Quintanilla-Vázquez, PG.; Beltrán Martínez, MC.; Escriche Roberto, MI.; Molina Pons, MP. (2021). Enrofloxacin Treatment on dairy goats: Presence of antibiotic in milk and impact of residue on technological process and characteristics of mature cheese. Food Control. 123:1-9. https://doi.org/10.1016/j.foodcont.2020.107762

Enrofloxacin Treatment on dairy goats: Presence of antibiotic in milk and impact of residue on technological process and characteristics of mature cheese

Quintanilla, P.[a]; Beltrán, M. C.[a]; Molina, M. P.[a]; Escriche, I.[b*]

[a]*Institute for Animal Science and Technology. Universitat Politècnica de València. Camino de Vera, s/n. 46022, Valencia, Spain (UPV)*

[b]*Institute of Food Engineering for Development. Food Technology Department. Universitat Politècnica de València. Camino de Vera, s/n. 46022, Valencia, Spain (UPV)*

* Corresponding author: Isabel Escriche: iescrich@tal.upv.es

Abstract

Lately quinolones, particularly enrofloxacin, have been incorporated as a veterinary treatment of small ruminants, like goats, whose milk is highly appreciated for the manufacture of traditional cheeses. This study aims to evaluate the influence of the presence of enrofloxacin in milk (from goats previously treated with this antibiotic), on the characteristics (chemical composition; colour, texture, volatile profile and sensory evaluation) of mature cheese at 0, 30 and 60 days. Three batches of cheeses were made from milk obtained at three different times with respect to the animals' antibiotic administration (24 h before treatment, 24 h after treatment, and after the withdrawal period). The manufacture process of cheese is not affected by enrofloxacin in any way; therefore, it will remain unnoticed in the production line. A transfer of enrofloxacin, and its metabolite ciprofloxacin, to the cheese produced with milk obtained 24 h after treatment was observed. The presence of antibiotic residues does not produce significant changes in any of its compositional, texture and colour characteristics, when compared to the cheeses made before veterinary treatment with antibiotic-free milk, with the only

exception of some compounds of the volatile fraction. Enrofloxacin and its metabolite show high stability during the cheese maturation (51% of both remain after 60 days of ripening). In general, the presence of this antibiotic has lesser effects on all the characteristics of the cheese compared to those modifications produced during the maturation time. Sensory measures of odour, colour, appearance and texture attributes, as well as the global preference, are also not affected by this antibiotic, which would imply a risk for the population since its presence would go undetected.

Keywords: Goat milk, enrofloxacin, ciprofloxacin, mature-cheese.

1. Introduction

The human exposure to antibiotics is being considered as a public health problem. The exposure of low but constant amounts of these substances is causing allergies, intestinal microbiota alterations and emergence of antibiotic resistance (EFSA, 2016; WHO, 2018).

The use of antimicrobial agent, especially antibiotics, in livestock is a necessary and widespread practice for the treatment of infectious pathologies (EMA, 2019). However, the irresponsible use of drugs in dairy farms is mainly due to the presence of antibiotic residues in milk (EMA, 2014) and also to their presence in the environment (Economou & Gousia, 2015).

Among the antibiotic substances, quinolones are part of an expanding group of synthetic antibiotics, as they are very effective in veterinary treatments due to their broad spectrum of activity. In particular in dairy goats, fluoroquinolones, especially enrofloxacin, are provided for the treatment of gastrointestinal, respiratory and mammary diseases (Menzies & Ramanoon, 2001; Papich, 2016).

In order to ensure public health and to ensure food safety, the European Union has established Maximum Residue Limits (MRLs) for different pharmacologically active

substances in food of animal origin (European Commission, 2010). This regulation specifies a MRL of 100 µg/kg for enrofloxacin, in milk of any species. However, referring to dairy products, at present, no limits have been set for antibiotics. However, the WHO Technical Report (FAO/WHO, 2004) recommended including MRL for fat-soluble veterinary substances such as quinolones, in dairy products with a high fat content (butter or cheese), since a greater concentration of these substances could be retained.

The lack of good livestock practices may result in the presence of residues of these substances in milk and its transmission to the production chain, and therefore to the consumer (Zhang et al., 2014; Zheng et al., 2013). Researches on the transfer of quinolones from milk to dairy derivates have been very limited. Thus, Beltrán, Morari-Pirlog, Quintanilla, Escriche, & Molina (2018) observed that between 75 and 99% of enrofloxacin initially present in goat's milk remained in yogurt throughout its shelf life. Also, in a study of different antibiotics spiked in goat milk destined for fresh cheese, the result showed that a high concentration of quinolones was retained in the curd, observing retention in the cheese of enrofloxacin (51.1%) and ciprofloxacin (57.3%) after 24 hours of drainage (Quintanilla, Doménech, Escriche, Beltrán, & Molina, 2019). In another research paper on mature cheeses, Quintanilla, Beltrán, Molina, Escriche, & Molina (2019) found that at the beginning of cheese maturation there was retention of 40% enrofloxacin and 56% ciprofloxacin with respect to the presence of these antibiotics in the milk used for its manufacture. Furthermore, the same authors also observed that both antibiotics were highly stable during maturation time.

In addition to the adverse effects on human health, the presence of antibiotic residues in milk intended for the production of dairy products, although these residues are below the established levels of MRL in milk, they can cause negative technological effects. Antibiotics can act as inhibitory substances that slow down or modify the development

of biochemical processes during the production and maturation of dairy products and therefore, could alter their final characteristics (Cabizza et al., 2017; Quintanilla, Beltrán, et al., 2019). Several studies have reported results in this line, although these were focused only on laboratory experiments, adding the active substance directly to goat or sheep milk and without considering the metabolization of these substances on the animal (Cabizza et al., 2018; Quintanilla, Doménech, et al., 2019).

Goat milk production is less than that of cow, however, this type of milk is highly appreciated for manufacturing traditional cheeses, especially in Mediterranean European countries (FAOSTAT, 2020). For the veterinary treatment of these animal species there is a limited availability of registered antibiotics, especially for goats (Clark, 2013). In recent years, quinolones such as enrofloxacin have been incorporated since they are not allowed for human treatments (López-Cadenas et al., 2013).

Therefore, the objective of this study was to quantify the enrofloxacin residues in raw milk from goats treated with this antibiotic, to evaluate their influence on the cheese-making process and the characteristics of these cheeses (chemical composition, colour, texture, volatile profile and sensory) during ripening.

2. Materials and Methods

2.1. Experimental procedure

This study was carried out using the experimental herd of goats (Murciano-Granadina) of Institute of Animal Science and Technology at Universitat Politècnica de València (UPV, Valencia, Spain). The Ethics Committee of UPV approved the animal management protocols. Thirty healthy goats were used, each weighing 45-55 kg, were randomly allocated into two groups of 15 animals each, being in mid-lactation and not having

98 received any antimicrobial substances either before the experimental period. Machine
99 milking was performed once a day in the morning.

100 The veterinary drug used was Baytril® (Bayer Hispania, S.L, Barcelona, Spain), 50
101 mg/mL of enrofloxacin, dose: 1 mL/10 kg body weight on three consecutive days and the
102 withdrawal period considered was four days after the last drug administration, as
103 stipulated the manufacturer's specification sheet. The antibiotic was administrated after
104 morning milking by the intramuscular route.

105 For each animal group, three batches of ripened cheese were made at different times
106 (hereafter "cheese-making timeframe"): one day before the antibiotic treatment was
107 applied (pre-treatment cheeses: PT-cheeses, which were then used as reference), 24 hours
108 thereafter of the last dose of antibiotic (after treatment cheeses: AT-cheeses), and after
109 the recommended safety period (after withdrawal period cheeses: AW-cheeses). In all
110 cases, bulk milk samples (100 mL) were analysed prior to the cheese production.

111 *2.2. Cheese manufacture*

112 The cheese was made at the UPV pilot plant, following the artisanal making-process for
113 mature *Tronchón* cheese. Cheeses were manufactured according to the mature *Tronchón*
114 cheese making-process as described by Quintanilla, Beltrán, et al. (2019) and ripened for
115 60 days. The kinetic acidification of the milk curd was checked periodically during the
116 cheesemaking using a pH-meter (model Basic 20, Crison, Barcelona, Spain) with a
117 penetration probe 5232 (Crison). Eight cheeses were obtained from each vat, which were
118 sampled in duplicate at 0, 30 and 60 days of ripening for further analysis (from now
119 onwards it will be titled as "ripening time").

120 *2.3. Milk and cheese analysis*

121 The gross composition of the milk samples (fat, protein and total solids) was analysed by
122 MilkoScan FT 6000 (Foss, Hillerød, Denmark). Also, somatic cell count (SCC); and the
123 total bacterial count (TBC) by Fossomatic 5000 (Foss), and Bactoscan FC (Foss)
124 respectively.

125 The milk pH value was measured by a conventional pH-meter (Crison). Cheese samples
126 were analysed during ripening stages at 0, 30 and 60 days by assessing quality variables
127 such as gross composition, pH, colour (CIELab coordinates and ΔE) and texture
128 parameters, as described by Quintanilla, Beltrán, et al. (2019).

129 The Free Fatty Acids (FFA) concentration (meq/100 g of fat) and the Free Amino Acids
130 (FAA) content (mg of leucine/g of cheese) were determined in duplicate according to the
131 methodologies of Nuñez, García-Aser, Rodríguez-Martin, Medina, & Gaya (1986) and
132 Folkertsma & Fox (1992), respectively. FFA and FAA were used as indicators of lipolytic
133 and proteolytic activities in the cheeses during ripening, principal biochemical pathways
134 involved during maturation (McSweeney & Sousa, 2000).

135 *2.4. Antibiotic residue quantification*

136 Enrofloxacin and its metabolite ciprofloxacin were quantified (in milk and ripened
137 cheeses at 0, 30 and 60 days) using liquid chromatography tandem-mass spectrometry
138 (LC/MS-MS) method validated previously described by Quintanilla, Beltrán, et al.
139 (2019).

140 *2.5. Analysis of volatile compounds in cheese*

141 Volatile compounds were extracted by purge and trap procedure (45 °C for 30 min) with
142 nitrogen (120 mL/min) and a glass tube (Tenax TA, 20–35 mesh), desorbed (TurboMatrix
143 TD, Perkin ElmerTM, CT, USA) (220 °C, 10 min, at 10 mL/min helium flow) and
144 cryofocused (cold trap at −30 °C). After, they were transferred onto a GC-MS (Finnigan

145 TRACETM MS, TermoQuest, TX, USA) with a DB-WAX capillary column (SGE,
146 Australia) (60 m length, 0.32 mm i.d., 1.0 μm film thickness). In each repetition 10 g of
147 cheese (on a dry basis) was used and miliQ water was added to a total weight of 15 g.
148 This mixture was stirred 30 sec in Vortex and 2 min in ultraturrax and ethyl propionate
149 was added as internal standard (15 μL of 100 ppm) and again this mixture was stirred 30
150 seconds more in Vortex. The identification of the compounds was based on the works of
151 Hayaloglu, Yasar, Tolu, & Sahingil, 2013; Aminifar, Hamedi, Emam-Djomeh, &
152 Mehdinia, 2014; Tanleque-Alberto, Juan-Borrás, & Escriche, 2019. The variables used in
153 the statistical analysis corresponded to semi quantified compounds, (μg/100 g of cheese)
154 obtained with the amount of internal standard, the relative area between the peak areas of
155 each compound and the peak area of the internal standard, assuming a response factor
156 equal to one. Each sample was analysed twice.

157 *2.6. Sensory analysis*

158 An acceptance test (using a 9-point hedonic scale, from 1=dislike extremely to 9=like
159 extremely), was carried out by 100 un-trained consumers in order to evaluate the odour,
160 colour, appearance and texture attributes, as well as the global preference of the cheeses
161 ripened at 60 days (ISO 4121, 2003; ISO 5492, 2008). Representative wedges (0.5 cm
162 thick) of the PT-cheeses, AT-cheeses and AW-cheeses were prepared at room
163 temperature, coded with random three-digit numbers, and presented individually to the
164 tasters. Attributes related to the tasting of the product were not included, due to the
165 presence of the antibiotic in some samples. The evaluations were conducted in individual
166 booths in a homologated sensory room (ISO 8589, 2007).

167 *2.7. Statistical analysis*

168 A multifactor analysis of variance (ANOVA) (using Statgraphics Centurion XVI.II,
169 Statpoint Technologies, Inc. The Plains, VA, USA) was carried out to study the influence

of the "cheesemaking timeframe" (PT: pre-treatment, AT: 24 hours after treatment, and AW: after the withdrawal period) and the "ripening time" (0, 30 and 60 days) on the gross composition, pH, free fatty acids, free amino acids, colour, texture, and volatile compounds. The method used for multiple comparisons was the LSD test (Least Significant Difference) with a significance level α=0.05. Additionally, a Principal Component Analysis (PCA) was applied by means of the software Unscrambler X.10.5 CAMO (Camo ASA, Oslo, Norway) to evaluate the relationship between the volatile compounds and the different "cheesemaking timeframe" and "ripening time". The data were centred (mean) and scaled (standard deviation) before the PCA cross validation analysis.

3. Result and discussion

3.1. Gross composition and hygienic characteristics of milk

The first step was to check the gross compositional and the hygienic characteristics of the three batches of milk used to elaborate "cheesemaking timeframe" (PT, AT and AW). The ANOVA results (data not shown) demonstrated that no significant differences were observed in any case. These gross compositional average values (expressed as g/100 g of milk) were the following: total solids content = 15.06±0.46; fat content = 5.66±0.37 and protein = 3.95±0.17, and the mean pH was 6.70±0.03. Regarding the hygienic characteristics of the different batches of milk used, the somatic cell count was 909,333±221,131 cells/mL and the total bacterial count was 18,167±5,250 cfu/mL.

The mean gross composition and hygienic quality of goat milk were among the usual values for this type of milk and Murciano-granadina breed (fat = 5.94 g/100 g and protein = 4.03 g/100 g) (Beltrán et al., 2018). The three batches of raw milk used for cheese production in the present work showed a normal somatic cell count in healthy dairy goats (Raynal-Ljutovac, Pirisi, de Crémoux, & Gonzalo, 2007). Furthermore, the bacterial

195 count was lower than the limit set by the legislative framework (500,000 cfu/mL) on the
196 hygiene of foodstuffs for human consumption (European Commission, 2004).

197 *3.2. Enrofloxacin residues in milk and cheese*

198 Table 1 shows the residual concentration of enrofloxacin and its metabolite
199 (ciprofloxacin) found in milk after treatment (AT) and in the respective cheeses at
200 different days of maturation (0, 30, 60). This table also shows the sum of both compounds,
201 as established by the legislation regarding the quantification of total enrofloxacin
202 (Regulation (EU) Nº 37/2010: European Commission, 2010). This is mainly due to the
203 metabolisation of enrofloxacin to ciprofloxacin and therefore both compounds can be
204 simultaneously present in the samples (Dorival-García, Junza, Zafra-Gómez, Barrón, &
205 Navalón, 2016; Saha & Paul, 2013).

206 In milk pre-treatment (PT), none of these substances were detected. Regarding AT-milk,
207 a mean concentration of enrofloxacin (17.3±0.3 µg/kg) and ciprofloxacin (60.3±3.1
208 µg/kg) below the MRL (100 µg/kg) was observed. However, in the cheeses at 0-day of
209 ripening, the mean level of both compounds was tripled (231.7±32.9 µg/kg) with that in
210 the starting milk (77.6±2.9 µg/kg). This is due mainly to the concentration effect that takes
211 place during the manufacture of the cheese, where, apart from the proper components
212 present in the starting milk (such as fat and proteins), there are also concentrations of
213 other substances such as residues of antibiotics (Quintanilla, Beltrán, Peris, Rodríguez, &
214 Molina, 2018).

215 In addition, Table 1 shows the mean percentage of the retention of enrofloxacin,
216 ciprofloxacin and the sum of both compounds in the cheeses at 30 and 60 days of ripening,
217 calculated with respect to the value obtained at day 0. The lipophilic nature of these
218 compounds fundamentally explains the ability of quinolones to be retained in the fat
219 fraction of milk and therefore of cheese (Giraldo, Althaus, Beltrán, & Molina, 2017;

Quintanilla, Doménech, et al., 2019). The antibiotic concentration in the cheeses decreases during ripening. However, at the end of maturation, the residual antibiotic (enrofloxacin+ciprofloxacin) still remains up to 51% with respect to the initial concentration in the cheese, being from 231.7 μg/kg at 0-day to 117.5 μg/kg at 60 days of ripening.

In an in vitro study, the "concentration effect" and persistence of a residual level of this antibiotic during the ripening of this type of cheese was observed (Quintanilla, Beltrán, et al., 2019). There, enrofloxacin and ciprofloxacin was added (at the MRL concentration) to goat's milk and subsequently cheeses were produced and ripened at 60 days. The same authors reported that in cheese at 0-day of maturation enrofloxacin had been concentrated 2.87 times and ciprofloxacin 3.63 times, with respect to the level present in milk (MRL). They also observed the stability of both compounds (51% and 69%) after 60 days of maturation.

In the case of after withdrawal (AW) milk and cheeses, enrofloxacin and ciprofloxacin were not detectable. These results demonstrate that the withdrawal established for this antibiotic is adequate in reducing or eliminating the probability of the consumer being exposed since complying with its withdrawal period will avoid the presence of this quinolone in dairy derivatives.

3.3. Cheese making process, composition, lipolytic and proteolytic activity of cheeses

During the cheesemaking process, the influence of the antibiotic presence in the acidification stage of the milk and curd, was evaluated by measuring the pH values. Non-significant differences (data not shown) were observed between the three elaborations of "cheesemaking timeframe" (PT, AT and AW), showing that the presence of the antibiotic could go unnoticed. This same pH trend was observed during the making of yogurts from

goat milk to which different concentrations of enrofloxacin were spiked (in vitro). In that case, the decrease in pH during manufacturing was not affected by the presence of the antibiotic, even with a starting milk that contained 1.5 times the MRL. This shows that still at these concentrations, the antibiotic was not able to significantly inhibit the growth of the starter cultures (Beltrán et al., 2018).

Regarding the characteristic of cheeses, Table 2 shows the values (average, standard deviation and ANOVA multifactor results) of pH, moisture, fat, protein, NaCl, free amino acids (FAA) and free fatty acids (FFA) considering the "cheesemaking timeframe", and the "ripening time" factors. No significant differences were observed due to "cheese-making timeframe" factor (PT, AT and AW) for the analysed parameters, with the only exception of fat and protein. Higher fat and lower protein contents were obtained in PT-cheeses in comparison to cheeses after treatment (AT and AW cheeses), despite the milk composition having not been affected by the antibiotic treatment ($p>0.05$). These differences, although statistically significant, are not very important and could be attributed to the artisanal making-process of this type of cheese. According to the ripening time, most of the analysed parameters varied significantly over time, similar to what was observed in goat cheese by other authors (Ferrandini, López, Castillo, & Laencina, 2011; Salvador, Igual, Contreras, Martínez-Navarrete, & Camacho, 2014). In the present study, during the ripening period, a significant increase in FFA (average from 2.0 to 3.1 meq/100 g of fat) and especially in FAA (average from 0.7 to 2.9 mg of leucine/g of cheese) is noticeable. This last parameter being the most affected by maturation, as demonstrated by its higher F-ratio value. FFA and FAA contents are indicators of the degree of lipolysis and proteolysis of cheese ripening from triglycerides and via formation of peptides, respectively. The biochemical processes during cheese maturation, are very important in the development of characteristic flavour of each type of cheese (Boutoial et al., 2013).

269 The levels of these parameters were in the order reported by other authors at 60 days of
270 matured goat cheese: FFA content of 3.2 meq/100 g of fat (Quintanilla, Beltrán, et al.,
271 2019) and FAA content of 2.9 mg of leucine/g of cheese, at 60 days of ripening (Juan,
272 Zamora, Quevedo, & Trujillo, 2016).
273 Regarding the ANOVA interaction, no significant effect between the two factors
274 considered (TxR) was found in any case.
275 *3.4. Colour and texture characteristics*
276 The evolution of colour and texture parameters in cheeses considering the "cheese-
277 making timeframe" (PT, AT and AW) and the "ripening time" factors, as well as the
278 ANOVA multifactor results are shown in Table 3. Luminosity (L*), was the only colour
279 parameter that showed significant differences with respect to "cheese-making timeframe"
280 factor, being lower for AW-cheeses than to PT-cheeses and AT-cheeses. However,
281 considering the colour differences (calculated as ΔE with respect to the control sample,
282 PT-cheeses), for AT-cheeses and AW-cheeses theses value were 1.2 and 2.2. Taking into
283 account that the human eye only appreciates differences in colour when $\Delta E > 3$, the colour
284 variation observed in this work between cheeses at a different "timeframe" would go
285 unnoticed by the consumer (Salvador et al., 2014). Cohesiveness was the only texture
286 parameter that showed a significant difference considering the "cheese-making
287 timeframe" factor. However, the range of variability of this parameter, between
288 treatments, was not relevant (from 0.4 to 0.5).

289 Regarding the effect of the ripening time, a significant reduction in luminosity (L*) and
290 a* coordinate, and an increment in the b* were observed. The evolution of colour
291 indicates that the cheeses, with increasing maturation, become less lightness and more
292 yellowness. The cheeses evaluated showed relative colour parameter trends as those
293 reported by Salvador et al. (2014) in goat cheese under similar conditions. With respect

294 to “ripening time” in the texture characteristics, hardness and adhesiveness significantly
295 increased, while springiness, cohesiveness and chewiness decreased. In general, these
296 changes are consistent with previous results obtained under similar conditions but
297 different type of cheese (Delgado, González-Crespo, Cava, & Ramírez, 2012; Salvador
298 et al., 2014).

299 No significant effect between the two factors considered was found in any case for colour
300 and texture parameters.

301 *3.5. Volatile compounds*

302 Table 4 shows the mean values of the volatile compounds (expressed as μg/100 g cheese),
303 as well as their standard deviations (SD) in cheeses with different treatments (PT, AT,
304 AW) and ripening days (0, 30, 60). The total average value for each chemical group
305 (obtained considering all the compounds found in each family) together with its relative
306 weight (expressed as percentage) is also shown. In this way, it can be evaluated how the
307 different chemical groups are globally affected by the factors hereby considered. In
308 addition, this table indicates the ANOVA multifactor obtained for every compound.

309 Twenty-two compounds including acids, alcohols, aldehydes, ketones and others were
310 found. The “cheese-making timeframe” factor had a significant effect mainly for the
311 group of alcohols and ketones ($p<0.001$). On the whole, the highest concentrations of
312 alcohols and ketones were found for PT-cheeses, and AW-cheeses. For AT-cheeses, in
313 general, a lower development of volatile compounds was observed. This is probably
314 because the presence of antibiotic residue in the milk used for producing the cheeses could
315 have an inhibitory effect on the microbiota of the milk, or on the lactic bacteria employed
316 during the manufacture of the cheese (Katla, Kruse, Johnsen, & Herikstad, 2001). These
317 lactic acid bacteria are partly responsible for the biochemical processes that contribute to
318 the development of the aromatic compounds (McSweeney & Sousa, 2000).

319 The “ripening time” has a significant effect over all the volatile compounds, with a
320 gradual increase over time reaching a maximum at 60 days. This fact coincides with other
321 authors who observed similar evolution in mature goat cheese (Castillo, Calvo, Alonso,
322 Juárez, & Fontecha, 2007; Mulet, Escriche, Rossello, & Tarrazó, 1999). It is worth noting
323 the important increase in the acetic and butanoic acids being typical flavor components
324 perceived as a goat-like smell (Delgado, González-Crespo, Cava, & Ramírez, 2011). The
325 most abundant alcohol at the end of maturation was 2-pentanol, in agreement with other
326 studies about the same type of goat cheese (Quintanilla, Hettinga, Beltrán, Escriche, &
327 Molina, 2020). The aldehydes were found in lower concentrations compared to the
328 presence of the other volatile compounds. This could be attributed to the transience of
329 aldehyde compounds in the maturation, since they are transformed into acids or are
330 reduced to alcohols (Andiç, Tunçtürk, & Boran, 2015).

331 The most noticeable effect of the “ripening time” is shown by ketones with a value that
332 reaches more than 74.27% of the total volatile profile at 60 days, similar to what was
333 reported by Castillo et al., (2007). At that time of maturation, the more abundant ketone
334 was 2-pentanone, characteristic for smelling to orange peel, sweet and fruity (Curioni &
335 Bosset, 2002). The presence of 2-pentanone and limonene may be due to the feeding of
336 goats with orange pulp (Delgado et al., 2011), a typical by-product for animal feed in
337 Valencian Region. Among the other compounds, butanoic acid-ethyl ester, which is one
338 of the most important esters in other types of goat, milk cheeses such as Ibores (Delgado
339 et al., 2011) or Majorero cheese (Castillo et al., 2007).

340 Significant interactions between the two factors (TxR) were obtained for some volatile
341 compounds, suggesting that these compounds did not evolve in a similar way over time
342 regardless of the treatment. In these cases, the differences between the “cheese-making

343 timeframe" are practically undetectable at 0-day, but they do increase with the maturation
344 time.

345 A PCA was performed to evaluate the overall effect of the enrofloxacin treatment and the
346 ripening time on the volatile profile of the cheeses. Figure 1 shows the two principal
347 components, which explained 69% of the variability of the data set (PC1, 54% and PC2,
348 15%). The PC1 differentiates the cheese samples very well according to the maturation
349 time, locating the 0 day and 30 days cheeses in the left quadrant, while the cheeses with
350 60 days of ripening are in the right quadrant. In the loading plot, all the variables (volatile
351 compounds) are situated in the zone corresponding to the longest ripening time as
352 expected, since the concentration of these compounds is maximum at the end of the
353 maturation time.

354 The second component (PC2) only differentiates the samples according to "cheese-
355 making timeframe" at 60 days of ripening, showing PT-cheeses in the lower quadrant and
356 AT- and AW-cheeses in the upper one.

357 In general, enrofloxacin treatment influences the volatile profile becoming more patent
358 with the increase of the maturation time and reaching its maximum differentiation at 60
359 days. At the end of ripening, AW-cheeses showed more similar behaviour in terms of
360 volatile profile to AT-cheeses than to PT-cheeses. The location of AW-cheeses next to
361 AT-cheeses is not what was expected, being the logical outcome AW close to PT (since
362 none of them present antibiotics in milk). This could be attributable to fact that after the
363 treatment with the antibiotic, the animal's own gut microbiota (Panda et al., 2014), as well
364 as the native flora of the milk might be affected.

365 *3.6. Sensorial analysis*

366 The acceptance test carried out on the cheeses (PT, AT and AW) ripened for 60 days
367 showed that the "cheese-making timeframe" does not significantly affect (ANOVA result
368 not shown), neither to the evaluated attributes (whose range were: odour from 6.9 to 7.0,
369 colour from 6.5 to 6.9, appearance from 6.9 to 7.1 and texture from 6.7 to 6.8), nor to the
370 global preference (from 6.5 to 7). The consumers scored the sample cheeses similarly and
371 satisfactorily, without appreciable differences between the three "cheese-making
372 timeframes" considered: pre-treatment or control, after treatment and after withdrawal.

373 These results indicate that, although instrumental techniques have made possible the
374 detection of some differences in certain parameters such as composition, colour,
375 cohesiveness and some volatile compounds, they were not appreciated by the panelists
376 and, therefore, would go unnoticed by consumers. This shows that if the established
377 withdrawal period is not respected, the milk and cheeses could contain residual
378 concentrations of enrofloxacin and ciprofloxacin, which would remain even after the
379 ripening period, being a danger to public health.

380 **4. Conclusions**

381 The antibiotic enrofloxacin and its metabolite ciprofloxacin, especially due to its
382 lipophilic nature, can be transferred to goat cheese, if the milk contains these substances.

383 The manufacture process of cheese is not altered in any way by this antibiotic; therefore,
384 it will remain unnoticed in the production line.

385 In general, with the only exception of some specific volatile compounds, the presence of
386 this antibiotic in cheese barely modifies the compositional, texture and colour
387 characteristics, when compared to the cheeses made with antibiotic-free milk. Cheese
388 maturation does not alter the stability of enrofloxacin and its metabolite. However, the
389 maturation time has a greater effect on all the characteristics of the cheese than the
390 presence of this antibiotic.

If to all of the above, the evident absence of a sensory perception is added, then this would imply a certain risk for the general population since the presence of this antibiotic would go unnoticed.

Acknowledgements

This work is part of the AGL-2013-45147-R Project funded by Ministerio de Ciencia e Innovación (Madrid. Spain). P. Quintanilla thanks Universitat Politècnica de València for the grant received for the development of this work (PAID-2014, UPV).

References

Aminifar, M., Hamedi, M., Emam-Djomeh, Z., & Mehdinia, A. (2014). Investigation on proteolysis and formation of volatile compounds of Lighvan cheese during ripening. *Journal of Food Science and Technology*, 51(10), 2454–2462. https://doi.org/10.1007/s13197-012-0755-3

Andiç, S., Tunçtürk, Y., & Boran, G. (2015). Changes in volatile compounds of cheese. In V. Preedy (Ed.), *Processing and Impact on Active Components in Food*. (pp. 231-239). London: Elsevier Inc. https://doi.org/10.1016/B978-0-12-404699-3.00028-7

Beltrán, M. C., Morari-Pirlog, A., Quintanilla, P., Escriche, I., & Molina, M. P. (2018). Influence of enrofloxacin on the coagulation time and the quality parameters of goat's milk yoghurt. *International Journal of Dairy Technology*, 71(1), 105–111. https://doi.org/10.1111/1471-0307.12388

Boutoial, K., Alcántara, Y., Rovira, S., García, V., Ferrandini, E., & López, M. B. (2013). Influence of ripening on proteolysis and lipolysis of Murcia al Vino cheese. *International Journal of Dairy Technology*, 66(3), 366–372. https://doi.org/10.1111/1471-0307.12024

414 Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., Addis, M., Testa, M.
415 C., & Urgeghe, P. P. (2017). Transfer of oxytetracycline from ovine spiked milk to
416 whey and cheese. *International Dairy Journal*, 70, 12–17.
417 https://doi.org/10.1016/j.idairyj.2016.12.002
418 Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., Daga, E., Addis, M.,
419 Testa, M. C., Urgeghe, P. P. (2018). Impact of a thermisation treatment on
420 oxytetracycline spiked ovine milk: Fate of the molecule and technological implications.
421 *LWT - Food Science and Technology*, 96, 236–243.
422 https://doi.org/10.1016/j.lwt.2018.05.026
423 Castillo, I., Calvo, M. V., Alonso, L., Juárez, M., & Fontecha, J. (2007). Changes in
424 lipolysis and volatile fraction of a goat cheese manufactured employing a hygienized
425 rennet paste and a defined strain starter. *Food Chemistry*, 100(2), 590–598.
426 https://doi.org/10.1016/j.foodchem.2005.09.081
427 Clark, C. R. (2013). Antimicrobial drug use in sheep and goats. In S. Giguère, J. F.
428 Prescott, P. M. Dowling (Eds)., *Antimicrobial therapy in veterinary medicine.* 5th ed.
429 (pp. 529-539). Iowa: John Wiley & Sons.
430 Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as
431 determined by gas chromatography-olfactometry. *International Dairy Journal*, 12(12),
432 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3
433 Delgado, F. J., González-Crespo, J., Cava, R., & Ramírez, R. (2011). Formation of the
434 aroma of a raw goat milk cheese during maturation analysed by SPME–GC–MS. *Food*
435 *Chemistry*, 129(3), 1156–1163. https://doi.org/10.1016/j.foodchem.2011.05.096
436 Delgado, F. J., González-Crespo, J., Cava, R., & Ramírez, R. (2012). Changes in
437 microbiology, proteolysis, texture and sensory characteristics of raw goat milk cheeses

438 treated by high-pressure at different stages of maturation. *LWT - Food Science and*
439 *Technology*, 48(2), 268–275. https://doi.org/10.1016/j.lwt.2012.03.025
440 Dorival-García, N., Junza, A., Zafra-Gómez, A., Barrón, D., & Navalón, A. (2016).
441 Simultaneous determination of quinolone and β-lactam residues in raw cow milk
442 samples using ultrasound-assisted extraction and dispersive-SPE prior to
443 UHPLC−MS/MS analysis. *Food Control*, 60, 382–393.
444 https://doi.org/10.1016/j.foodcont.2015.08.008
445 Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of
446 antimicrobial-resistant bacteria. *Infection and Drug Resistance*, 49.
447 https://doi.org/10.2147/IDR.S55778
448 EFSA (2016). European Food Safety Authority. The European Union summary report on
449 antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and
450 food in 2014. *EFSA Journal*, 14: 4380.
451 EMA (2014). European Medicines Agency. Answers to the requests for scientific advice
452 on the impact on public health and animal health of the use of antibiotics in animals.
453 European Medicines Agency, Veterinary Medicines Division/CVMP/CHMP,
454 EMA/381884/2014.
455 EMA (2019). European Medicines Agency. Sales of veterinary antimicrobial agents in
456 31 European countries in 2017. Trends from 2010 to 2017. Ninth ESVAC Report,
457 EMA/294674/2019.
458 European Commission (2004). Regulation (EC) Nº 852/2004 of the European Parliament
459 and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Off. J. Eur. Union*,
460 L139: 1-54.
461 European Commission (2010). Commission Regulation (EU) No 37/2010 of 22
462 December 2009 on pharmacologically active substances and their classification

463 regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Union.*
464 L15: 1-72.
465 FAOSTAT (2019). Food and Agriculture Organization Food and agriculture data.
466 Available from: http://www.fao.org/faostat/en/#data/QL/visualize/ Accessed 21 April
467 2020.
468 FAO/WHO (2004). Evaluation of certain food additives and contaminants: Sixty-second
469 Report of the Joint FAO/WHO Expert committee on food additives. Technical Report
470 Series 925. Geneva, Switzerland.
471 Ferrandini, E., López, M. B., Castillo, M., & Laencina, J. (2011). Influence of an artisanal
472 lamb rennet paste on proteolysis and textural properties of Murcia al Vino cheese. *Food*
473 *Chemistry*, 124(2), 583–588. https://doi.org/10.1016/j.foodchem.2010.06.079
474 Folkertsma, B., & Fox, P. F. (1992). Use of the Cd-ninhydrin reagent to assess proteolysis
475 in cheese during ripening. *Journal of Dairy Research*, 59(2), 217–224.
476 https://doi.org/10.1017/S0022029900030466
477 Giraldo, J., Althaus, R. L., Beltrán, M. C., & Molina, M. P. (2017). Antimicrobial activity
478 in cheese whey as an indicator of antibiotic drug transfer from goat milk. *International*
479 *Dairy Journal*, 69, 40–44.
480 https://doi.org/http://dx.doi.org/10.1016/j.idairyj.2017.02.003
481 Hayaloglu, A. A., Yasar, K., Tolu, C., & Sahingil, D. (2013). Characterizing volatile
482 compounds and proteolysis in Gokceada artisanal goat cheese. *Small Ruminant*
483 *Research*, 113(1), 187–194. https://doi.org/10.1016/j.smallrumres.2013.01.001
484 ISO (2003). ISO Standard Nº 4121:2003. Sensory analysis Methodology. Guidelines for
485 the use of quantitative response scales. International Organization for Standardization
486 Publications, Geneva, Switzerland.

ISO (2007). ISO Standard Nº 8587:2007. Sensory Analysis, Methodology, Ranking. International Organization for Standardization Publications, Geneva, Switzerland.

ISO (2008). ISO Standard Nº 5492:2008. Sensory analysis. Vocabulary. International Organization for Standardization Publications, Geneva, Switzerland.

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

Katla, A. K., Kruse, H., Johnsen, G., & Herikstad, H. (2001). Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *International Journal of Food Microbiology*, 67(1–2), 147–152. https://doi.org/10.1016/S0168-1605(00)00522-5.

López-Cadenas, C., Sierra-Vega, M., Garcia-Vieitez, J. J., Diez-Liebana, M. J., Sahagun-Prieto, A., & Fernandez- Martinez, N. (2013). Enrofloxacin: pharmacokinetics and metabolism in domestic animal species. *Current Drug Metabolism*, 14(10), 1042–1058.

Menzies, P. I., & Ramanoon, S. Z. (2001). Mastitis of sheep and goats. *Veterinary Clinics of North America: Food Animal Practice*, 17: 333-358.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait*, 80(3), 293–324. https://doi.org/10.1051/lait:2000127

Mulet, A., Escriche, I., Rossello, C., & Tarrazó, J. (1999). Changes in the volatile fraction during ripening of Mahón cheese. *Food Chemistry*, 65(2), 219–225. https://doi.org/10.1016/S0308-8146(98)00209-X

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

511 Manchego cheese. *Food Chemistry*, 21(2), 115–123. https://doi.org/10.1016/0308-
512 8146(86)90156-1
513 Panda, S., El khader, I., Casellas, F., López Vivancos, J., García Cors, M., Santiago, A.,
514 Cuenca, S., Guarner, F., & Manichanh, C. (2014). Short-Term effect of antibiotics on
515 human gut microbiota. *PLoS ONE*, 9(4), e95476.
516 https://doi.org/10.1371/journal.pone.0095476
517 Papich M.G. (2016). Enrofloxacin. In *Saunders handbook of veterinary drugs: Small and*
518 *large animal*. 4th ed. (pp. 287-289). North Carolina: Elsevier Inc., Raleigh.
519 Quintanilla, P., Beltrán, M. C., Molina, A., Escriche, I., & Molina, M. P. (2019).
520 Characteristics of ripened Tronchón cheese from raw goat milk containing legally
521 admissible amounts of antibiotics. *Journal of Dairy Science*, 102(4), 2941–2953.
522 https://doi.org/10.3168/jds.2018-15532
523 Quintanilla, P., Beltrán, M. C., Peris, B., Rodríguez, M., & Molina, M. P. (2018).
524 Antibiotic residues in milk and cheeses after the off-label use of macrolides in dairy
525 goats. *Small Ruminant Research*, 167, 55–60.
526 https://doi.org/10.1016/j.smallrumres.2018.08.008
527 Quintanilla, P., Doménech, E., Escriche, I., Beltrán, M. C., & Molina, M. P. (2019). Food
528 safety margin assessment of antibiotics: Pasteurized goat's milk and fresh cheese.
529 *Journal of Food Protection*, 82(9), 1553–1559. https://doi.org/10.4315/0362-
530 028X.JFP-18-434
531 Quintanilla, P., Hettinga, K. A., Beltrán, M. C., Escriche, I., & Molina, M. P. (2020).
532 Short communication: Volatile profile of matured Tronchón cheese affected by
533 oxytetracycline in raw goat milk. *Journal of Dairy Science*, in press.
534 https://doi.org/10.3168/jds.2019-16510

535 Raynal-Ljutovac, K., Pirisi, A., De Crémoux, R., & Gonzalo, C. (2007). Somatic cells of
536 goat and sheep milk: Analytical, sanitary, productive and technological aspects. *Small*
537 *Ruminant Research*, 68(1–2), 126–144.
538 https://doi.org/10.1016/j.smallrumres.2006.09.012
539 Saha, D., & Paul, S. (2013). Pharmacokinetics of ciprofloxacin in animals. *Egyptian*
540 *Academic Journal of Biological Sciences, B. Zoology*, 5(1), 23–32.
541 Salvador, A., Igual, M., Contreras, C., Martínez-Navarrete, N., & Camacho, M. M.
542 (2014). Effect of the inclusion of citrus pulp in the diet of goats on cheeses
543 characteristics. *Small Ruminant Research*, 121(2–3), 361–367.
544 https://doi.org/10.1016/j.smallrumres.2014.06.012
545 Tanleque-Alberto, F., Juan-Borrás, M., & Escriche, I. (2019). Quality parameters, pollen
546 and volatile profiles of honey from North and Central Mozambique. *Food Chemistry*,
547 277, 543-553. https://doi.org/10.1016/j.foodchem.2018.11.007
548 WHO (2018). Antibiotic resistance. Available from: http://www.who.int/news-
549 room/fact-sheets/detail/antibiotic-resistance/ Accessed 12 October 2019.
550 Zhang, Y. D., Zheng, N., Han, R. W., Zheng, B. Q., Yu, Z. N., Li, S. L., Zheng, S. S.,
551 &Wang, J. Q. (2014). Occurrence of tetracyclines, sulfonamides, sulfamethazine and
552 quinolones in pasteurized milk and UHT milk in China's market. *Food Control*, 36(1),
553 238–242. https://doi.org/10.1016/j.foodcont.2013.08.012
554 Zheng, N., Wang, J., Han, R., Xu, X., Zhen, Y., Qu, X., Sun, P., Li, S., &Yu, Z. (2013).
555 Occurrence of several main antibiotic residues in raw milk in 10 provinces of China.
556 *Food Additives and Contaminants: Part B,* 6(2), 84–89.
557 https://doi.org/10.1080/19393210.2012.727189
558
559

560 **Figure caption**

561 **Figure 1.** PCA for the volatile profile of *Tronchón* cheese. Codes in the score plot refer
562 to the "ripening time" (0, 30 and 60 days), "cheese-making timeframe" (PT: one day
563 before the antibiotic treatment was applied, AT: 24 hours thereafter of the last dose of
564 antibiotic and AW: after the recommended safety period), number of cheese samples (1
565 and 2) and repetition of analysis (1 and 2).

566

567

568

569

570

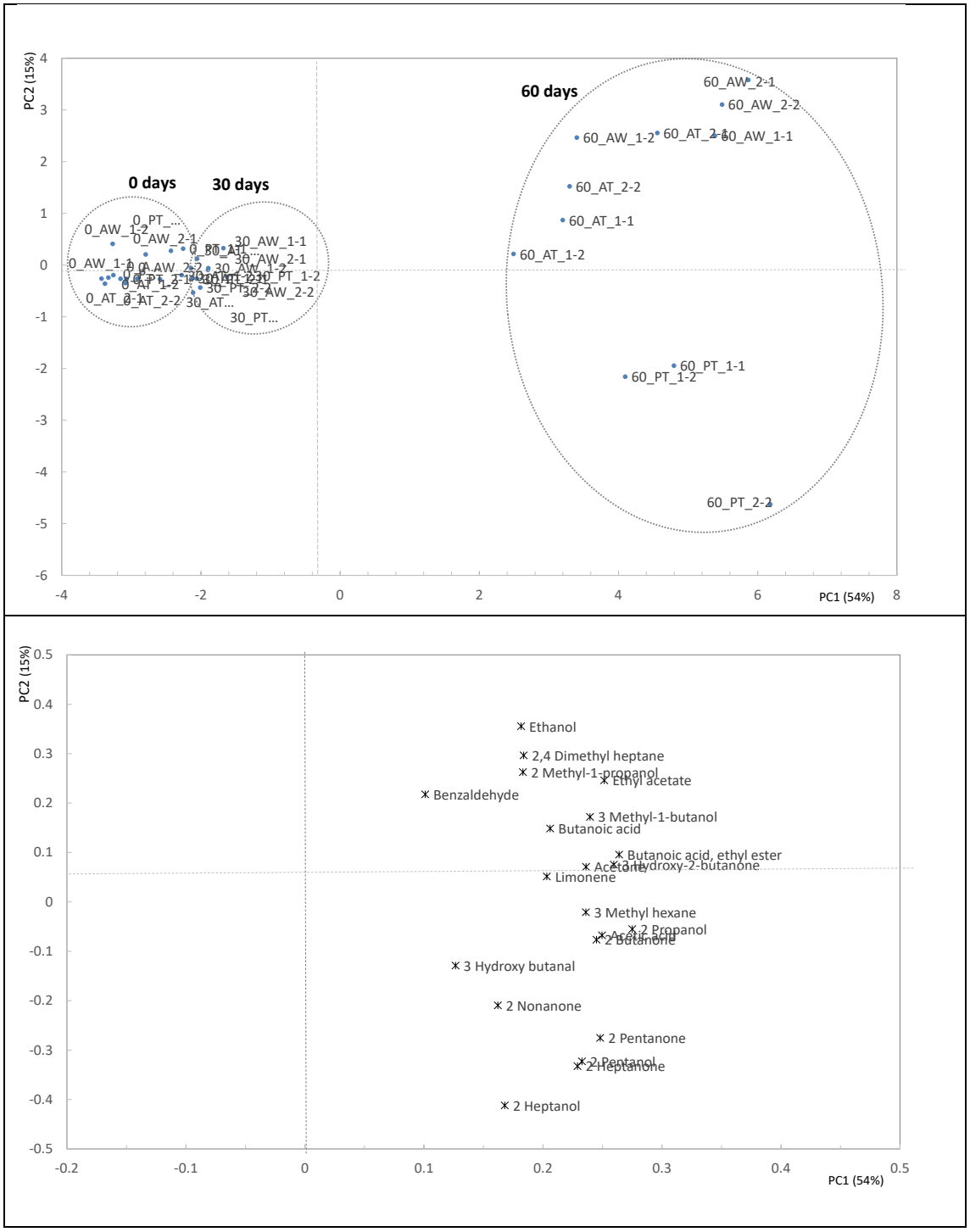
571

572

573

574

575



576 Figure 1

577

578

579

580

581

Table 1. Concentration of enrofloxacin and ciprofloxacin (mean±standard deviation) in milk after-treatment with enrofloxacin (AT-milk), and related cheeses (AT-cheeses) at different days of maturation (0, 30, 60).

Antibiotic	AT-milk	AT-cheeses (Ripening)			ANOVA
		0 days	30 days	60 days	
Enrofloxacin (µg/kg)	17.3±0.3	46.2±3.8^{c}	38.1±2.1^{b}	18.6±0.9^{a}	63.4**
Retention enrofloxacin (%)[1]			83.0±11	40.4±1.4	
Ciprofloxacin (µg/kg)	60.3±3.1	185.5±29.2^{b}	134.8±2.2^{ab}	98.8±1.6^{a}	13.25*
Retention ciprofloxacin (%)[1]			73.5±10	53.9±7.6	
Enro+Cipro (µg/kg)	77.6±2.9	231.7±32.9^{b}	172.9±0.2^{ab}	117.5±2.4^{a}	17.9*
Retención enro+cipro (%)[1]			75.4±11	51.1±6.2	

Enr + Cip: Sum of enrofloxacin and ciprofloxacin concentration;

[1] Percentage of the antibiotic retained in cheese at 30 and 60 days with respect to the content at 0 days.

[a, b, c:] Different letters in the same row indicate significant differences between days of ripening: ($P<0.05$); *$P<0.05$; ** $P<0.01$.

Table 2. Values (mean±standard deviation) of pH, gross composition, Free Fatty Acids (FFA) and Free Amino Acids (FAA) in cheeses with respect to the "cheeses-making timeframe" (T), and the "ripening time" (R). ANOVA results considering both factors.

Parameters	Cheese-making timeframe (T)			Ripening time (R)			ANOVA (f-ratio)	
	PT-cheeses	AT-cheeses	AW-cheeses	0 days	30 days	60 days	T	R
pH	5.36±0.05	5.35±0.09	5.37±0.08	5.42±0.05^{c}	5.30±0.05^{a}	5.36±0.07^{b}	0.9^{ns}	18.8***
Moisture (g/100g)	38.9±3.1	38.5±3.1	38.1±2.7	41.4±0.8^{c}	39.4±1.1^{b}	34.8±0.8^{a}	2.4^{ns}	181.9***
Fat (g/100g DM)	56.7±1.8^{c}	55.2±0.5^{b}	53.8±0.8^{a}	55.6±1.4	55.2±2.0	55.0±1.5	16.4*	0.8^{ns}
Protein (g/100g DM)	35.6±2.3^{a}	37.5±0.6^{b}	38.9±0.7^{c}	37.6±1.8	36.7±2.3	37.8±1.7	15.5*	2.2^{ns}
NaCl (g/100g DM)	3.2±0.2	3.2±0.2	3.1±0.2	2.9±0.1^{a}	3.3±0.1^{b}	3.3±0.1^{b}	2.9^{ns}	32.0***
FFA (meq/100 g of fat)	2.6±0.7	2.5±0.5	2.4±0.6	2.0±0.3^{a}	2.5±0.3^{b}	3.1±0.4^{c}	1.4^{ns}	25.5***
FAA (mg leucine/g of cheese)	2.0±1.0	2.0±1.0	2.1±0.9	0.7±0.1^{a}	2.5±0.2^{b}	2.9±0.2^{c}	1.8^{ns}	481.7***

"Cheese-making timeframe": PT-cheeses: Pre-treatment cheeses (one day before the antibiotic treatment was applied); AT-cheeses: After treatment cheeses (24 hours thereafter of the last dose of antibiotic); AW-cheeses: After withdrawal period cheeses (after the recommended safety period).

DM: Dry matter.

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

Table 3. Average values (mean±standard deviation) of the colour and texture parameters in cheeses with respect to the "cheeses-making timeframe" (T), and the "ripening time" (R). ANOVA results considering both factors.

Parameters	Cheese-making timeframe (T)			Ripening time (R)			ANOVA (f-ratio)	
	PT-cheeses	AT-cheeses	AW-cheeses	0 days	30 days	60 days	T	R
Colour								
L*	88.6±2.3^{b}	88.3±1.8^{b}	86.5±2.7^{a}	90.2±0.7^{c}	87.6±1.4^{b}	85.6±2.2^{a}	10.5***	43.1***
a*	-1.0±0.6	-0.9±0.6	-0.9±0.5	-0.3±0.1^{c}	-1.0±0.1^{b}	-1.5±0.2^{a}	0.64^{ns}	190.5***
b*	11.2±0.9	11.3±1.1	11.5±0.9	10.4±0.8^{a}	11.7±0.6^{b}	11.9±0.7^{b}	0.84^{ns}	18.3***
Texture								
Hardness (N)	28.0±4.4	29.6±4.6	30.9±6.2	26.6±6.3^{a}	28.9±2.7^{a}	33.1±3.8^{b}	1.2^{ns}	6.4**
Adhesiveness (N.s)	-1.5±0.7	-1.3±0.6	-1.3±0.5	-0.6±0.1^{b}	-1.8±0.2^{a}	-1.7±0.5^{a}	1.3^{ns}	51.4***
Springiness	0.7±0.1	0.6±0.1	0.7±0.1	0.8±0.1^{b}	0.6±0.1^{a}	0.6±0.1^{a}	3.0^{ns}	145.3***
Cohesiveness	0.4±0.2^{a}	0.4±0.2^{a}	0.5±0.2^{b}	0.7±0.1^{c}	0.4±0.1^{b}	0.3±0.1^{a}	10.4*	410.6***
Chewiness (N)	8.8±4.0	9.5±5.6	11.4±5.6	16.0±4.0^{b}	6.8±1.5^{a}	6.9±1.8^{a}	3.4^{ns}	50.7***

"Cheese-making timeframe": PT-cheeses: Pre-treatment cheeses (one day before the antibiotic treatment was applied); AT-cheeses: After treatment cheeses (24 hours thereafter of the last dose of antibiotic); AW-cheeses: After withdrawal period cheeses (after the recommended safety period).

Table 4. Volatile compounds (µg/100 g cheese: mean±standard deviations) in cheeses with different "cheese-making timeframe" (PT, AT, AW) and ripening days (0, 30, 60). Total average value for each chemical group and its relative weight. ANOVA multifactor (f-ratio).

Chemical group	Cheese-making timeframe (T)			Ripening time (R)			ANOVA (f-ratio)		
	PT-cheeses	AT-cheeses	AW-cheeses	0 days	30 days	60 days	T	R	TxR
Acids									
Acetic acid	1.25±1.36	1.21±1.10	1.13±1.00	0.47±0.22^{a}	0.58±0.17^{a}	2.54±1.01^{b}	0.12^{ns}	39.59***	0.59^{ns}
Butanoic acid	0.66±0.70	0.88±0.85	0.78±0.69	0.20±0.11^{a}	0.46±0.15^{a}	1.66±0.74^{b}	0.66^{ns}	32.95***	0.45^{ns}
Total average acids	**1.80±1.60**	**2.09±1.94**	**1.76±1.41**	**0.67±0.33^{a}**	**1.04±0.30^{a}**	**3.93±1.21^{b}**	**0.66^{ns}**	**66.03*** **	**0.76^{ns}**
Relative weight (%)	**2.57**	**5.08**	**3.62**	**3.71**	**4.41**	**3.15**			
Alcohols									
Ethanol	1.93±0.83^{a}	2.96±1.58^{b}	4.50±2.47^{c}	2.75±0.60^{b}	1.73±0.82^{a}	4.91±2.49^{c}	35.07***	54.88***	10.27***
2-propanol	0.62±0.49^{c}	0.31±0.29^{a}	0.49±0.41^{b}	n.d.	0.11±0.06^{a}	0.83±0.24^{b}	15.83***	256.39***	5.83*
3-methyl-1-butanol	1.82±1.06^{b}	0.93±0.62^{a}	2.40±1.56^{c}	n.d.	0.72±0.31^{a}	2.70±1.09^{b}	58.77***	296.42***	20.54***
2-methyl-1-propanol	0.83±0.10^{a}	1.00±0.24^{a}	1.66±0.13^{b}	n.d.	n.d.	1.23±0.42	19.55**		
2-pentanol	8.43±7.84^{c}	1.46±0.14^{a}	2.50±2.13^{b}	n.d.	0.68±0.49^{a}	7.63±6.08^{b}	107.20***	274.26***	79.21***
2-heptanol	0.77±0.93	0.08±0.05	0.15±0.15	n.d.	0.04±0.03^{a}	0.75±0.80^{b}	4.71^{ns}	6.71*	4.27^{ns}
Total average alcohols	**9.66±10.0^{c}**	**4.90±4.03^{a}**	**8.56±7.16^{b}**	**2.75±0.60^{a}**	**3.21±1.09^{a}**	**17.14±5.92^{b}**	**46.44*** **	**498.74*** **	**32.74*** **
Relative weight (%)	**10.99**	**13.47**	**16.66**	**15.99**	**12.58**	**12.56**			
Aldehydes									
3-hydroxy-butanal	0.25±0.21	0.23±0.11	0.23±0.11	0.16±0.07^{a}	0.14±0.05^{a}	0.42±0.14^{b}	0.15^{ns}	28.64***	1.82^{ns}
3-methyl-butanal	0.07±0.03^{a}	n.d.	0.14±0.02^{b}	n.d.	0.11±0.04	n.d.	17.81**		

Benzaldehyde	0.21±0.13	0.20±0.16	0.23±0.15	0.13±0.05^{a}	0.08±0.02^{a}	0.43±0.09^{b}	0.65^{ns}	67.60***	0.14^{ns}
Total average aldehydes	**0.38±0.25^{a}**	**0.39±0.22^{a}**	**0.51±0.20^{b}**	**0.29±0.11^{a}**	**0.29±0.10^{a}**	**0.71±0.17^{b}**	**3.82***	**37.48*****	**0.29^{ns}**
Relative weight (%)	**0.68**	**1.26**	**1.33**	**1.68**	**1.16**	**0.43**			

Table 4 *(Cont.)*

Chemical group	**Cheese-making timeframe (T)**			**Ripening time (R)**			**ANOVA (f-ratio)**		
	PT-cheeses	**AT-cheeses**	**AW-cheeses**	**0 days**	**30 days**	**60 days**	**T**	**R**	**TxR**
Ketones									
2-propanone	2.78±3.08	2.38±2.65	2.94±2.81	0.97±1.03^{a}	1.26±0.60^{a}	5.86±2.47^{b}	0.32^{ns}	28.59***	0.22^{ns}
2-butanone	2.12±3.21	2.11±2.90	1.51±1.91	0.22±0.04^{a}	0.32±0.08^{a}	5.06±2.42^{b}	0.53^{ns}	41.05***	0.57^{ns}
2-pentanone	47.63±57.27^{b}	15.78±19.65^{a}	18.41±19.77^{a}	3.33±1.13^{a}	8.55±5.68^{a}	69.94±41.37^{b}	67.20***	294.91***	45.65***
2-heptanone	6.55±7.97^{b}	2.03±2.13^{a}	1.93±2.10^{a}	0.13±0.08^{a}	0.75±0.43^{a}	7.78±6.15^{b}	9.01**	38.43***	9.43***
3-hydroxy-2-butanone	11.02±4.63^{b}	8.32±5.98^{a}	11.35±6.48^{b}	6.50±2.79^{a}	7.42±1.14^{a}	16.77±5.08^{b}	3.79*	44.41***	2.29^{ns}
2-nonanone	0.27±0.38	0.15±0.17	0.11±0.08	n.d.	0.04±0.01^{a}	0.34±0.31^{b}	1.45^{ns}	5.30*	1.50^{ns}
Total average ketones	**68 99±73.16^{b}**	**30.17±31.51^{a}**	**35.85±32.09^{a}**	**11.06±4.30^{a}**	**18.32±6.78^{a}**	**105.64±47.18^{b}**	**52.03***	**327.84***	**29.35***
Relative weight (%)	**76.55**	**60.21**	**66.30**	**59.49**	**69.31**	**74.27**			
Other compounds									
Ethyl acetate	0.47±0.37^{a}	0.66±0.86^{ab}	0.87±0.98^{b}	0.19±0.05^{a}	0.23±0.07^{a}	1.58±0.74^{b}	3.77*	6.40***	4.33**
Butanoic acid ethyl ester	0.38±0.42^{a}	0.46±0.45^{a}	0.66±0.75^{b}	0.04±0.01^{a}	0.18±0.04^{b}	1.17±0.39^{c}	11.67***	205.74***	9.95***
Limonene	3.05±2.90^{a}	5.23±3.64^{b}	3.22±2.28^{a}	2.53±0.65^{a}	1.98±1.13^{a}	6.99±3.46^{b}	5.19*	26.57***	1.67^{ns}
3-methyl-hexane	1.10±0.95	0.83±0.84	1.07±1.30	0.37±0.16^{a}	0.52±0.22^{a}	2.11±1.12^{b}	0.51^{ns}	21.79***	0.13^{ns}
2,4-dimethyl-heptane	0.53±0.38	0.44±0.34	0.66±0.87	0.20±0.05^{a}	0.22±0.08^{a}	1.21±0.71^{b}	1.02^{ns}	22.84***	1.13^{ns}
Total average others	**4.95±3.85**	**7.09±5.24**	**5.81±5.10**	**3.27±0.63^{a}**	**2.94±1.06^{a}**	**11.63±4.05^{b}**	**2.67^{ns}**	**56.20***	**1.44^{ns}**
Relative weight (%)	**9.20**	**19.98**	**12.09**	**19.13**	**12.53**	**9.59**			

“cheese-making timeframe”: PT-cheeses: Pre-treatment cheeses (one day before the antibiotic treatment was applied); AT-cheeses: After treatment cheeses (24 hours thereafter of the last dose of antibiotic); AW-cheeses: After withdrawal period cheeses (after the recommended safety period).

n.d.: not detected; [a, b, c:] Superscript letters in the same row for factor indicate significant differences ($p<0.05$); *P< 0.05; **P<0.01; ***P<0.001; ns: non-significant ($p>0.05$).