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Interpretive summary:

Characteristics of ripened Tronchón cheese from raw goat's milk containing legally admissible amounts of antibiotics

Quintanilla

Antibiotic residues in milk can be a problem for public health, and for the dairy industry, especially in fermented products. Raw goat's milk spiked with legally admissible amounts of antibiotics was used to make Tronchón cheeses. The cheese-making and the cheese quality parameters were only slightly affected by the presence of antibiotics in the milk. However, varying amounts of antibiotics were retained in the cheeses before maturation. Although their concentrations decrease along time, antibiotics such as quinolones and oxytetracycline were detected in the cheeses after 60 days of maturation, making it necessary to assess the risk for consumer.

Running headline: **ANTIBIOTICS IN GOAT'S CHEESE**

Characteristics of ripened Tronchón cheese from raw goat's milk containing legally admissible amounts of antibiotics

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ABSTRACT

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

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

Abbreviation key: EU = European Union, **MRL** = maximum residue limit, **AF** = antibiotic-free, **SM** = spiked milk, **LOQ** = limit of quantification, **LOD =** limit of detection, **FAA** = free amino acids, **FFA** = free fatty acids.

INTRODUCTION

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

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

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

Antibiotics could be retained in milk curd to a greater or lesser extent, depending on the physicochemical properties of these substances and their ability to interact with the fat and protein fraction of the matrix (Giraldo et al., 2017; Shappell et al., 2017). The World Health Organization (WHO) suggested establishing MRLs of liposoluble antibiotics in milk products such as milkfat and cheese, being apprehensive that such substances might reach levels far above the initial contents in milk and, thus, possibly posing a risk for consumers (FAO/WHO, 2004). However, EU-legislation has established only an MRL for raw milk, while safety limits for related products have not been fixed.

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

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

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

Studies on the retention of the antibiotic during dairy manufacturing processes are crucial to prevent the negative implications related to the presence of such substances in milk products. Therefore, the aim of the present work was to evaluate the transfer of the most widely used antibiotics in dairy goats from milk to cheese, as well as their effect on the cheese-making process and the cheese characteristics during ripening.

MATERIALS AND METHODS

Milk samples and antibiotics

Antibiotic-free milk was obtained from the experimental herd of Murciano-Granadina goats of Universitat Politècnica de València (UPV, Valencia, Spain). Animals did not receive any antimicrobial substances neither before nor along the experimental period. The milk chemical composition was analyzed by MilkoScan 6000 (Foss, Hillerød, Denmark), somatic cell count by Fossomatic 5000 (Foss), and total bacterial count by Bactoscan FC (Foss). The milk pH value was measured by a conventional pH-meter (model Basic 20, Crison, Barcelona, Spain). Screening test Eclipse 100 (Zeulab, Zaragoza, Spain) was used to detect inhibitors in milk.

The goat's milk composition (g/100 g) had an average (mean ± SD) total solids content of 14.4 ± 0.7, fat content 5.3 ± 0.5, and protein 3.7 ± 0.8. The somatic cell count and total bacterial count were 6.08 log cells/mL and 4.76 log cfu/mL, respectively, and the mean pH was 6.80 ± 0.05.

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

Cheese-making process

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

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

Analysis of antibiotic residues in cheese

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

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

Antibiotics were analyzed using a chromatography system consisting of an LC/MS-MS Alliance 2695 with a diode-array detector (Waters Chromatography Division) and a

Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters Chromatography Division). An XBridge™ C_{18} column (100 x 34.6 x 2.1 mm, particle size of 3.5 µm) was used (Waters Chromatography Division). Chromatographic separation was carried out with a mobile phase A consisting of 0.1% (v/v) formic acid in water and mobile phase B consisting of 0.1% formic acid in acetonitrile (LC gradient grade, Scharlau). The solvent gradient conditions of the mobile phase for the antibiotics (except oxytetracycline) were as follows: time (t, min) t_0, 95% A and 5% B; t_8, A = 25%; t_{14}, A = 5%; t_{15}, A = 95%; t_{20}, A = 95%. In the case of oxytetracycline analyses, the mobile phase gradient profile was: t_0, 85% A and 15% B; t_6, A = 82%; t_8, A = 50%; t_{10}, A = 50%; t_{15}, A = 85%; t_{20}, A = 85%. The flow rate was 0.2 mL/min. The operating parameters for the mass spectrometer were needle voltage 3.0 kV, lens voltage 0.2 V; source block temperature 140 °C; desolvation temperature 450 °C. Desolvation and cone gas (nitrogen) was 750 and 50 L/h, respectively. Analytes were detected using electrospray ionization in the positive ion mode. The MassLynx 4.0 software (Waters Chromatography Division) was used to calculate the antibiotic concentrations in goat's cheeses. The typical recoveries were approximately between 85-100% for the β-lactams and tetracyclines, 80-95% for the macrolides, and 90-110% for the quinolones.

The calibration curves had previously been established for each antibiotic considered. The limit of quantification (**LOQ**) being equal to 2 µg/kg for amoxicillin and benzylpenincillin, 10 µg/kg for oxytetracycline and erythromycin, 15 µg/kg for cloxacillin and 50 µg/kg for ciprofloxacin and enrofloxacin.

Analysis of cheese samples

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

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

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

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

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

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

where ΔL^*, Δa^* and Δb^* are the differences between the two samples in L^*, a^* and b^*, respectively. The perception of the color difference ΔE^* varied according to the observed color and the sensitivity of the human eye. $\Delta E^* < 1$ color differences could not be detected by the human eye; $1 < \Delta E^* < 3$ minor color differences could be detected by the human eye, and $\Delta E^* > 3$ color differences could be detected by the human eye.

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

Sensory evaluation

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

Statistical analyses

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

RESULTS AND DISCUSSION

Antibiotic residues in goat's milk cheeses

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

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

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

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

In all cases, the antibiotic concentration in the fresh cheeses decreases during maturation (Table 1). Thus, β-lactam drugs and erythromycin were not detected in cheeses having ripening for 30 days. However, quinolones and oxytetracycline were detected even after a period of 60 days although the residual concentrations of oxytetracycline in ripened cheeses was 95% lower than at the beginning of maturation possibly due to the lower stability of this substance under refrigerated conditions (Roca et al., 2008). Instead, quinolones were more stable showing a lower reduction rate along maturation (30-45%) which also implies a higher concentration of these drugs in the final products making it necessary to assess their risk for consumer health. Also, the transfer of antibiotic residues from cheese to whey (Giraldo et al., 2017; Gajda et al., 2018), could have negative implications for humans, animals and environmental safety as this byproduct is used in the manufacture of foodstuffs for human consumption, animal feeding and agricultural applications, among other uses (Carvalho et al., 2013).

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

Effect of the antibiotics on cheese-making

The cheese-making process was unaffected by the presence of β-lactams and quinolones at safety levels in raw goat's milk. However, the time required for cheese production using milk containing admissible amounts of erythromycin and oxytetracycline (40 and 100 µg/kg, respectively) increased ($p = 0.003$; $p = 0.013$, respectively). As shown in Figure 2, the kinetic of acidification of the cheeses during cheese production was considerably affected by the presence of these antibiotics, requiring additional time to reach the final pH (5.30 ± 0.05) in the cheeses made from milk containing erythromycin (122 ± 29 min) and oxytetracycline (108 ± 25 min) with respect to the control cheeses. This suggests that the activity of the starter cultures was strongly inhibited by the presence of these antibiotics, leading to a lower acidification rate than in the control cheeses, especially for erythromycin which was also able to increase ($p = 0.006$) the heating and stirring time (25 ± 5 min) prior to the draining off and molding of the curd (Figure 2.A). Similarly, Cabizza et al. (2017, 2018) also observed delays, ranging from 60 to 78 min, in the acidification process during ewe's cheese manufacture from milk with oxytetracycline at safety level (100 µg/kg). Moreover, a concentration below MRL for erythromycin (16 µg/L) reported by Katla et al. (2001) was able to reduce by 50% the activity of *Streptococcus* spp., isolated from dairy products (yoghurt, sour cream, fermented milk, whey and cheese), commercial and starter cultures.

As expected, the results herein suggest that admissible amounts of some antibiotic residues in milk could interfere in the metabolism of the microbiota present in the initial stages of the cheese production of ripened cheese, which could also affect its capacity to develop the complex biochemical processes necessary during maturation, and the quality characteristics of the mature cheeses could, therefore, be affected. On the other hand, a low activity of the starter culture could result in more favorable environmental growth conditions for undesirable bacteria, like coliforms (Cabizza et al., 2018) during cheese-making, which could affect the microbiological safety of the cheeses (Choi et al., 2016).

Physicochemical parameters of the cheeses

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

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

Proteolytic and lipolytic activities in the cheeses

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

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

In general, the FAA content in the experimental cheeses was in the order of those reported by other authors for cheeses of 60 days ripened (Juan et al., 2016), and the FFA content showed a similar trend that the data presented by Buffa et al. (2001) in mature cheese made from raw goat's milk. It should be noted that proteolysis and lipolysis play a major role in the development of texture and flavor in most cheese varieties during ripening, directly contributing to flavor, via formation of peptides and FAA (Fenelon et al., 2000), as well as FFA from the lipolysis of triglycerides (Collins et al., 2003).

Color evaluation of the cheeses

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

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

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

Textural properties of the cheeses

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

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

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

Sensorial analysis of the cheeses

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

As shown in Figure 3.A, the SM-cheeses with amoxicillin had lower scores for the odor attribute than the reference cheeses ($p = 0.032$). This could be related to the lower concentration of FFA in the cheeses with the antibiotic (Table 3). The FFA content is closely related to the characteristic aroma to goat's milk cheeses and precursors of other high-flavored compounds, such as methyl ketones and lactones (McSweeney and Sousa, 2000; Collins et al., 2003). Regarding mature SM-cheeses from goat's milk containing erythromycin at safety level (Figure 3.B), the sensory analysis results indicated significant differences for the attributes odor ($p = 0.001$) and overall preference (p =

0.002), which were better valued than the control cheeses by the untrained consumers. Despite these differences, the SM-cheeses with these antibiotics were evaluated with high scores (overall preference: 7.3 and 7.1 for amoxicillin and erythromycin, respectively) suggesting a high degree of acceptance by the panelists.

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

CONCLUSIONS

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

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

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REFERENCES

Adetunji, V.O. 2011. Effects of processing on antibiotic residues (streptomycin, penicillin−g and tetracycline) in soft cheese and yoghurt processing lines. Pakistan J. Nutr. 10:792–795. https://doi.org/10.3923/pjn.2011.792.795.

Arias, M., M.S. García-Falcón, L. García-Río, J.C. Mejuto, R. Rial-Otero, and J. Simal-Gándara. 2007. Binding constants of oxytetracycline to animal feed divalent cations. J. Food Eng. 78:69–73. https://doi.org/10.1016/j.jfoodeng.2005.09.016.

Beltrán, M.C., R.L. Althaus, A. Molina, M.I. Berruga, and M.P. Molina. 2015. Analytical strategy for the detection of antibiotic residues in sheep and goat’s milk. Spanish J. Agric. Res. 13. http://dx.doi.org/10.5424/sjar/2015131-6522.

Berruga, M.I., A. Molina, R.L. Althaus, and M.P. Molina. 2016. Control and prevention of antibiotic residues and contaminants in sheep and goat’s milk. Small Rumin. Res. 142:38–43. https://doi.org/10.1016/j.smallrumres.2016.02.023.

Bodart, M., R. de Peñaranda, A. Deneyer, and G. Flamant. 2008. Photometry and colorimetry characterisation of materials in daylighting evaluation tools. Build. Environ. 43:2046–2058. https://doi.org/10.1016/j.buildenv.2007.12.006.

Buffa, M., B. Guamis, M., Pavia, and A. J. Trujillo. 2001. Lipolysis in cheese made from raw, pasteurized or high-pressure-treated goats' milk. Int. Dairy J. 11: 175–179. https://doi.org/10.1016/S0958-6946(01)00044-9.

Cabizza, R., N. Rubattu, S. Salis, M. Pes, R. Comunian, A. Paba, M. Addis, M.C. Testa, and P.P. Urgeghe. 2017. Transfer of oxytetracycline from ovine spiked milk to whey and cheese. Int. Dairy J. 70:12–17. https://doi.org/10.1016/j.idairyj.2016.12.002.

Cabizza, R., N. Rubattu, S. Salis, M. Pes, R. Comunian, A. Paba, E. Daga, M. Addis, M.C. Testa, and P.P. Urgeghe. 2018. Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications. LWT - Food Sci. Technol. 96:236–243. https://doi.org/10.1016/j.lwt.2018.05.026.

Carreira, A., K. Dillinger, F. Eliskases-Lechner, V. Loureiro, W. Ginzinger, and H. Rohm. 2002. Influence of selected factors on browning of Camembert cheese. J. Dairy Res. 69:281–292. https://doi.org/10.1017/S0022029902005393.

Carvalho, F., A.R. Prazeres, and J. Rivas. 2013. Cheese whey wastewater: Characterization and treatment. Sci. Total Environ. 445–446:385–396. https://doi.org/10.1016/j.scitotenv.2012.12.038.

Chen, S.X., J.Z. Wang, J.S. Van Kessel, F.Z. Ren, and S.S. Zeng. 2010. Effect of somatic cell count in goat milk on yield, sensory quality, and fatty acid profile of semisoft cheese. J. Dairy Sci. 93:1345–1354. https://doi.org/10.3168/jds.2009-2366.

Chilliard, Y., A. Ferlay, J. Rouel, and G. Lamberet. 2003. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. J. Dairy Sci. 86:1751–1770. https://doi.org/10.3168/jds.S0022-0302(03)73761-8.

Choi, K.H., H. Lee, S. Lee, S. Kim, and Y. Yoon. 2016. Cheese microbial risk assessments - A review. Asian-Australasian J. Anim. Sci. 29:307–314. https://doi.org/10.5713/ajas.15.0332.

Collins, Y.F., P.L.H. McSweeney, and M.G. Wilkinson. 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int. Dairy J. 13:841–866. https://doi.org/10.1016/S0958-6946(03)00109-2.

Delgado, F.J., J. González-Crespo, R. Cava, and R. Ramírez. 2011. Proteolysis, texture and colour of a raw goat milk cheese throughout the maturation. Eur. Food Res. Technol. 233:483–488. http://doi.org/10.1007/s00217-011-1536-3.

Dethlefsen, L., S. Huse, M.L. Sogin, and D.A. Relman. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLOS Biol. 6:1–18. https://doi.org/10.1371/journal.pbio.0060280.

European Union. 2002. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. L221:8–36.

European Union. 2009. Regulation (EC) Nº 470/2009 of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) Nº 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) Nº 726/2004 of the European Parliament and of the Council. Off. J. Eur. Union L152:11–22.

European Union. 2010. Regulation (EU) Nº 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union L15:1–72.

Everett, D.W., and M.A.E. Auty. 2008. Cheese structure and current methods of analysis. Int. Dairy J. 18:759–773. https://doi.org/10.1016/j.idairyj.2008.03.012.

FDA. 2018. Food and Drug Administration Center for Veterinary medicine. MI-18-09: Tolerance and/or target testing levels of animal drug residues in milk. Accessed July 24, 2018. https://www.fda.gov/downloads/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Milk/UCM596680.pdf

FAOSTAT. 2018. Food and Agriculture Organization of the United Nations. Food and agriculture data. Accessed July 26, 2018. http://www.fao.org/faostat/en/#data/QL/visualize.

FAO/WHO. 2004. Evaluation of Certain Food Additives and Contaminants: Sixty-Second Report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, Switzerland.

Fenelon, M.A., P. O'Connor, and T.P. Guinee. 2000. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. J. Dairy Sci. 83:2173–2183. https://doi.org/10.3168/jds.S0022-0302(00)75100-9.

Ferrandini, E., M.B. López, M. Castillo, and J. Laencina. 2011. Influence of an artisanal lamb rennet paste on proteolysis and textural properties of Murcia al Vino cheese. Food Chem. 124:583–588. https://doi.org/10.1016/j.foodchem.2010.06.079.

Folkertsma, B., and P.F. Fox. 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.

Freitas, C., and F.X. Malcata. 2000. Microbiology and biochemistry of cheeses with Appélation d'Origine Protegée and manufactured in the Iberian Peninsula from ovine and caprine milks. J. Dairy Sci. 83:584–602. https://doi.org/10.3168/jds.S0022-0302(00)74918-6.

Fresno, M., and S. Álvarez. 2012. Chemical, textural and sensorial changes during the ripening of Majorero goat cheese. Int. J. Dairy Technol. 65:393–400. https://doi.org/10.1111/j.1471-0307.2012.00842.x.

Gajda, A., E. Nowacka-Kozak, M. Gbylik-Sikorska, and A. Posyniak. 2018. Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 35:66–76. https://doi.org/10.1080/19440049.2017.1397773.

Giguère, S. 2013. Antimicrobial Drug Action and Interaction. Pages 1-10 in Antimicrobial therapy in Veterinary Medicine (5th ed.). S. Giguère, J.F. Prescott, and P.M. Dowling, ed. Wiley-Blackwell. Ames, Iowa. https://doi.org/10.1002/9781118675014.ch1.

Giraldo, J., R.L. Althaus, M.C. Beltrán, and M.P. Molina. 2017. Antimicrobial activity in cheese whey as an indicator of antibiotic drug transfer from goat milk. Int. Dairy J. 69:40–44. http://doi.org/10.1016/j.idairyj.2017.02.003.

Grunwald, L., and M. Petz. 2003. Food processing effects on residues: penicillins in milk and yoghurt. Anal. Chim. Acta 483:73–79. https://doi.org/10.1016/S0003-2670(02)01405-8.

Haenlein, G.F.W. 2004. Goat milk in human nutrition. Small Rumin. Res. 51:155–163. https://dx.doi.org/10.1016/j.smallrumres.2003.08.010.

ISO 4121. 2003. International Organization for Standardization Publications. Sensory analysis Methodology. Guidelines for the use of quantitative response scales. ISO 4121: 2003. Available at: www.iso.org

ISO/IDF. 2003. Milk and milk products. Guidelines for a standardized description of microbial inhibitor tests. IDF Standard Nº 183:2003. Brussels, Belgium.

Jeong, S.H., Y.K. Song, and J.H. Cho. 2009. Risk assessment of ciprofloxacin, flavomycin, olaquindox and colistin sulfate based on microbiological impact on human gut biota. Regul. Toxicol. Pharmacol. 53:209–216. https://doi.org/10.1016/j.yrtph.2009.01.004.

Juan, B., A. Zamora, J.M. Quevedo, and A.J. Trujillo. 2016. Proteolysis of cheese made from goat milk treated by ultra high pressure homogenisation. LWT - Food Sci. Technol. 69:17–23. https://doi.org/10.1016/j.lwt.2015.12.013.

Katla, A.K., H. Kruse, G. Johnsen, and H. Herikstad. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int. J. Food Microbiol. 67:147–152. https://doi.org/10.1016/S0168-1605(00)00522-5.

Lees, P., and P.L. Toutain. 2011. Pharmacokinetics, Distribution, Bioavailability, and Relationship to Antibiotic Residues. Pages 61-109 in Chemical Analysis of

Antibiotic Residues in Food. J. Wang, J.D. MacNeil, and J.F. Kay, ed. Wiley & Sons, Hoboken, NJ. https://doi.org/10.1002/9781118067208.ch2.

Lucas, A., E. Rock, J.F. Chamba, I. Verdier-Metz, P. Brachet, and J.B. Coulon. 2006. Respective effects of milk composition and the cheese-making process on cheese compositional variability in components of nutritional interest. Lait 86:21–41. https://doi.org/10.1051/lait:2005042.

McSweeney, P.L.H., and M.J. Sousa. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Lait 80:293–324. https://doi.org/10.1051/lait:2000127.

Merin, U., G. Fleminger, J. Komanovsky, N. Silanikove, S. Bernstein, and G. Leitner. 2008. Subclinical udder infection with Streptococcus dysgalactiae impairs milk coagulation properties: The emerging role of proteose peptones. Dairy Sci. Technol. 88:407–419. https://doi.org/10.1051/dst:2008022.

Nuñez, M., C. García-Aser, M.A. Rodríguez-Martin, M. Medina, and P. Gaya. 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.

Oliver, S.P., S.E. Murinda, and B.M. Jayarao. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. Foodborne Pathog. Dis. 8:337–355. https://doi.org/10.1089/fpd.2010.0730.

Park, Y. 2017. Goat milk – Chemistry and nutrition. Pages 42-83 in Handbook of Milk of Non-Bovine Mammals (2nd ed.). Y.W. Park, G.F.W. Haenlein, and W.L.

Wendorff, ed. Wiley-Blackwell, Oxford, UK. https://doi.org/10.1002/9781119110316.ch2.2.

Reeves, P.T. 2011. Antibiotics: Groups and Properties. Pages 1-60 in Chemical Analysis of Antibiotic Residues in Food. J. Wang, J.D. MacNeil, and J.F. Kay, ed. Wiley & Sons, Hoboken, NJ. https://doi.org/10.1002/9781118067208.ch1.

Revilla, I., J.M. Rodríguez-Nogales, and A.M. Vivar-Quintana. 2007. Proteolysis and texture of hard ewes' milk cheese during ripening as affected by somatic cell counts. J. Dairy Res. 74:127–136. https://doi.org/10.1017/S0022029906002342.

Roca, M. I. 2008. Termoestabilidad de sustancias antimicrobianas en la leche. PhD Thesis. Universitat Politècnica de València, Valencia (Spain). https://riunet.upv.es/handle/10251/3022

Roca, M., L. Villegas, M.L. Kortabitarte, R.L. Althaus, and M.P. Molina. 2011. Effect of heat treatments on stability of β-lactams in milk. J. Dairy Sci. 94:1155–1164. https://doi.org/10.3168/jds.2010-3599.

Salvador, A., M. Igual, C. Contreras, N. Martínez-Navarrete, and M.M. Camacho. 2014. Effect of the inclusion of citrus pulp in the diet of goats on cheeses characteristics. Small Rumin. Res. 121:361–367. https://doi.org/10.1016/j.smallrumres.2014.06.012.

Shappell, N.W., W.L. Shelver, S.J. Lupton, W. Fanaselle, J.M. Van Doren, and H. Hakk. 2017. Distribution of animal drugs among curd, whey, and milk protein fractions in spiked skim milk and whey. J. Agric. Food Chem. 65:938–949. https://doi.org/10.1021/acs.jafc.6b04258.

Stolker, A.A.M., and U.A.T. Brinkman. 2005. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals-a review. J. Chromatogr. A 1067:15–53. https://doi.org/10.1016/j.chroma.2005.02.037.

Tejada, L., R. Gómez, and J. Fernández-Salguero. 2007. Sensory characteristics of ewe milk cheese made with three types of coagulant: calf rennet, powdered vegetable coagulant and crude aqueous extract from Cynara cardunculus. J. Food Qual. 30:91–103. https://doi.org/10.1111/j.1745-4557.2007.00108.x.

Thierry, A., Y.F. Collins, M.C. Abeijón Mukdsi, M.G. Wilkinson, and H.E. Spinnler. 2017. Lipolysis and Metabolism of Fatty Acids in Cheese. Pages 423-444 in Cheese: Chemistry, Physics and Microbiology (4th ed.). P.L.H. McSweeney, P.F. Fox, C.P. D., and E.D. W., ed. Academic Press, London, UK. https://doi.org/10.1016/B978-0-12-417012-4.00017-X.

WHO, FAO, and OIE. 2018. World Health Organization, Food and Agriculture Organization of the United Nations and World Organisation for Animal Health. Monitoring Global Progress on Addressing Antimicrobial Resistance (AMR). Analysis report of the second round of results of AMR country self-assessment survey. Geneva, Switzerland. Accessed October 24, 2018. http://apps.who.int/iris/bitstream/handle/10665/273128/9789241514422-eng.pdf

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

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

[1]EU-MRL: European Union Maximum Residue Limit in raw milk (Commission Regulation (EU) No. 37/2010); [2]tr = traces (LOD < result < LOQ); [3]nd = not detected (result < LOD); LOD = limit of detection; LOQ = limit of quantification; [4]Sum of enrofloxacin and ciprofloxacin.

Table 2. Effect of antibiotic concentration and ripening time on the physicochemical

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

characteristics of Tronchón cheese made from goat's milk.

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

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

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

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

Table 4. Effect of antibiotic concentration and ripening time on the color coordinates

Antibiotic	Parameters	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	L*	87.5	88.0	0.26	90.4^{b}	86.9^{a}	86.0^{a}	0.31
	a*	-1.21	-1.30	0.058	-0.34^{c}	-1.55^{b}	-1.88^{a}	0.071
	b*	11.5	11.5	0.21	10.7^{a}	12.2^{b}	11.7^{b}	0.26
Benzylpenicillin	L*	89.6	89.5	0.16	91.0^{c}	89.3^{b}	88.3^{a}	0.19
	a*	-1.43^{b}	-1.54^{a}	0.030	-0.43^{c}	-1.74^{b}	-2.29^{a}	0.037
	b*	10.1	10.2	0.18	9.3^{a}	10.7^{b}	10.4^{b}	0.22
Cloxacillin	L*	87.5	88.5	0.34	90.6^{b}	87.1^{a}	86.4^{a}	0.41
	a*	-1.21^{b}	-1.34^{a}	0.033	-0.30^{c}	-1.59^{b}	-1.94^{a}	0.041
	b*	11.5	11.5	0.21	10.6^{a}	12.2^{b}	11.7^{b}	0.254
Erythromycin	L*	89.8	89.2	0.19	90.2^{b}	89.7^{b}	88.6^{a}	0.23
	a*	-1.16^{b}	-1.23^{a}	0.018	-0.29^{c}	-1.61^{b}	-1.69^{a}	0.022
	b*	10.60	10.27	0.117	9.74^{a}	10.52^{b}	11.1^{c}	0.14
Ciprofloxacin	L*	89.4^{b}	88.3^{a}	0.16	90.1^{c}	88.7^{b}	87.8^{a}	0.19
	a*	-1.51	-1.47	0.030	-0.43^{c}	-1.81^{b}	-2.23^{a}	0.037
	b*	12.4	12.7	0.15	10.9^{a}	13.1^{b}	13.7^{c}	0.18
Enrofloxacin	L*	88.3	87.7	0.25	90.0^{b}	87.3^{a}	86.5^{a}	0.30
	a*	-1.27	-1.26	0.028	-0.20^{c}	-1.61^{b}	-1.97^{a}	0.035
	b*	12.1	12.1	0.19	10.7^{a}	12.4^{b}	13.0^{c}	0.23
Oxytetracycline	L*	88.3	88.9	0.26	90.8^{b}	88.0^{a}	87.1^{a}	0.31
	a*	-1.85	-1.77	0.042	-0.43^{c}	-2.17^{b}	-2.81^{a}	0.052
	b*	12.5^{b}	12.0^{a}	0.13	10.0^{a}	12.9^{b}	13.9^{b}	0.16

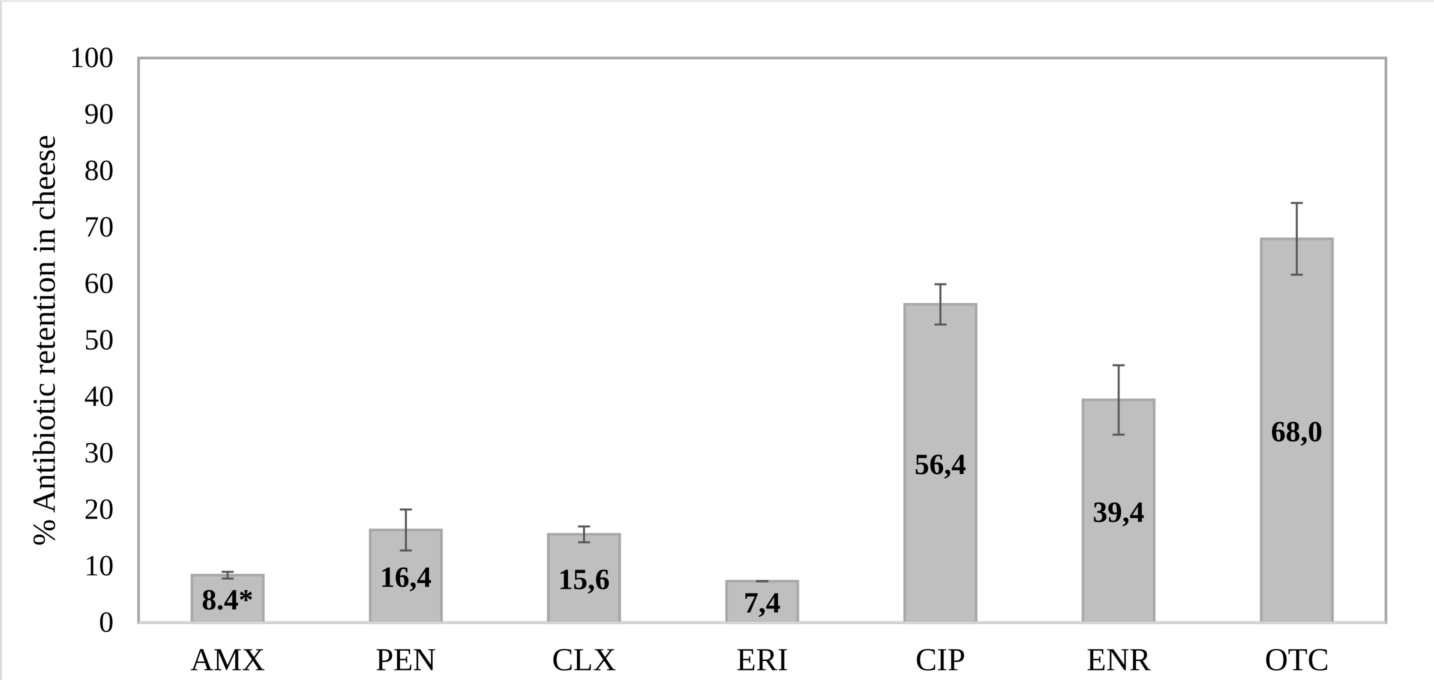
(CIE L* a * b*) of Tronchón cheese made from goat's milk.

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

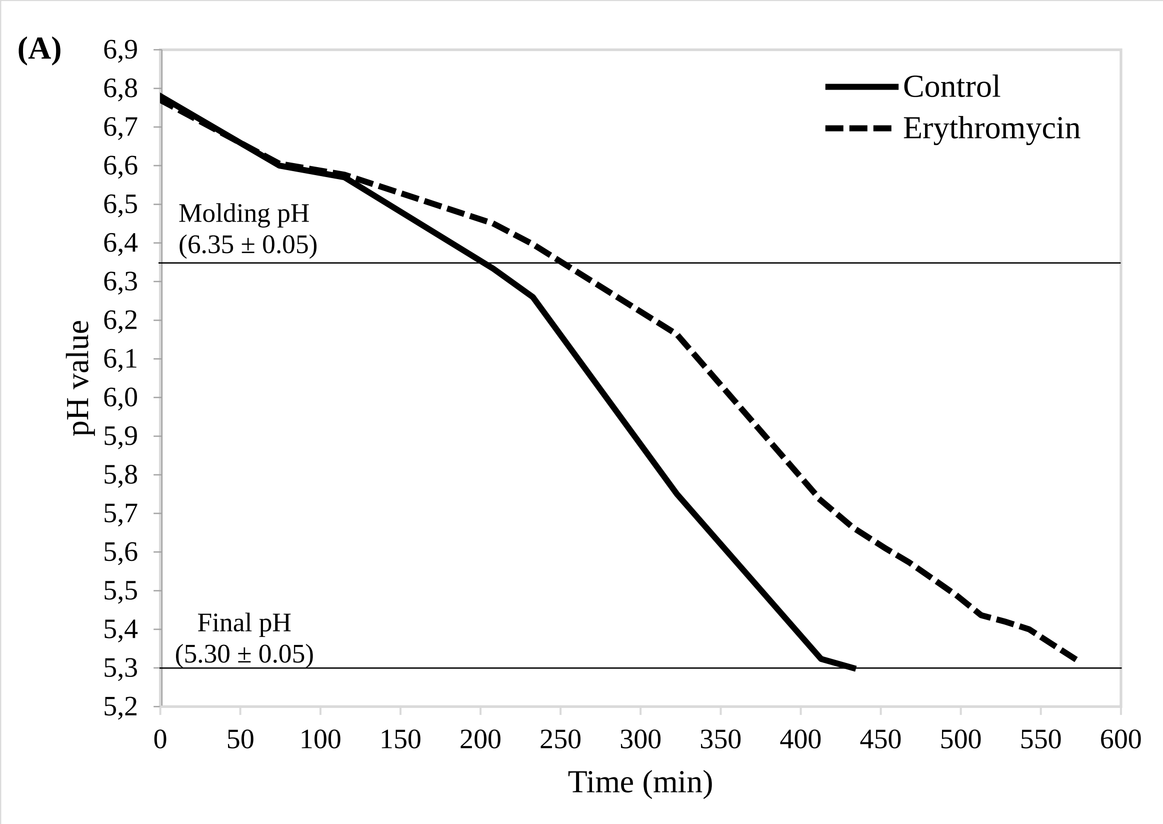
Table 5. Effect of antibiotic concentration and ripening time on the texture profile of Tronchón cheese made from goat's milk.

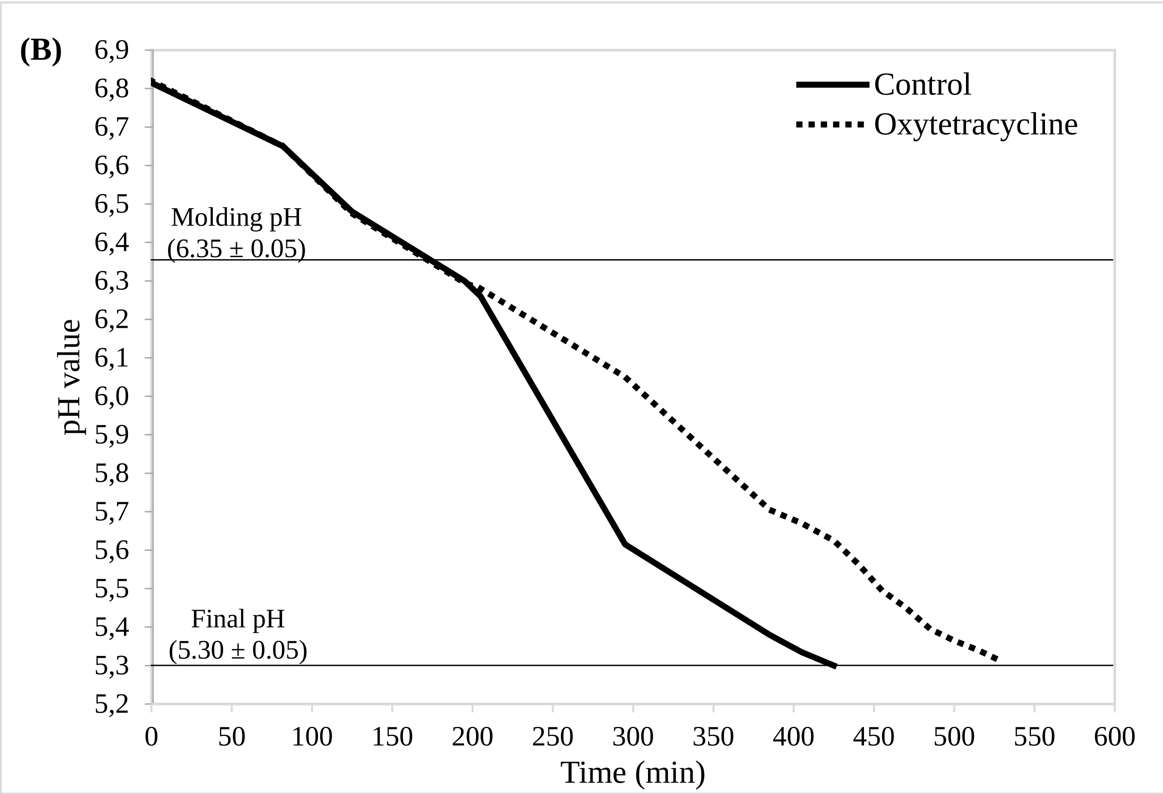
Antibiotic	Parameters	Antibiotic concentration[1]			Ripening time (days)			
		AFC (n = 12)	SMC (n = 12)	SEM[2]	0 (n = 8)	30 (n = 8)	60 (n = 8)	SEM
Amoxicillin	Hardness (N)	20.8	20.9	0.99	28.0^{b}	18.6^{a}	15.9^{a}	1.22
	Adhesiveness (N*s)	-0.93	-0.96	0.047	-0.61^{b}	-1.09^{a}	-1.13^{a}	0.057
	Springiness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

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



Quintanilla. Figure 1.





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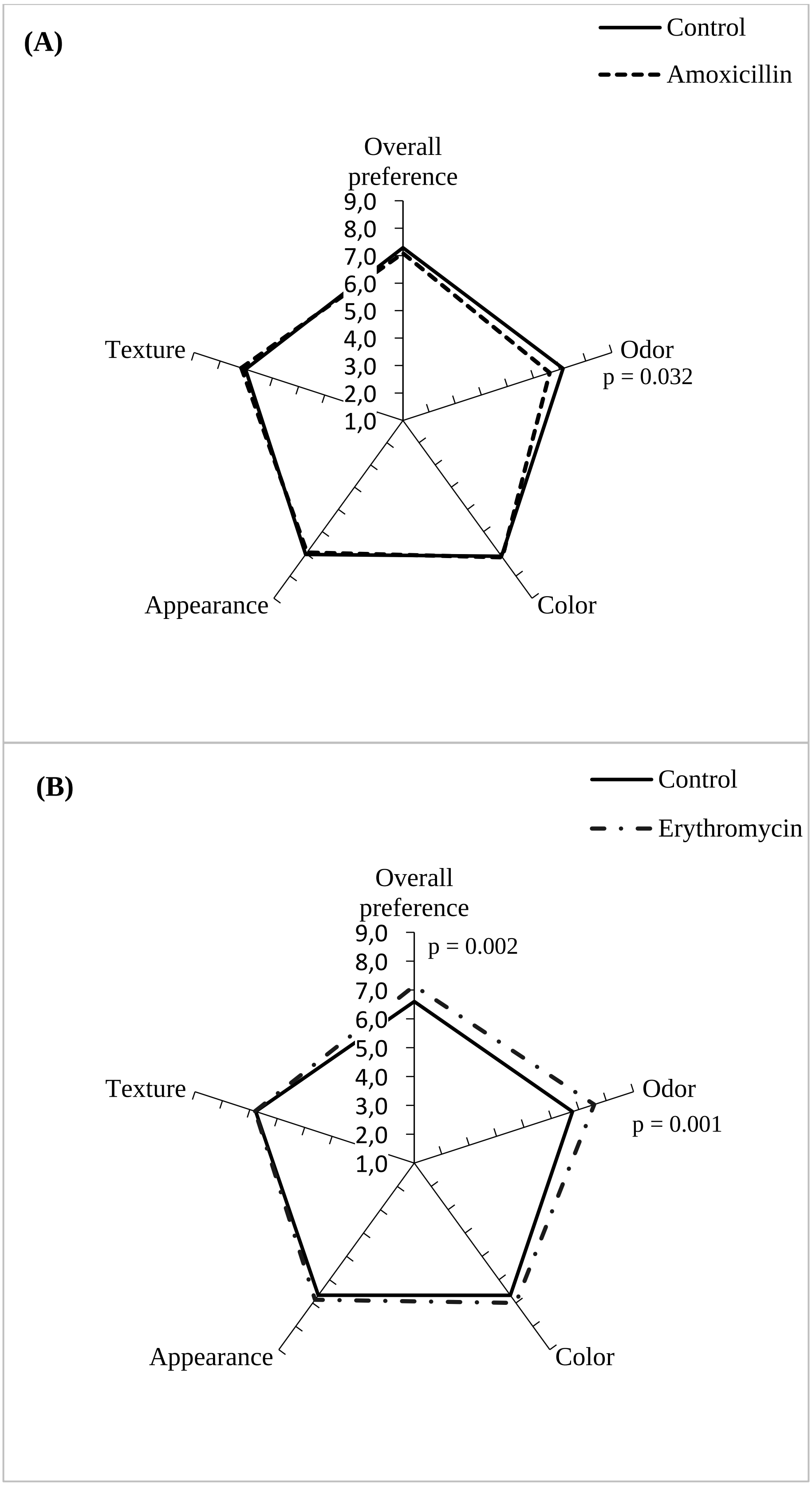

Quintanilla. Figure 3.

Figure captions

Figure 1. Retention rate (%) of antibiotic Tronchón cheese before ripening made from goat's milk spiked with antibiotics at EU-MRL concentration.

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

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

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