Influence of enrofloxacin on the coagulation time and the quality parameters of goat's milk yoghurt

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Running headline: Enrofloxacin in goat's milk yoghurt

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ABSTRACT

Three batches of yoghurts were made from goat's milk with different enrofloxacin concentrations (0, 50, 100 and 150 μg/kg). Quality parameters were analysed at 1, 7, 14 and 28 days at 5ºC. Drug residues were also quantified by HPLC. Coagulation time and most yoghurt properties remained unaffected by the presence of enrofloxacin in goat's milk. However, quality parameters were affected by the storage period. 74.9-99.2% of enrofloxacin initially added to goat's milk remained in the yoghurt throughout its entire shelf life, potentially posing a risk to consumer health. Therefore, an enrofloxacin Maximum Residue Limit in yoghurt should be established.

Key words: caprine milk, yoghurt, antibiotic, storage

INTRODUCTION

Enrofloxacin is a synthetic antimicrobial agent belonging to the fluoroquinolone group, widely used in veterinary medicine due its effectiveness against the infectious diseases produced by Gram-negative and Gram-positive bacteria as well as mycoplasma (Elsheikh *et al*. 2002). In dairy goats, enrofloxacin is usually administered by veterinarians in the treatment of gastrointestinal, respiratory and mammary diseases (Menzies and Ramanoon 2001), often being applied in an off-label manner given the scarcity of drugs indicated for the use in this species, which is likely to increase the risk of the presence of antibiotic residues in milk.

Drug residues in milk pose a potential risk for consumer health as they may lead to allergies or the generation of microbial resistance, among other reactions (Tollefson and Karp 2004; Sanders *et al*. 2011) and as a consequence, a Maximum Residue Limits (MRLs) have been established for these substances in milk and other foodstuff of animal origin by European legislation (Regulation UE 37/2010). It should be noted that MRLs are not established for

dairy products widely consumed like cheeses and yoghurts. However, some of these antimicrobial substances are hardly affected by heat treatments usually carried out by the dairy industry (Zorraquino *et al*. 2008; Roca *et al*. 2010) or by the manufacture processes themselves (Grunwald and Petz 2003; Adetunji 2011) and therefore, variable amounts of drug residues could remain in the final products, if present in raw milk.

Also, the presence of antibiotics could have negative technological effects as the activity of starters employed in the manufacture of fermented products could be totally or partially inhibited even at or below safety levels. In this sense, a significant delay in the coagulation time has been reported in ewe's milk yoghurts spiked with penicillins (Berruga *et al*. 2007) and cephalosporins (Berruga *et al*. 2008) at or below their respective MRLs. Consequently, the physicochemical and organoleptic characteristics of fermented products could also be affected by drug residues in milk, leading to significant economic losses as the commercial quality of these products is lowered. Thus, for example, the presence of oxytetracycline at or below MRL has been related to lower firmness values in sheep milk yoghurts (Novés *et al*. 2012).

On the other hand, goat's milk production is traditionally destined to the manufacture of cheeses and other milk products such as yoghurts. The production of goat's milk yoghurt has increased considerably in the last decades given the growing consumer interest in these products as they can be more easily digested and are more suitable for individuals with allergic reactions to cow milk protein (Haenlein 2004; Park 2005). Moreover, these products are often made in a traditional way and are destined for a gourmet-type market, fetching higher prices owing to their additional value (Ribeiro and Ribeiro 2010).

There is very little information available related to the effect of the presence of enrofloxacin in milk on the manufacture process and the organoleptic characteristics of

yoghurts. Neither is the amount of enrofloxacin residues known that could remain in yoghurts made from contaminated milk, nor the effect of the refrigeration period on the drug residues in the product.

Therefore, the aim of this study was to evaluate the effect of enrofloxacin in goat's milk on the production and quality parameters of yoghurt, as well as the antibiotic residual concentration in the final products.

MATERIALS AND METHODS

Yoghurt production

Goat's milk yoghurts were manufactured at pilot plant-scale using antibiotic-free milk from the experimental flock of Murciano-Granadina breed goats of the Universitat Politècnica de València (Valencia, Spain). Three batches of yoghurts were made on three different days with different concentrations (0, 50, 100 and 150 µg/Kg) of enrofloxacin (33699, Sigma-Aldrich, Madrid, Spain) close to MRL (100 µg/Kg). Raw goat's milk (2 L) was heat treated at 80 ºC for 30 minutes in a Thermomix (Vorwerk, Wuppertal, Germany). After heating, the milk was cooled to 45 ºC and then, inoculated with a yoghurt starter culture containing *Streptoccocus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus* (FD-DVS YF-L812 Yo-Flex®, CHR-Hansen, Madrid, Spain) following the manufacturer's instructions. Inoculated milk was poured into polystyrene containers (60 mL) and incubated at 43±1 ºC in a thermostatized water bath until a pH of 4.60±0.05 was reached. Thereafter, the yoghurts were immediately cooled and stored at 5 ºC to be analysed on days 1, 7, 14 and 28 post-production.

Physicochemical analysis

The pH of the inoculated milk samples was monitored every 15 minutes during fermentation, using a conventional pH-meter (Crison, Barcelona, Spain). The time required

to complete the acidification process, expressed in minutes, was recorded as coagulation time.

Postacidification of yoghurts along the refrigerated storage period was evaluated by measuring the pH value and also by determining the titratable acidity, expressed as lactic acid percentage, using NaOH 0.111N (Panreac, Barcelona, Spain), and phenolphthalein (Panreac) as indicator.

The colour in yoghurts was determined in triplicate using a spectrocolorimeter Minolta CM-3600D (Minolta, Tokyo, Japan). Colour coordinates CIE L*, a* and b* were obtained using observer 10º and illuminant D65. Chromatic parameters chroma (C), hue (h) and whiteness index (WI) were obtained from these coordinates using the SpectraMagic v. 3.60 G software

Rheological and mechanical properties

The mechanical characterization of the yoghurt samples was carried out by means of a Texture Analyser (TA.XT Plus, Stable Micro Systems, Surrey, UK) equipped with a 50 kg load cell. A plunger with a diameter of 35 mm was used at a speed of 120 mm min^{-1}. The yoghurt samples were held in a plastic cup and placed on a flat holding plate at 12±1ºC. A maximum sample strain of 50% was employed. Firmness (N) (the maximum force reached during the compression cycle) and the adhesiveness (N*s) (negative force area) were calculated from the resulting curve. Ten replicates of each analysis were carried out for each condition and storage time.

The rheological behavior of the samples was determined at 12±1ºC using a controlled shear stress rheometer with a coaxial cylinders (Z34 DIN) sensor system coupled to a thermostatic bath (Thermo Electron Co., Haake RheoStress 1, Germany). A relax time of

300 s was chosen for the sample before running the test. The shear rate, $\dot{\gamma}$ (s^{-1}), was increased from 0 to 150 s^{-1} (duration step 300 s) and shear stress, σ (Pa), was recorded. Four tests were carried out for each yoghurt sample. For each sample, the mean value of apparent viscosity (Pa*s) was reported at 100 s-1.

Bacterial counts

Cell populations of starter cultures in yoghurts during cold storage were counted by the pour plate technique, and results expressed as the logarithm of colony-forming units per gram of sample.

The selective count of *Str. thermophilus* was made using M17 agar (Biokar Diagnostics, Allone, France) supplemented with lactose (Scharlau, Barcelona, Spain) after aerobic incubation at 37 °C for 48 hours. For the *L. delbruekii* ssp. *bulgaricus* count, acidified (pH= 5.6) MRS agar (Biokar Diagnostics) and anaerobic incubation at 37ºC for 72 hours were used. Anaerobic conditions were produced applying the Thermo Scientific Oxoid Anaerogen system (Thermo Scientific. Madrid, Spain).

Antibiotic residue quantification

The extraction and purification of enrofloxacin from yoghurt samples was conducted using a procedure, described as follows, in accordance with the protocols established and validated at the Instituto Lactológico de Lekunberri (Lekunberri, Pamplona), using ISO standard 17025 (ISO/IEC, 2005): a yoghurt sample (10±0.5 g) was weighed, and 20±0.01 g trisodium citrate (20% w/w) at 40ºC, were added to the sample and the mixture was shaken for 90 s, twice. The mixture (10±0.01g) was centrifuged for 10 min at 9000 g. Two mL of the supernatant were purified by solid-phase extraction (SPE) using an Oasis HLB cartridge (Baker, 200 mg, 3 ml) previously conditioned with 1mL of methanol and 1 mL of

distillated water. After the extract had passed through the cartridge, it was rinsed with 2 mL of water, and it was eluted with 2mL of methanol and dried under vacuum. Finally residues were resuspended in 500 µL of 0.1% formic acid. The solution was mixed using a vortex mixer, homogenized in the ultrasonic bath 5 min, filtered into a chromatographic vial using a 0.45-µm polyvinylidene fluoride filter. Twenty mL of this mixture were injected into the HPLC system.

An Alliance 2695 high-performance liquid chromatograph with a diode-array detector from Waters (Waters Chromatography Division PA, USA) was used. Analytical separation of drugs was achieved on a XBridgeTM C18 column (100 mm, 34.6 mm, 2.1 mm) whit a particle size of 3.5 µm and a pore size of 3.5 Å. The mobile phase consisted of A (0.1% formic acid) and B (acetonitrile). The solvent gradient conditions of the liquid chromatography mobile phase were as follows: time (t)= 0–8 min, 95% A and 5% B; t= 8–14 min, 25% A and 75% B; t= 14–15 min, 5% A and 95% B and t= 15-20 min, 95% A and 5% B. The flow rate was 0.2 mL/min.

Mass spectral analyses were performed on a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (Waters Chromatography división, Milford, MA). The analytes were detected using electrospray ionization in the positive ion mode The needle voltage was typically set at 3.0 kV and the rf lens voltage at 0.2 V. Source block and desolvation temperature were set at 120 and 350°C, respectively. Nitrogen gas was used as desolvation gas at a flow rate of 60 L/h. For quantitation calibration curves were had previously been established and the MassLynx 4.0 sofware (Waters) was used to calculate the enrofloxacin amounts in goats milk yoghurt.

Statistical analysis

A multifactor analysis of variance (ANOVA) (using Statgraphics Centurion XVI.II) was carried out to study the influence of enrofloxacin concentration (0, 50, 100 and 150 μg/kg) and cold storage (1, 7, 14 and 28 days) on the different parameters analysed. The interactions between factors were considered. Multiple comparisons were made using the LSD test (least significant difference) with a significance level of $\alpha = 0.05$. Furthermore, the data were analysed using a principal component analysis (PCA) applying the Unscrambler X.10.3 software. The variables were weighted with the inverse of the standard deviation of all objects in order to compensate for the different scales of the variables.

RESULTS AND DISCUSSION

Antibiotic-free goat's milk employed for yoghurt production showed a good hygienic quality and similar physico-chemical characteristics to those reported by other authors for Murciano-Granadina breed goats (Beltrán *et al*. 2015). The gross chemical composition (g/100 g) was: total solids 15.32, fat 5.94, protein 4.03. Somatic cell count and total bacterial count were 610,000 cells/mL and 62,000 cfu/mL, respectively; the pH value was 6.72.

As shown in Fig. 1, the fermentation kinetics was similar for all the experimental yoghurts. Therefore, the coagulation time required for yoghurt production (250±6.12 min) was unaffected by the presence of enrofloxacin in goat's milk ($p>0.05$), suggesting that antibiotic concentrations used in this study are not able to significantly inhibit the growth of the starter cultures.

Table 1 shows the average values of acidity, colour, mechanical, rheological and microbiological properties. In addition, this table shows the ANOVA results (F-ratio and

165 significant differences) obtained for the two factors considered: antibiotic concentration and
166 days of refrigerated storage.
167 The presence of enrofloxacin in goat's milk at concentrations of up to 150 µg/kg does not
168 substantially modify ($p>0.05$) most of the variables analysed. Only the titratable acidity
169 slightly increased in the yoghurts containing the highest antibiotic concentrations. These
170 results could be related to larger *L. delbruekki* ssp. *bulgaricus* populations present in these
171 yoghurts. Although the differences were not statistically significant ($p>0.05$), it is well
172 established that *L. delbruekki* ssp. *bulgaricus* is more effective in the production of lactic
173 acid from sugars present in milk than *Str. thermophilus* (Tamine and Robinson 1999).
174 Nevertheless, the variation in the acid lactic content found in the four types of yoghurt
175 could be considered irrelevant.
176 The average hue values are similar to those reported by Vargas *et al*. (2008). There is, in
177 general, no information available about the effect of the presence of antibiotics on the
178 chromatic characteristics on dairy products.
179 As shown in Table 1, there are no significant interactions between the two factors
180 considered in any case. All the yogurts evolved similarly modifying significantly their
181 initial characteristics along the cold storage period ($p<0.05$).
182 In all yoghurt samples, the pH value decreased significantly ($p< 0.05$) during the 28 days of
183 cold storage most likely related to the production of organic acids in this period. Thus, the
184 titratable acidity was also affected by time ($p< 0.05$). It should be noted that the
185 acidification level in the yoghurts was lower than that reported by others authors for goat's
186 milk yoghurts (Stelios and Anifantakis 2004; Ranadheera *et al*. 2012). Differences could be
187 attributed to the properties of the commercial starter cultures used in this study which are

188 recommended by manufacturers for the elaboration of yoghurt with a very mild flavour,
189 extra high viscosity and very low post-acidification.
190 With respect to the chromatic parameters evaluated, luminosity (L*) and whiteness index
191 (WI) decreased along time, while chroma (C) increases presenting the highest values on
192 days 7 and 14 of cold storage.
193 Mechanical and rheological parameters were also affected by the storage time. The
194 hardness of yoghurts increases during storage as a consequence of post-acidification
195 occurring in this period. On the other hand, adhesiveness and viscosity of yoghurts remains
196 more stable.
197 Regarding bacterial counts in goat's milk yoghurts (Table 1) the *Str. thermophilus*
198 population was similar for the different days considered ($p>0.05$). However, the *L.*
199 *delbruekii* ssp. *bulgaricus* count decreased significantly ($p<0.01$) during cold storage. The
200 decline in the viable lactobacilli population in yoghurt along time has been reported by
201 several authors (Güler and Akın 2007; Ranadheera *et al*. 2012), being also the subject of
202 numerous studies aiming at the prolongation of the viability of these lactobacilli and other
203 probiotics usually employed to produce yoghurts and other fermented milk products
204 (Moayednia *et al*. 2009; Sah *et al*. 2015).
205 In order to evaluate the global effect of time of storage and enrofloxacin concentration on
206 the different parameters evaluated from a descriptive point of view, a principal component
207 analysis (PCA) was performed. Fig. 2 shows the PCA test results (a: scores of the samples,
208 and b: loading). This analysis was carried out considering the average values of each
209 parameter obtained from each sample (the code for each point in the figure corresponds to
210 time of storage–concentration). In the score plot, proximity between samples reflects
211 similarity in relation to the analysed parameters. Two principal components explained 68%

of the variations in the data set: PC1 (43%) and PC2 (25%). The first principal component differentiates the samples with respect to storage time. There was a clear differentiation between day 1, day 7 (placed in the right quadrants), and the rest of refrigerated storage time (14 and 28) placed on the left, without differences between them. Differences between samples were strongly influenced by storage time. However, the enrofloxacin concentration clearly did not exert any effect on the variables analysed as the samples were grouped according to the storage time and not to antibiotic concentration. The loading plot shows that certain parameters are largely responsible for this differentiation, namely the largest values of pH, L* and WI at shortest storage times (1 and 7 days) and the largest firmness and acidity at longer storage times (14 and 28 days).

Finally, despite the intense heat treatment inherent to the yoghurt production process (80 ºC-30 min), the residual amounts of enrofloxacin in yoghurts one day after production were 97-100% of the drug initially added to the goat's milk (Fig. 3). These results are undoubtedly related to the high heat stability of the quinolones reported by several authors (Lolo *et al*. 2006; Roca *et al*. 2010).

Enrofloxacin residues in the goat's milk yoghurts decrease along cold storage being approx.16.3- 25% lower after 28 days at 5 ºC. However, after that period, they still remained at 74.9-99.2% of those initially present in goat's milk. There is no information available related to the residual amounts of quinolones in yoghurts or other dairy products made from milk containing these antibiotics and, therefore, our results cannot be compared.

It is noteworthy that enrofloxacin residues are not detected at MRL by the microbial inhibitor tests usually employed for screening antibiotics in raw milk (Sierra *et al.* 2009; Beltrán *et al.* 2015). Thus, the presence of such substances in raw milk may remain undetected in the screening phase and finally reach the dairy industry where, in spite of the

236 treatments applied in the production and storage process, elevated amounts of this antibiotic
237 may be found in yoghurts.

238 **CONCLUSIONS**

239 The presence of enrofloxacin in goat's milk of up to 150 µg/kg did not lead to technical
240 failures in the yoghurt production nor to detectable quality alterations along time and
241 therefore yoghurts made from contaminated milk might reach consumer. It should be noted
242 that large amounts of drug residues could remain in the yoghurts throughout its entire shelf
243 life. It would be convenient to improve the detection of this substance in the screening of
244 raw milk as well as to establish a safety levels for dairy products in order to guarantee the
245 consumer health.

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Table 1. Average values of parameters analysed in samples and ANOVA F-ratio for each of the two factors: antibiotic concentration (C) and storage period (days) and their respective interaction (C*D)

Parameter	**Antibiotic concentration (µg/kg)**					**Refrigerated storage (days)**					**ANOVA F-ratio**		
	0	**50**	**100**	**150**	**SE**	**1**	**7**	**14**	**28**	**SE**	**C**	**D**	**C*D**
Acidity													
pH	4.53	4.50	4.50	4.50	0.04	4.68^{b}	4.57^{b}	4.39^{a}	4.38^{a}	0.03	0.15^{ns}	15.56^{***}	0.10^{ns}
Dornic Acidity (% lactic acid)	0.84^{a}	0.86^{ab}	0.87^{b}	0.89^{b}	0.01	0.77^{a}	0.84^{b}	0.93^{c}	0.92^{c}	0.01	3.88^{*}	45.86^{***}	1.58^{ns}
Colour													
L*	90.29	90.17	90.16	90.24	0.05	90.34^{b}	90.62^{c}	90.01^{a}	89.88^{a}	0.05	1.60^{ns}	44.84^{***}	1.12^{ns}
Chroma (C_{ab})	8.13	8.10	8.09	8.08	0.03	7.88^{a}	8.26^{c}	8.18^{c}	8.08^{b}	0.03	0.47^{ns}	26.66^{***}	0.18^{ns}
Hue (h)	102.11^{b}	101.97^{a}	102.01^{ab}	102.12^{b}	0.04	102.48^{d}	101.57^{a}	102.27^{c}	101.89^{b}	0.03	3.84^{**}	126.80^{***}	0.97^{ns}
Whitness index (CIE)	39.78	39.60	39.83	40.17	0.32	40.25^{b}	42.73^{c}	38.13^{a}	38.27^{a}	0.26	0.53^{ns}	34.84^{***}	0.34^{ns}
Mecanical and rheological properties													
Firmness (N)	1.25	1.25	1.29	1.23	0.02	1.10^{a}	1.16^{b}	1.41^{d}	1.35^{c}	0.02	2.22^{ns}	54.85^{***}	0.83^{ns}
Adhesiviness (N*s)	-1.31	-1.35	-1.25	-1.24	0.04	-1.35^{a}	-1.19^{b}	-1.34^{a}	-1.27^{ab}	0.04	1.43^{ns}	3.23^{*}	1.56^{ns}
Viscosity (Pa*s)	0.246	0.248	0.252	0.247	0.002	0.256^{b}	0.243^{a}	0.239^{a}	0.255^{b}	0.002	1.18^{ns}	14.00^{***}	1.43^{ns}
Microbiology													
S. thermophilus (Log ufc/g)	8.83	8.89	8.81	8.88	0.03	8.88	8.82	8.90	8.81	0.03	1.64^{ns}	2.23^{ns}	0.49^{ns}
L. delbruekii ssp. *bulgaricus (*Log ufc/g)	6.76	6.81	6.90	6.90	0.07	6.99^{b}	6.82^{ab}	6.91^{b}	6.64^{a}	0.07	0.98^{ns}	4.36^{**}	1.05^{ns}

Fig. 1. Fermentation kinetics of the experimental goat's milk yoghurts

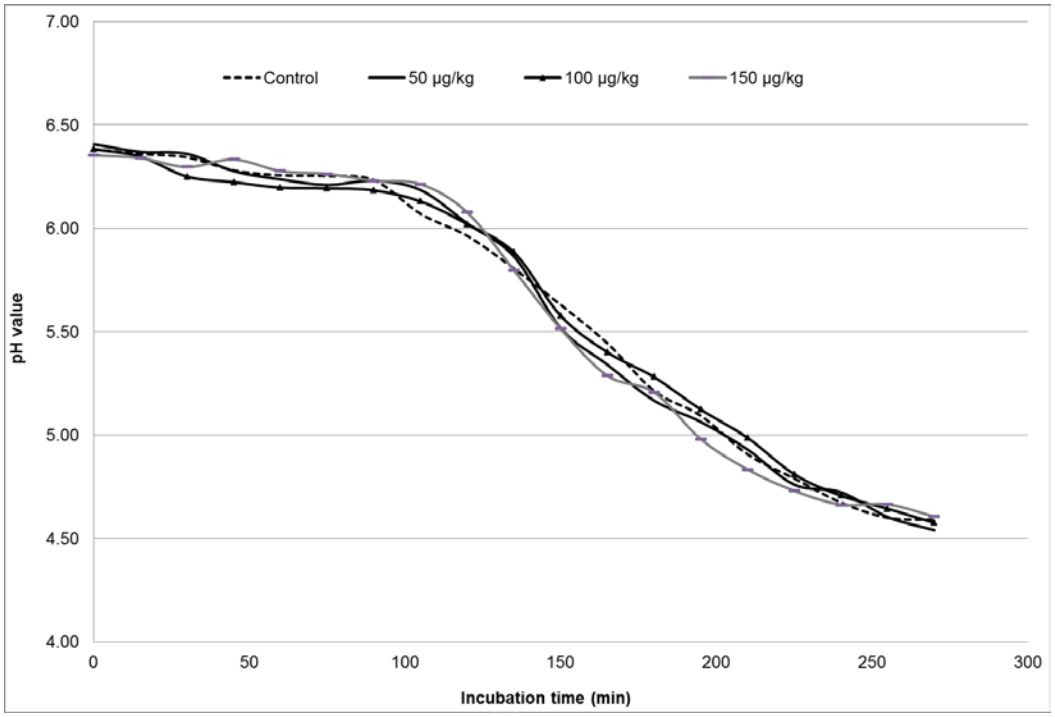

Fig. 2. PCA plots. A: Plot of the two principal component scores (the code for each point in the figure corresponds to: time of storage–concentration), B: Plot of the two principal component loadings.

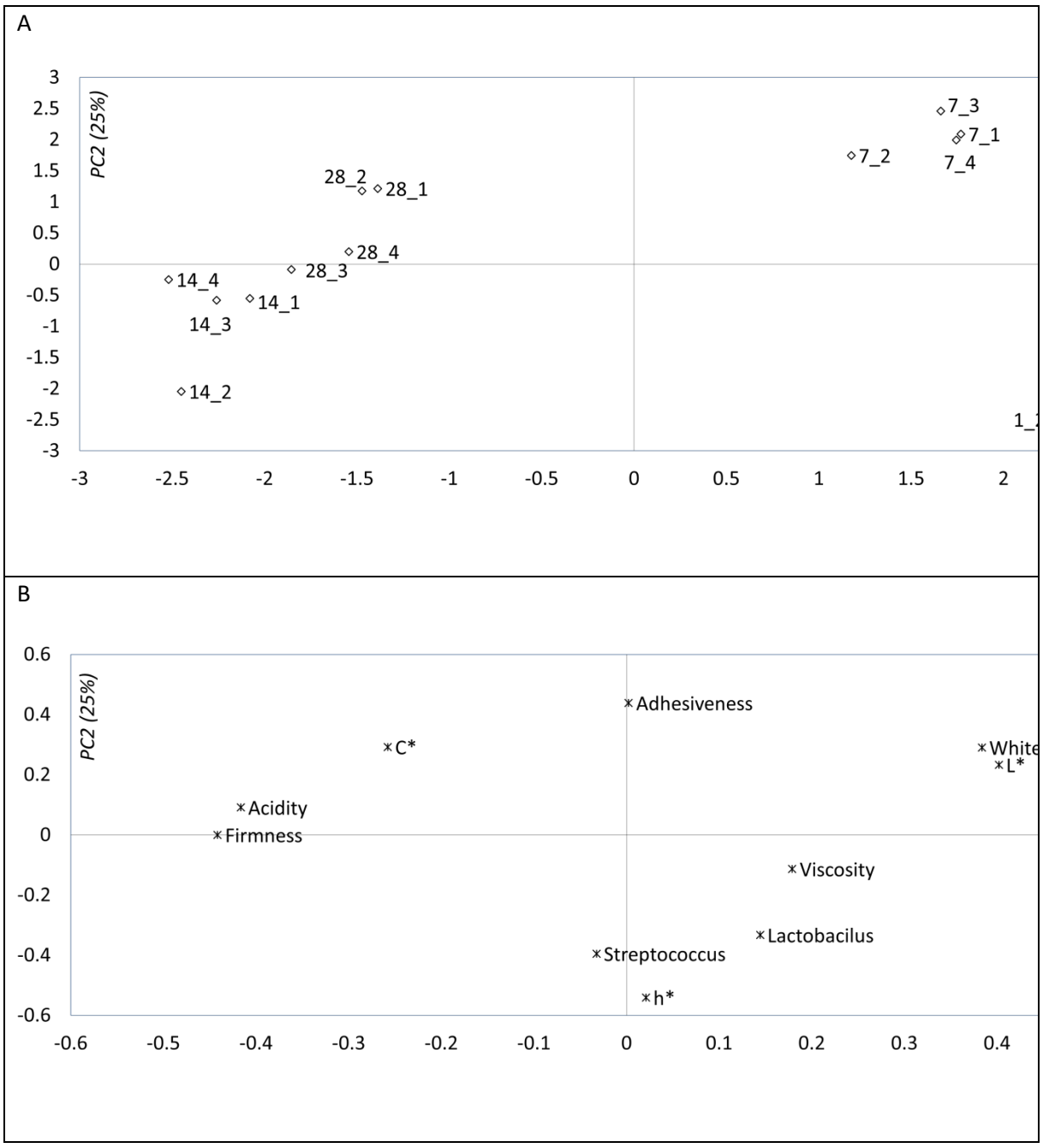

Fig. 3. Enrofloxacin residues in goat's milk yoghurts