycin (234.9±52.7 µg/kg), tylosin (198.7±57.8 µg/kg) and spiramycin (1539.8±469.4 µg/kg) were found in goat's milk 24 hours after the last drug administration. In all cases, the residues decreased markedly along time becoming undetectable in milk 3-5 days after completing antibiotic therapy, except for spiramycin, whose residues were quantified in milk until the eighth day of the withdrawal period (Fig. 1).

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

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

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

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

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

235 Results for the antibiotic treatment with tylosin (Table 2) indicate that cheeses made
236 from milk after the last drug administration (AT-cheeses) showed higher hardness values
237 ($P<0.05$) and a lower FAA concentration ($P<0.01$) than the other two types of cheese. As

shown in Fig. 2, statistical differences in the FAA concentration were only detected at the end of the 60-day ripening period (interaction $C_i \times R_j, P<0.05$) evidencing a lower proteolytic activity in the AT-cheeses during maturation. On the other hand, both lower lipolytic and proteolytic activities were also observed in cheeses made in the second experimental replicate ($P<0.05$) although the related interactions ($C_i \times G_k$ and $R_j \times G_k$) were non-significant.

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

4. Discussion

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

cultures activity. Cabizza et al. (2017) observed a delay of 60 minutes in the completion of the acidification process of ripened cheeses from sheep milk spiked with 100 µg/kg of oxytetracycline, in comparison to the cheese made from antibiotic-free sheep milk, due to the inhibitory effect of this substance. It should be noted that an increase in the acidification time to reach the final pH in the cheese poses a risk to consumer health as high pH values could facilitate the growth of pathogenic or undesirable microorganisms (Fox and McSweeney, 2017). In our study, the inhibition of the starter bacteria by the erythromycin and the spiramycin residues was so pronounced that the pH values of the curd remained at 6.4-6.5 along the entire production process, impeding the maturation of the cheeses.

However, the presence of high concentrations of tylosin in goat's milk collected 24 hours after drug administration (198.7±57.8 µg/kg) did not affect the cheese-making processes. Nevertheless, tylosin residues were detected in AT-cheeses along the entire ripening period which could be related to the lower proteolytic activity in such cheeses containing lower FAA at the end of this period. Results herein suggest that 48.5 % of the antibiotic retained in the soft cheeses remain in the final product, the rest being degraded during maturation. The lower stability of this substance in acidic conditions (Papich and Riviere, 2001) could be related to the antibiotic losses in cheeses which presented a pH final ranging from 5.1 to 5.3. In any case, macrolides show a low protein binding ability due to their low degree of ionization. Thus, considering a mean cheese yield value for mature cheese like Tronchón of 8 (kg of milk/kg of cheese), the antibiotic retained in the cheese could represent 5-6 % of the drug initially present in the milk supply. It should be noted that the information related to the presence of macrolide residues in cheeses is practically non-existent.

After the legally established withdrawal period, seven days, erythromycin and tylosin residues were not detected in bulk milk from treated goats. Hence, considering that after 48 h the residues of these substances in bulk milk are lower than the MRLs (European Commission, 2010) prescribed, the shortening of the legal withdrawal period could be considered. However, spiramycin residues can be found in milk until the eighth day of this period although being below the MRL established for this substance (200 μg/kg). These results are in agreement with those observed by other authors when studying the pharmacokinetics of macrolide antibiotics in dairy goats. Thus, while erythromycin and tylosin, given their lipophilic nature, are rapidly eliminated from the animal's organism by excretion in milk during the first hours after their systemic administration (Ambros et al., 2007; Atef et al., 2009), spiramycin requires a longer elimination period. The lower absorption rate of spiramycin could be related to its higher pKa value, possibly a result of the high degree of ionization in acidic conditions making the excretion in milk slower (DrugBank, 2018), Therefore, in spite of the fact that even after seven days, the antibiotic is detected below the MRL, further pharmacokinetic studies on spiramycin are recommended to establish its adequate withdrawal period to avoid negative implications on the consumer health.

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

5. Conclusions

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

Conflict of interest statement

The authors declare that the research has no conflict of interest.

Acknowledgments

This work is part of the AGL-2013-45147-R funded by Ministerio de Ciencia e Innovación (Madrid. Spain). The authors thank the 'Program of Support for Research and Development' (PAID-2014, UPV) for support P. Quintanilla PhD studies at Universitat Politècnica de València.

References

Al-Wabel, N.A., 2008. The pharmacokinetics and milk residual behaviour of tylosin in lactating Najdi ewes. Iran. J. Vet. Res. 9, 138–143. https://doi.org/10.22099/ijvr.2008.537

Ambros, L., Montoya, L., Kreil, V., Waxman, S., Albarellos, G., Rebuelto, M., Hallu, R., San Andres, M.I., 2007. Pharmacokinetics of erythromycin in nonlactating and lactating goats after intravenous and intramuscular administration. J. Vet. Pharmacol. Ther. 30, 80–85. https://doi.org/10.1111/j.1365-2885.2007.00819.x

337 Amer, A.M.M., Constable, P.D., Goudah, A., El Badawy, S.A., 2012. Pharmacokinetics
338 of tulathromycin in lactating goats. Small Rumin. Res. 108, 137–143.
339 https://doi.org/10.1016/j.smallrumres.2012.07.003
340 Atef, M., Ramadan, A., Darwish, A.S., Fahim, A.M.M., 2009. Effect of albendazole
341 administration on pharmacokinetic aspects of tylosin in lactating goats. Drug Metab.
342 Lett. 3, 137–143. https://doi.org/10.2174/187231209789352076
343 Avci, T., Elmas, M., 2014. Milk and Blood Pharmacokinetics of Tylosin and Tilmicosin
344 following Parenteral Administrations to Cows. Sci. World J. 2014, Article ID 869096,
345 6 pages. http://dx.doi.org/10.1155/2014/869096
346 Berruga, M.I., Novés, B., Molina, M.P., Román, M., Molina, A., 2008. Influence of
347 cephalosporins on the coagulation time of yogurt made from ewes' milk. Int. J. Dairy
348 Technol. 61, 372–378. https://doi.org/10.1111/j.1471-0307.2008.00421.x
349 Blasco, E., Gomez, E.A., Vicente, C., Vidal, G., Peris, C., 2016. Factors affecting milking
350 speed in Murciano-Granadina breed goats. J. Dairy Sci. 99, 10102–10108.
351 https://doi.org/10.3168/jds.2016-10869
352 Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., Addis, M., Testa,
353 M.C., Urgeghe, P.P., 2017. Transfer of oxytetracycline from ovine spiked milk to
354 whey and cheese. Int. Dairy J. 70, 12–17.
355 https://doi.org/10.1016/j.idairyj.2016.12.002
356 Cao, X.Y., Dong, M., Shen, J.Z., Wu, B.B., Wu, C.M., Du, X.D., Wang, Z., Qi, Y.T., Li,
357 B. Y., 2006. Tilmicosin and tylosin have anti-inflammatory properties via modulation
358 of COX-2 and iNOS gene expression and production of cytokines in LPS-induced
359 macrophages and monocytes. Int. J. Antimicrob. Agents 27, 442–449.
360 https://doi.org/10.1016/j.ijantimicag.2005.12.010

361 Cárceles, C. M., Font, A., Espuny, A., Fernández-Varón, E., Serrano, J. M., Escudero, E.,
362 2005. Pharmacokinetics of azithromycin after intravenous and intramuscular
363 administration to goats. J. Vet. Pharmacol. Therap. 28, 51–55.
364 http://dx.doi.org/10.1111/j.1365-2885.2004.00626.x
365 Clothier, K.A., Kinyon, J.M., Griffith, R.W., 2012. Antimicrobial susceptibility patterns
366 and sensitivity to tulathromycin in goat respiratory bacterial isolates. Vet. Microbiol.
367 156, 178–182. https://doi.org/10.1016/j.vetmic.2011.10.025
368 DrugBank, 2018. A knowledge base for drugs, drug actions, and drug targets. Spiramycin.
369 https://www.drugbank.ca/drugs/DB06145/ (accessed 31 January 2018).
370 EFSA, 2016. The European Union summary report on antimicrobial resistance in
371 zoonotic and indicator bacteria from humans, animals and food in 2014. EFSA J.
372 14:4380. doi:10.2903/j.efsa.2016.4380
373 EMA, 2017. European Medicines Agency, European Surveillance of Veterinary
374 Antimicrobial Consumption, 2017. 'Sales of veterinary antimicrobial agents in 30
375 European countries in 2015'. (EMA/184855/2017)
376 European Commission, 2010. Commission Regulation (EU) No 37/2010 of 22 December
377 2009 on pharmacologically active substances and their classification regarding
378 maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union L15, 1–72.
379 http://data.europa.eu/eli/reg/2010/37(1)/oj (accessed 02 January 2018).
380 European Parliament and the Council of the European Union, 2001. Directive
381 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on
382 the Community code relating to veterinary medicinal products. Off. J. Eur.
383 Communities L311, 1–66.
384 European Parliament and the Council of the European Union, 2004. Directive
385 2004/28/EC of the European Parliament and of the Council of 31 March 2004

amending Directive, 2001/82/EC on the Community code relating to veterinary medicinal products. Off. J. Eur. Union L136, 58–84.

Ferrini, A.M., Trenta, S., Mannoni, V., Rosati, R., Coni, E., 2010. Depletion of Long-Acting Ampicillin in Goat Milk following Intramuscular Administration. J. Agric. Food Chem 58, 12199–12203. https://doi.org/10.1021/jf102351p

Folkertsma, B., Fox, P.F., 1992. Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. J. Dairy Res. 59, 217–224. https://doi.org/10.1017/S0022029900030466

Fox, P.F., McSweeney, P.L.H., 2017. Chapter 1 - Cheese: An Overview, in: McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W. (Eds.), Cheese (Fourth Edition). Academic Press, San Diego, pp. 5–21. https://doi.org/https://doi.org/10.1016/B978-0-12-417012-4.00001-6

Gómez-Martín, A., Amores, J., Paterna, A., De la Fe, C., 2013. Contagious agalactia due to Mycoplasma spp. in small dairy ruminants: Epidemiology and prospects for diagnosis and control. Vet. J. 198, 48–56. https://doi.org/10.1016/j.tvjl.2013.04.015

Graham, F., Paradis, L., Bégin, P., Paradis, J., Babin, Y., Des Roches, A., 2014. Risk of allergic reaction and sensitization to antibiotics in foods. Ann. Allergy, Asthma Immunol. 113, 329–330. https://doi.org/10.1016/j.anai.2014.06.029

Nuñez, M., García-Aser, C., Rodríguez-Martin, M.A., Medina, M., Gaya, P., 1986. The effect of ripening and cooking temperatures on proteolysis and lipolysis in Manchego cheese. Food Chem. 21, 115–123. https://doi.org/10.1016/0308-8146(86)90156-1

Papich, M. G., Riviere, J. E., 2001. Chloramphenicol and derivatives, macrolides, lincosamides, and miscellaneous antimicrobials, in: Adams, H. R. (Ed.), Veterinary Pharmacology and Therapeutics, (Eighth Edition). Iowa State University Press, Ames, Iowa, pp. 868–897.

411 Taha, A.A., Elsheikh, H.A., Khalafalla, A.E., Osman, I.A.M., Abdullah, A.S., 1999.
412 Disposition kinetics of tylosin administered intravenously and intramuscularly in
413 desert sheep and nubian goats. Vet. J. 158, 210–215.
414 https://doi.org/10.1053/TVJL.1999.0374
415 Young, G., Smith, G. W., Leavens, T. L., Wetzlich, S. E., Baynes, R. E., Mason, S. E.,
416 Riviere, J. E., Tell, L. A., 2011. Pharmacokinetics of tulathromycin following
417 subcutaneous administration in meat goats. Res. Vet. Sci., 90(3), 477-479.
418 https://doi.org/10.1016/j.rvsc.2010.06.025

Table 1

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

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

[1] The manufacture of 24 h after treatment cheeses (AT-cheeses) was not possible.

PT-cheeses: Pre-treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error.

a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/100 g cheese).

*P< 0.05 indicate significant difference; ns: non-significant.

Table 2

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

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

PT-cheeses: Pre-treatment cheeses; AT-cheeses: After treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error.

a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/100 g cheese).

$^{a, b:}$ Different letters in the same row indicate significant differences ($P< 0.05$); ***$P< 0.001$; **$P< 0.01$; *$P< 0.05$; ns: non-significant.

Table 3

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

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

[1] The manufacture of 24 h after treatment cheeses (AT-cheeses) was not possible.

PT-cheeses: Pre-treatment cheeses; AW-cheeses: After withdrawal period cheeses; SE: standard error.

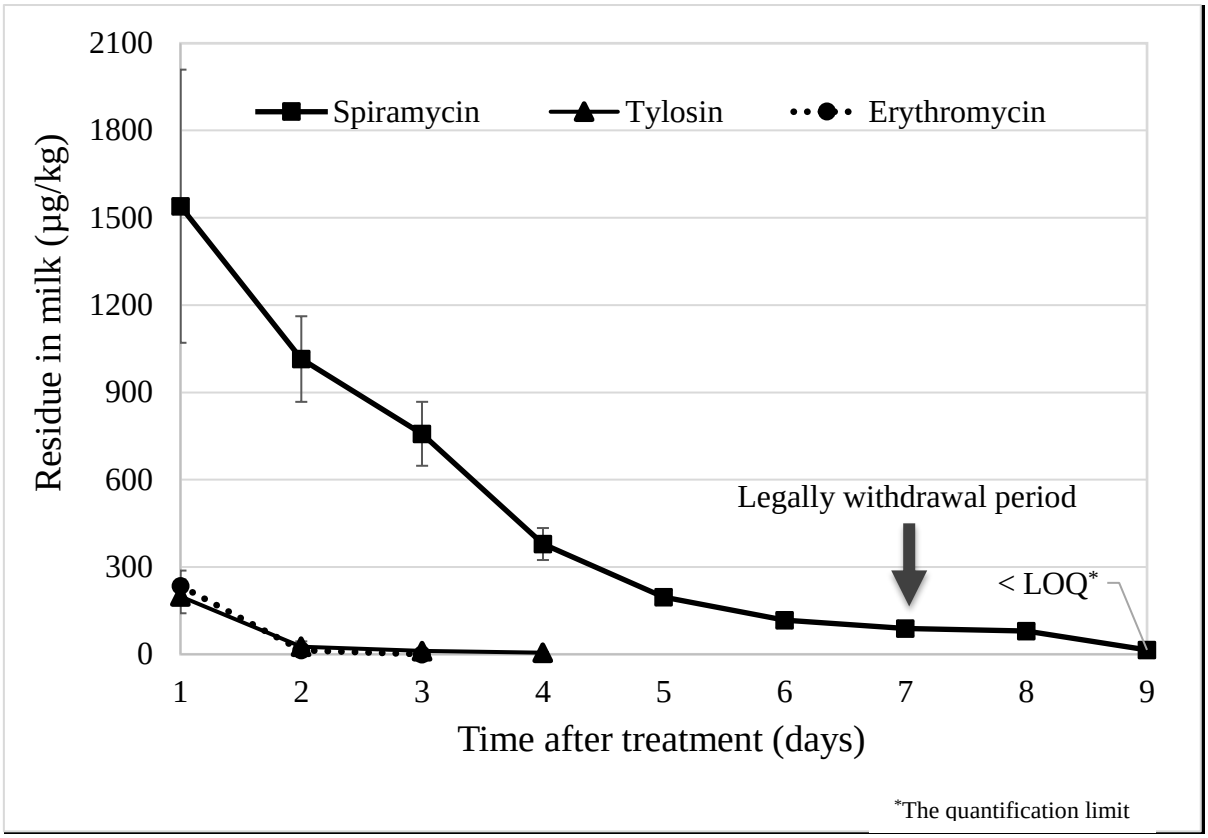
a_w: water activity; FFA: Free Fatty Acids (meq/100 g of fat); FAA: Free Amino-Acids (mg leucine/100 g cheese).

Significant differences ($P< 0.05$); ***$P< 0.001$; **$P< 0.01$; *$P< 0.05$; ns: non-significant.

Figure legends

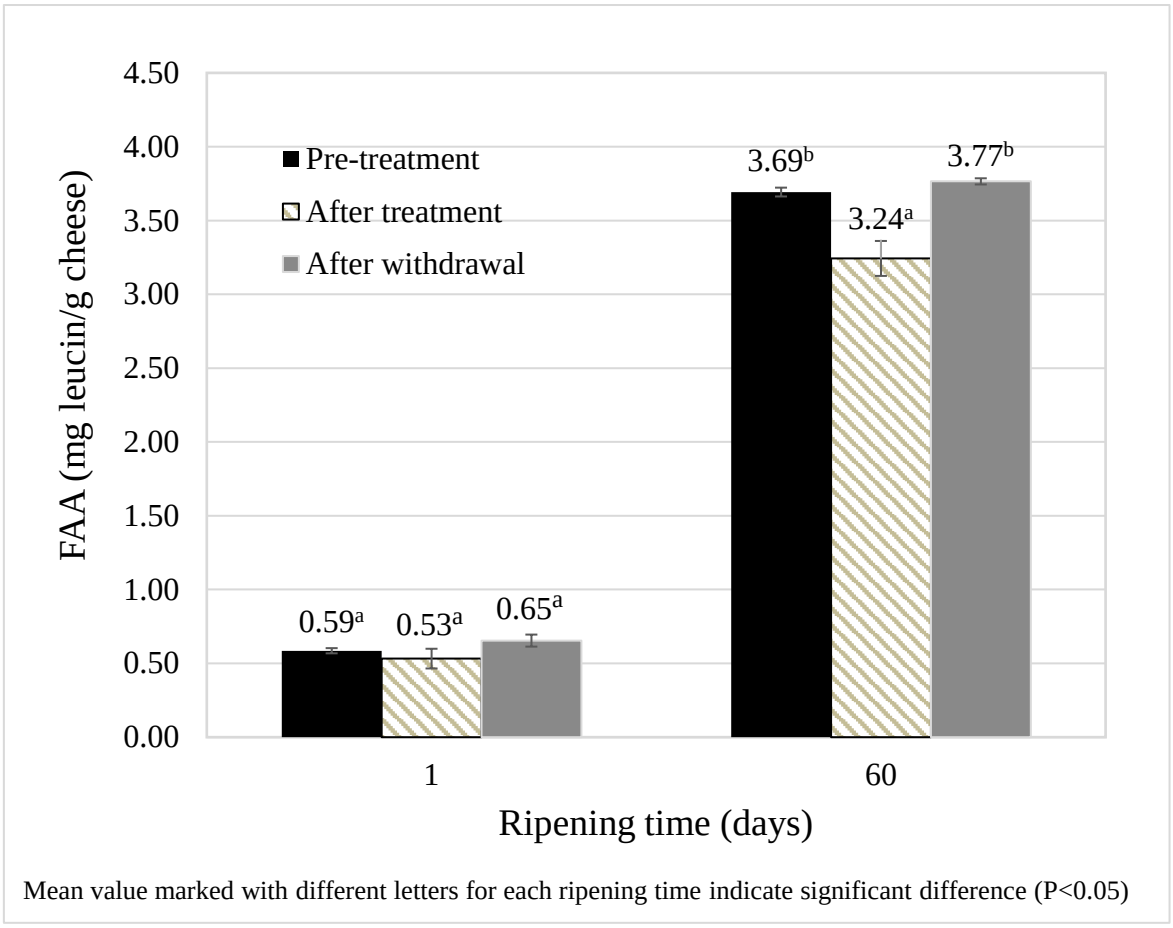
Fig. 1. Concentration (µg/kg) of macrolides in goat's milk at different time after antibiotics treatment.

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


2100
1800
1500
1200
900
600
300
0
Residue in milk (µg/kg)
Spiramycin
Tylosin
Erythromycin
Legally withdrawal period
< LOQ*
1
2
3
4
5
6
7
8
9
Time after treatment (days)
*The quantification limit

445

446 **Fig. 1.**



Mean value marked with different letters for each ripening time indicate significant difference (P<0.05)

447

448 **Fig. 2.**