Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com

Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications

Roberto Cabizza[a], Nicolino Rubattu[b], Severyn Salis[b], Massimo Pes[c], Roberta Comunian[c], Antonio Paba[c], Elisabetta Daga[c], Margherita Addis[c], M. Cecilia Testa[b], P. Paolo Urgeghe[a, *]

[a] Dipartimento di Agraria, Università degli Studi di Sassari, Viale Italia, 39, 07100 Sassari, Italy
[b] Struttura Complessa Sicurezza Alimentare, Istituto Zooprofilattico Sperimentale della Sardegna, 07100, Via Vienna, 2, Sassari, Italy
[c] AGRIS Sardegna, loc. Bonassai, km 18.600 SS291, 07100 Sassari, Italy

ARTICLE INFO

Keywords:
Oxytetracycline
Ovine milk
Thermisation
Antibiotic residues
Starter LABs

ABSTRACT

A few studies have been published on the distribution of oxytetracyline (OTC) residues present in milk among cheese, whey, and milk protein fractions, throughout cheese-making, most of them, focused on the effect of pasteurization and Ultra High Temperature (UHT) treatments, and carried out on cow milk. This study aimed to investigate the impact of a thermisation treatment of ovine milk spiked with oxytetracycline at MRL (maximum residue limit) and half MRL, on the fate of the molecule, and the effect of OTC residues on starter culture development and cheese composition. The antibiotic recovery and partition from milk into whey and cheese were assessed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Starter and non-starter microflora development was monitored by viable plate counts. Milk thermisation did not affect OTC recovery, partition and cheese chemical composition. On a dry matter basis, an OTC reduction between 15 and 19% was calculated in 60-day cheese, at MRL and half MRL, respectively. OTC caused a dose-dependent difference in the time required to reach pH 5.60, which was significantly higher ($P < 0.05$) at MRL level compared to half MRL and control, allowing coliform bacteria to reach 6 log CFU g^{-1} in 1-day MRL OTC cheeses.

1. Introduction

Antimicrobial agents are an important tool to treat and prevent diseases and as metaphylaxis in food producing animals. However, their presence as residues in foods of animal origin may lead to the possible induction of allergic reactions in consumers (Adetunji, 2011), and contribute to the development of antimicrobial resistance phenomena (AMR) (European Food Safety Authority EFSA, 2017).

Antibiotics residues occur in milk mainly as a result of incorrect veterinary treatments, such as the adoption of uncorrected withdrawal periods, or the unregulated use of extra label molecules (Beyene, 2016). Sometimes, these wrong practices can lead to overcome the maximum residue limit (MRL) adopted for milk by the international authorities in order to protect the consumers.

Moreover, some authors (Berruga, Beltrán Martínez, Novés, Molina, & Molina, 2011) reported that residual sub-MRL concentrations of antibiotics may be able to affect the manufacturing process of dairy products. Microorganisms continuously exposed to antibiotics at sub-inhibitory concentrations can undergo a specific transcriptional change that will modify their metabolic activity (Broszat & Grohmann, 2014). For instance, residues of OTC in yogurt and cheese may cause a significant delay in the pH decrease rate and defects in the development of lactic acid bacteria (Berruga, Battacone, Molina, Román, & Molina, 2008; Cabizza et al., 2017; Suhren, 1998), and interfere with the coagulation and ripening phases affecting the sensory properties of cheeses (Berruga, Molina, Althaus, & Molina, 2016; Nagel et al., 2009).

A few studies have been published on the distribution of antibiotics residues among cheese, whey, and milk protein fractions, during cheese-making. Shappell et al. (2017), working in tubes, at a laboratory scale, found that only 14% of oxytetracycline (OTC) added to the milk was recovered in the curd, and, based on lipophilicity characteristics of various type of animal drugs molecules, provided an empirical model for predicting their distribution between cow skim milk curd, whey and associations with proteins. However, the actual process conditions and the cheese-making technology applied should be taken into account, since they could affect the molecules distribution. Indeed,

* Corresponding author.
Email address: paolou@uniss.it (P.Paolo Urgeghe)

https://doi.org/10.1016/j.lwt.2018.05.026
Received 31 October 2017; Received in revised form 8 May 2018; Accepted 9 May 2018
Available online xxx
0023-6438/

Cabizza et al. (2017), who studied the transfer of oxytetracycline added in ovine raw milk at MRL level (100 μg kg^{-1}), showed that the cheese was able to retain most of the molecule added to the milk and the presence of the molecule produced a delay during cheese-making in the early acidification phase.

The thermal treatment of milk is a common practice before cheese-making. Indeed, most of the ovine milk undergoes thermisation or pasteurization in order to contrast the development of pathogen and spoilage bacteria, preventing possible technological defects in cheese. Therefore, the ability of these treatments to reduce the antibiotics concentrations should be also assessed taking into account the specific technological process. Some authors conducted studies on the effect of heat treatment on the stability of β-lactams, quinolones, aminoglycosides in milk (Zorraquino, Althaus, Roca, & Molina, 2011, 2009; Roca, Castillo, Marti, Althaus, & Molina, 2010; Roca, Villegas, Kortabitarte, Althaus, & Molina, 2011; Zorraquino, Roca, Castillo, Althaus, & Molina, 2008a; Zorraquino, Roca, Fernandez, Molina, & Althaus, 2008b).

The available studies on the effect of milk thermal treatments on OTC degradation were mainly carried out on cow milk and focused on pasteurization, while no data are available on ovine milk and the effect of thermisation. Shahani (1958) reported a 23.6% degradation of OTC, in milk spiked at 320–900 μg L^{-1}, after a long time low temperature (LTLT) treatment (61.7 °C, 30 min). Kellnerová, Navrátilová, and Borkovcová (2015) reported a 15.3% degradation of OTC in 1.5 MRL spiked milk, after high pasteurization treatment (85 °C, 3 s), while an ultra-high-temperature sterilization (UHT) was shown to be effective to completely degrade the OTC spiked in McIlvaine buffer (Hassani, Lázaro, Pérez, Condón, & Pagán, 2008). Zorraquino, Althaus, Nagel, Roca, and Molina (2010) investigated the effect of different heat treatments on the antimicrobial activity of milk samples spiked with OTC by antimicrobial assay. The thermal treatments produced a high loss of antimicrobial activity with classic sterilization (120 °C, 20 min), while LTLT treatment and UHT produced a little loss of antimicrobial activity. However, most of these studies were focused on hardly comparable OTC concentrations ranges and time-temperature combinations, performed in laboratory conditions, and often on cow milk or other matrices. Moreover, the impact on the microbiota is even less studied and, to date, a study assessing the effect of low-dosage antibiotics on starter lactic acid bacteria development during cheese-making lacks.

The aim of this work was to assess the effect of a thermisation treatment of ovine milk spiked with oxytetracycline, both at half MRL and MRL, on the recovery and partition of OTC residues in resulting cheese and whey, and investigate their possible effect on starter culture development, and composition of 1- and 60-day-old cheeses.

2. Material and methods

2.1. Chemicals and reagents

Oxytetracycline hydrochloride (OTC, purity ≥ 96.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA); 4-epi-oxytetracycline (EOTC, purity ≥ 97%), α-apo-oxytetracycline (α-apo-OTC) and β-apo-oxytetracycline (β-apo-OTC) were purchased from Acros Organics (Geel, Belgium). All solvents used were liquid chromatography-mass spectrometry (LC-MS) grade from Carlo Erba (Milan, Italy), and LC-MS grade water was produced with an Advantage System (Millipore, Billerica, MA, USA). Stock and spiking solutions were prepared as previously described (Cabizza et al., 2017).

2.2. Cheese-making process

Ovine milk was collected from an experimental flock of Sarda breed sheep at AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy). Sheep were in good health and did not undergo antibiotics treatments. The milk was divided into 3 jacketed vats of 12 kg each. The first one was the control (not spiked), the second and the third one were spiked with OTC in order to obtain milk at half MRL (50 μg kg^{-1}) and at MRL level (100 μg kg^{-1}), respectively. Subsequently, raw milk was heated up to 63 °C, and immediately cooled until 38 °C. Thermal treatment was performed in batch using circulating water jacket at 68 °C and 12 °C during the increasing and the decreasing step, respectively. The temperature of milk during the treatment was simultaneously measured every minute in each vat by a thermocouple. The average heat penetration curve is reported in Fig. 1.

Then, the starter culture was inoculated (6 log CFU g^{-1}, CHOOZIT® Su Casu LYO, Danisco, Denmark) and calf rennet added (500 IMCU kg^{-1}, Bellucci, Italy), following the cheese-making process described in Fig. 2, in order to obtain uncooked, hard, pecorino cheeses ripened until 60 days. The experiment was replicated three times in a short period (3 consecutive weeks, in January 2016) to minimize the possible effect of milk composition.

Data from the heat penetration curves obtained in the present work were used to calculate the cook value or C value (Awuah, Ramaswamy, & Economides, 2007), as reported in Equation (1):

$$C = \int_0^t 10^{(T-T_r)/z_r} dt \quad (1)$$

where t is the duration of treatment (min), T is the temperature, T_r is the reference temperature and z_r is the reference temperature resistance coefficient. The trapezoidal rule was used for integration. The same procedure was used to estimate C values of thermal treatment performed by Shahani (1958) and Kellnerová et al. (2015).

2.3. Milk and cheese composition, mass balance and component recoveries (fat and protein)

The parameters of milk composition were evaluated by using MilkoScan FT+ (Foss, Hillerød, Denmark) as described in ISO 9622 (ISO, 2013). pH was measured by pH meter (Knick 911, Knick, Berlin, Germany), equipped with a InLab® Solids electrode (Mettler Toledo, Ohio, USA). The pH of cheeses was measured every 60 min until 300 min and then every 30 min until pH 5.5. Total solids were measured according to the ISO method 6731 (ISO, 2010). Cheeses, at 1 and 60 days of ripening, were analysed for many parameters: pH (pH meter Crison Basic 20+, equipped with a 5232 Puncture electrode); dry matter in accordance with ISO 5534 (ISO, 2004); fat (Soxhlet, 1879), lipolysis index (Nuñez, García-Aser, Rodríguez-Martin, Medina, & Gaya, 1986); protein in accordance with ISO 8968-1(ISO, 2014); pH 4.6-soluble N, 12% trichloroacetic acid soluble N, 5% phosphotungstic acid-soluble N as described by Gripon, Desmazeaud, Le Bars, and Bergere (1975); total ash by gravimetric analysis after ashing the sample at 550 °C for 12 h according to ISO 27:1964 (ISO, 1964); NaCl according to ISO 5943:2006 (ISO, 2006). Each analysis was performed in duplicate. Mass balance and components recoveries (fat and protein) in 1-day cheese were expressed as indicated in Cabizza et al. (2017).

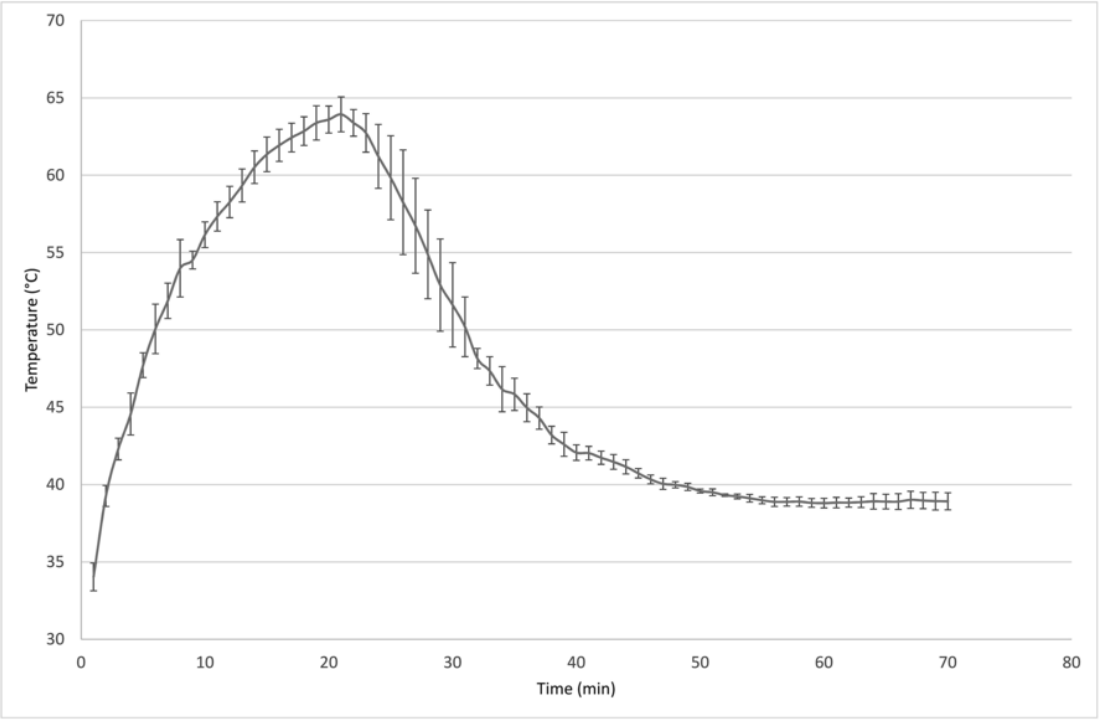

Fig. 1. Heat penetration curve of the adopted thermal treatment. The points are mean ± standard deviation of the average temperature of the three vats during each cheese-making session (n = 3).

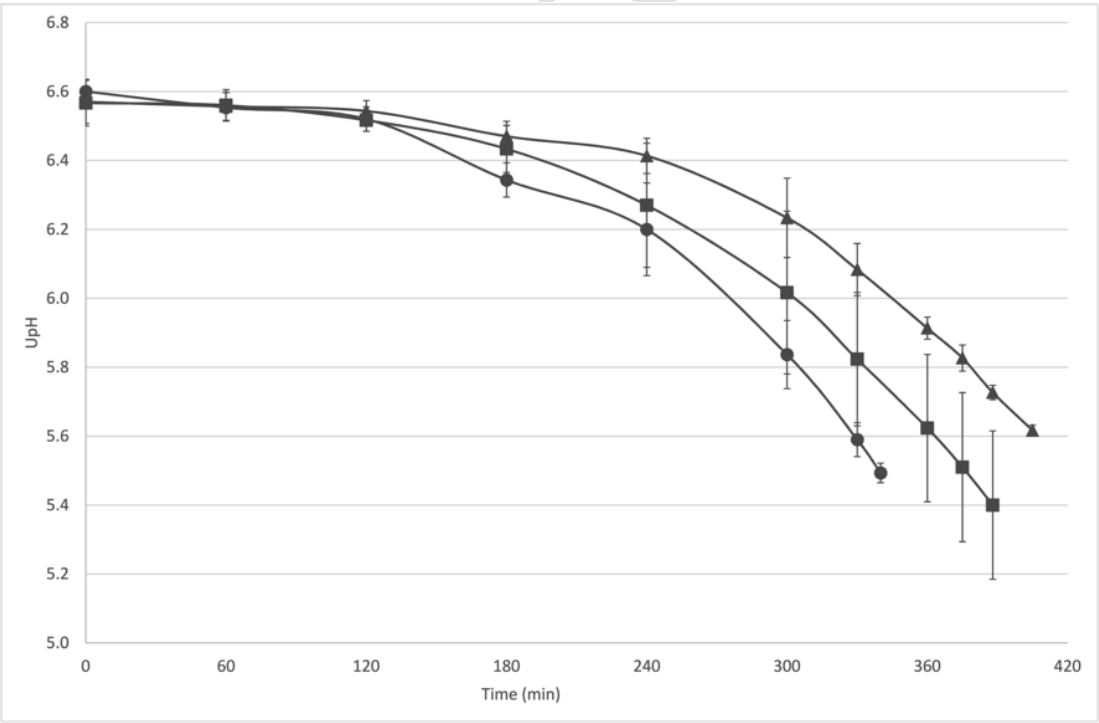

Fig. 2. Cheese-making process.

2.4. Measurement of OTC levels in milk, whey and cheese

2.4.1. Sample extraction and solid phase extraction parameters

Milk and whey samples (2.0 ± 0.1 g) were weighed into a 25 mL centrifuge tube; 10 mL of Mc Ilvaine EDTA buffer (pH 4) were then added and mixture was agitated using a vortex (Classic Advanced Vortex Mixer, Velp Scientifica, Usmate Velate, MB) and centrifuged at 12800 × g for 10 min at 0 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The purification and extraction phases were achieved as described in Cabizza et al. (2017), with except to final volume of the eluates after reconstitution in 14% MeOH (2 mL). Similarly, the whole

preparation protocol of cheese samples was as described in Cabizza et al. (2017).

2.4.2. Liquid chromatography-tandem mass spectrometry analysis

Liquid chromatography-tandem high-resolution mass spectrometry (LC-HRMS) analysis was performed using an UPLC Ultimate 3000 (Thermo Fisher-Dionex San Jose, CA, USA) system was coupled by a HESI-II electrospray source to a Q-Exactive Orbitrap™-based mass spectrometer (all Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed on Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm particles) column preceded by a guard column BEH Shield RP18 (5 mm × 2.1 mm, 1.7 μm particles), gradient elution with H_2O with 0.1% (v/v) formic acid (A) and MeCN (B). The injection volume was 2 μL, the flow rate was 0.400 mL min^{-1}, and the adopted gradient varied the % of A to B as follows: T 0.0 min, 80%; T 0.50 min, 80%; T 5 min, 0%; T 6.50 min, 0%; T 6.60 min, 80%; T 8.00 min, 80%; the target column temperature was 45 °C.

Q-Orbitrap HRMS with HESI-II electrospray source was operated in positive mode.

The following ionization parameters were applied: electrospray voltage 4 kV for positive mode, capillary temperature 300 °C, Aux gas heater temp 330 °C, sheath gas (N_2) 40 arbitrary units (arb), auxiliary gas (N_2) 10 (arb), and S-Lens RF level at 50 (arb). LTQ Velos ESI positive-ion calibration solutions (Thermo Scientific, San Jose, CA). The acquisition was achieved in full scan/dd-MS^2 mode. Full MS mode: mass range m/z 150–500, resolution of 70000 FWHM (m/z 200), AGC target 3.0E6, maximum injection time of 100 ms; dd-MS^2 mode: resolution of 17500 FWHM (m/z 200), AGC target was set at 1.0E5 ions, maximum injection time of 75 ms, isolation window of m/z 2.0.

The obtained retention time (RT) of analytes of OTC, EOTC, α-apo-OTC and β-apo-OTC were 2.56, 2.21, 3.04 min, respectively. MS monitored masses in HESI + mode (m/z) were: OTC, precursor 461.1555, fragments 426.1 and 337.1; EOTC, precursor 461.1555, fragments 426.1 and 444.1; α-apo-OTC and β-apo-OTC, precursor 443.1449, fragment 426.1 (fragment ions are used only for qualitative purposes).

2.4.3. Method validation

The method performance was assessed by an in-house validation protocol applied to the milk, whey and cheese, as described in Cabizza et al. (2017). The values obtained for each criteria adopted are reported below. The trueness obtained, expressed as the percent of recovery, was 102 for milk and whey and 80 for cheese. The limit of quantification was 1 μg kg^{-1} for milk and whey, and 10 μg kg^{-1} for cheese. The repeatability, expressed as the relative standard deviation "within-lab" repeatability (RSDwr; %), was 9% for milk and whey and 10% for cheese. The extended uncertainty was 22% and 25% for milk, whey and cheese, respectively.

2.5. Microbiological analysis

Samples of thermised milk, before and after the inoculum of the starter culture, 1 and 60 days ripened cheeses were prepared according to the IDF standard 122C (1996). Viable counts were carried out to enumerate: starter lactic acid bacteria (SLAB), i.e. thermophilic cocci and lactobacilli, on M17 agar and MRS agar media (Microbiol, Cagliari, Italy), respectively, incubated at 45 °C, in anaerobiosis for 72 h; non-starter lactic acid bacteria (NSLAB), i.e. mesophilic lactobacilli, on FH agar medium (Isolini, Grand, & Glaettli, 1990), incubated at 37 °C, in anaerobiosis for 72 h; Enterococci on KAA medium (Microbiol), incubated at 42 °C, in aerobiosis, for 18–24 h; coliform bacteria, on VRBA MUG (Microbiol), incubated at 37 °C, in aerobiosis for 18–24 h.

2.6. Statistical analysis

Statistical analysis was performed by Statgraphics Centurion XVI for Windows software package (version 16.2.04; Statpoint Technologies, Inc. Warrenton, Virginia, VA, USA). Analysis of variance (ANOVA) was carried out using the general linear model (GLM) to determine the effects of treatment and replicate on each response variable. Tukey's multiple comparison test was used for paired comparison of treatment means ($P < 0.05$).

3. Results and discussion

3.1. Milk and cheese composition, mass balance and components recoveries

The physical-chemical characteristics of the milk (mean ± s.d.) were: total solids 15.1 ± 0.1; protein, 4.8 ± 0.1; fat, 4.9 ± 0.1; casein, 3.5 ± 0.1; and lactose, 4.8 ± 0.1 (g 100 g^{-1} of milk). The pH was 6.7 ± 0.1.

OTC spiked milk (half MRL and MRL) showed a dose-dependent delay during the early acidification phase (Fig. 3), until reaching pH 5.60 (35 ± 11 and 78 ± 26 min, at half MRL and MRL levels). In fact, the time required to reach pH 5.60 was significantly higher at the MRL level (406 ± 2 min), compared to the control (328 ± 24 min, $P = 0.0050$) and markedly higher, even not significantly, compared to half MRL (363 ± 30 min, $P = 0.0666$).

These results are in accordance with those previously noticed for raw milk by Cabizza et al. (2017), as milk thermisation did not affect OTC activity and its influence on starter culture acidification performance.

The delay in acidification profiles did not result in a different mass balance (weights of sweet whey, draining whey and cheese, as proportions of total processed milk weight) between control and experiments (Table 1). Control and OTC (both half MRL and MRL) cheese yields, calculated as ratio between produced cheese at 1-day and milk used for cheese-making, were comparable. Similarly, the same component recoveries in control and experimental cheeses were observed, as already noticed for cheese obtained from raw milk (Cabizza et al., 2017). Furthermore, no differences in pH, composition and soluble N levels, between control and experimental cheeses, both at 1 and 60 days of ripening, were observed (Table 2). The chemical characteristics of 60-day ripened cheeses were close to those of Pecorino Sardo cheese (Pirisi, Comunian, Urgeghe, & Scintu, 2011).

3.2. Measurement of OTC levels in fractions

The thermal treatment of spiked milk samples did not reduce the concentration of OTC both in half MRL (47 ± 6 μg kg^{-1} and 42 ± 1 μg kg^{-1}, raw vs. thermised) and MRL (90 ± 11 μg kg^{-1} and 90 ± 13 μg kg^{-1}, raw vs. thermised) spiked milk samples. Using for OTC a z_{ref} value of 8.88 °C and a T_{ref} of 71.1 °C, as provided by Shahani (1958) for cow milk, in the pasteurization temperature range, we obtained a C value of 1.64 ± 0.13. This value should be equivalent to about 84% of that estimated (C value of 1.84) using the conditions applied (85 °C, 3 s) by Kellnerová et al. (2015), which resulted in a 15.3% antibiotic drop. On this basis, a residual concentration of 36.4 ± 0.5 μg kg^{-1} and 78 ± 11 μg kg^{-1} should have been expected for samples spiked at half MRL and MRL, respectively. However, the heat degradation of oxytetracycline is reported to be also dependent from the total solid content of the milk, with a lower level of degradation as the solid content, particularly fat, increases (Moats, 1999; Tian, Khalil, & Bayen, 2017). Ovine milk, whose total solid, especially fat, content is

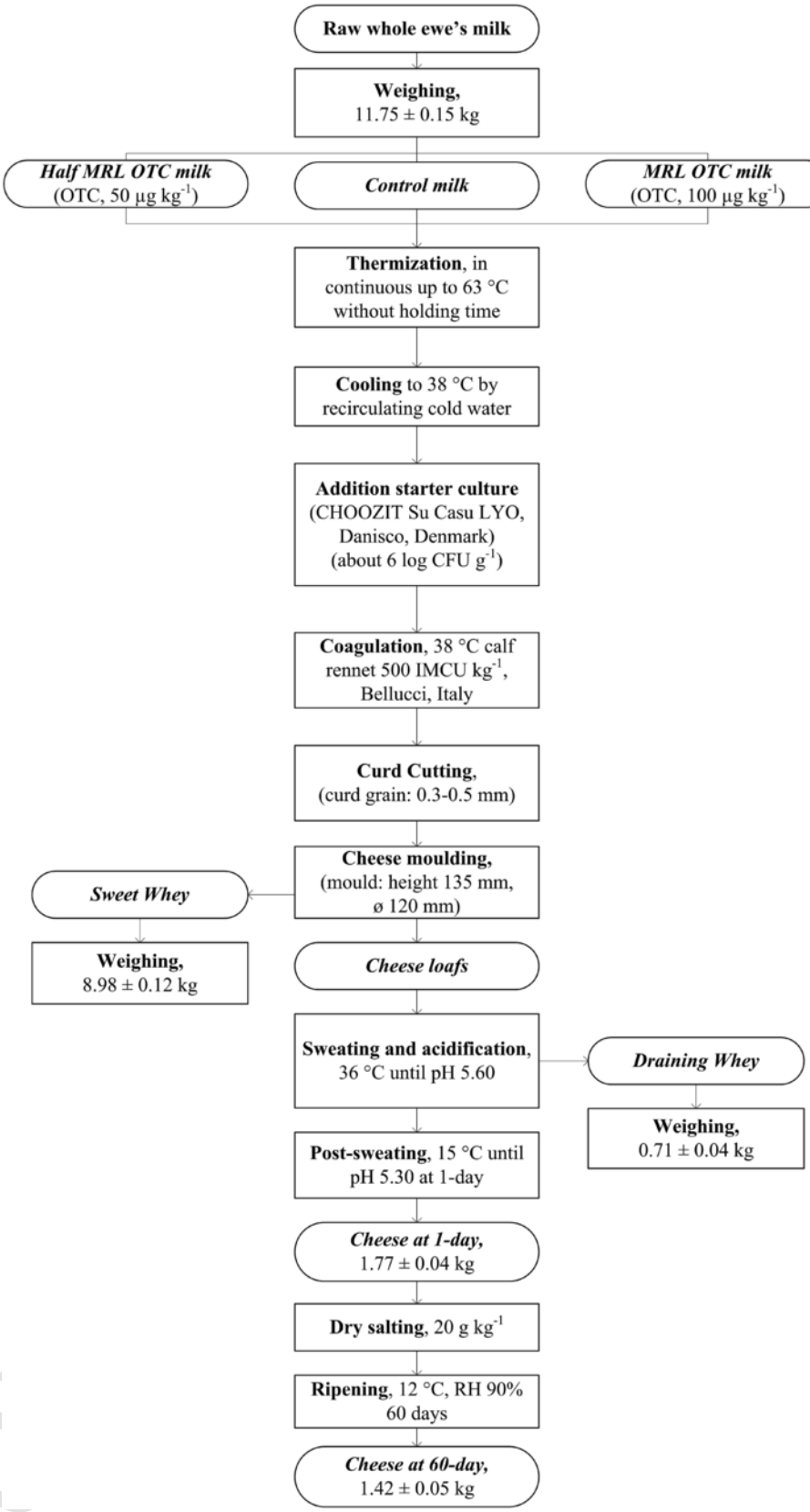

Fig. 3. Acidification curves. The points of each curve are mean values ± standard deviation (n = 3). Control (—●—), Half MRL OTC (—■—) and MRL OTC (—▲—).

Table 1
Technological parameters.

Parameter	Control			Half MRL OTC			MRL OTC		
Time to reach pH 5.60 [A]	328[b]	±	24	363[b]	±	30	406[a]	±	2
Mass balance [B]									
Sweet whey	76[a]	±	1	76.6[a]	±	0.2	76.4[a]	±	0.7
Draining whey	6.1[a]	±	0.4	6.0[a]	±	0.3	6.0[a]	±	0.2
1-day cheese	15.1[a]	±	0.1	15[a]	±	0.1	15.1[a]	±	0.5
Recovery (%) [C]									
Fat	84[a]	±	4	84[a]	±	3	84[a]	±	6
Protein	73[a]	±	1	73[a]	±	2	74[a]	±	2

[A] Time expressed in minutes during the acidification phase to reach pH 5.60.
[B] Values (g $100\,g^{-1}$ of milk) are means ± standard deviation (n = 3). Mass balance defined as outputs (sweet whey, draining whey, cheese) as a percentage of milk total weight.
[C] Recovery in 1-day cheese, defined as percentage of fat and protein weight in cheese on fat and protein weight in milk, respectively. Level of spike with oxytetracycline (OTC): Half MRL, $50\,\mu g\,kg^{-1}$; MRL, $100\,\mu g\,kg^{-1}$. Values are means ± standard deviation (n = 3); values within a row not sharing common superscript letters were significantly different ($P < 0.05$).

higher than cow milk (Park, Juárez, Ramos, & Haenlein, 2007), could have protected OTC from degradation during the thermal treatment applied in this study. The adopted time-temperature profiles in cheese-making practices usually are milder than that reported in the above-cited literature, and the treatment is often performed at the plant by continuous systems. Further, as many cheeses derived from milk undergone to thermisation, particularly those made from ovine milk (Pirisi & Pes, 2011), it can expect that the thermal treatment of ovine milk, in cheese-making conditions usually applied at dairy plant, should produce a poor or negligible degradation of OTC.

The distribution between wheys (sweet and draining) and cheese of the OTC added in milk is reported in Table 3. The partition between the obtained fractions appeared to be independent from the spike level, and was similar to that previously observed in the experiments performed with raw milk (Cabizza et al., 2017), except for the cheese fraction, which was able to retain a greater amount of the added molecule (about 80%) than the cheese made from raw milk (about 60%). The overall molecule recovery, close to 100%, obtained in this study, could be ascribable to the better performances provided by the adopted analytical method, involving LC-HRMS, indicating that the unrecovered amount (17%) obtained by Cabizza et al. (2017) could be attributable to the cheese.

The concentration of OTC in cheese, for both the studied levels, did not decrease with ripening, (Table 3), differing from what previously observed for cheese obtained from raw milk (Cabizza et al., 2017). Actually, in absence of degradation phenomena, an increase in concentration of OTC should be expected, as for the other components, due to the reduction of moisture. However, considering the concentration on a dry matter basis, it was possible to calculate a reduction of OTC with ripening, from $413 \pm 11\,\mu g\,kg^{-1}$ to $335 \pm 3\,\mu g\,kg^{-1}$ of dry matter (19 ± 2%), and from $832 \pm 57\,\mu g\,kg^{-1}$ to $707 \pm 25\,\mu g\,kg^{-1}$ of dry matter (15 ± 4%), in half MRL and MRL cheeses, respectively. Some consequences of the thermal treatment of milk, such as the modification of the enzymatic pattern and the reduction of the redox potential (McSweeney & Fox, 2009) could have contributed to the stability of OTC during ripening. In our opinion this could be a reason of the lower reduction of OTC (about 50%) compared to that previously observed in raw milk cheese (Cabizza et al., 2017), even if, in that case, cheese underwent to a longer ripening period.

3.3. Microbiological analysis

Results of viable counts, carried out to verify the effect of OTC on microflora development in 1-day and 60-day cheeses, are reported in Table 4.

No significant differences were detected between viable counts of the microbial groups searched in control and experimental thermised milk samples, both before and after the starter inoculum, whose level was confirmed to be about 6 log CFU mL^{-1}. NSLAB, Enterococci and coliform bacteria counts were < 1 log CFU g^{-1} in all the thermised milk samples analysed.

In 1-day cheese, SLAB (thermophilic lactobacilli and thermophilic streptococci) reached the same average log CFU g^{-1} in control and experimental samples, despite a pronounced delay during the acidification phase. Whereas, coliform bacteria counts were significantly higher ($P < 0.05$) in MRL level, than in half MRL and control samples. This difference is attributable to the acidification delay, which resulted in more favourable environmental growth conditions (higher pH in OTC cheeses than in control ones) for coliform bacteria that were able to reach 6 log CFU g^{-1}. This is a crucial issue to be taken into account, since high levels of coliform bacteria could have negative technological implications during the cheese-making, resulting into cheese defects, as well as being a safety concern for human health, particularly in short-ripened cheeses.

In 60-day cheese, thermophilic lactobacilli decreased about 1 log CFU g^{-1}, in all samples, while thermophilic streptococci remained stable in both OTC spiked samples, and decreased about 1 log CFU g^{-1} in the control, whose counts resulted significantly different ($P < 0.05$) from both OTC samples. In fact, as reported by Broszat and Grohmann (2014), a higher ability to survive could be acquired by microorganism as stress response to sub-inhibitory concentrations of antibiotics that could modify their metabolic activity. As expected, NSLAB, whose important role during ripening is well known, though were almost undetectable in 1-day cheese, increased in all samples, until 4 log CFU g^{-1}. Enterococci remained constant, while coliform bacteria decreased during ripening (about 4 log CFU g^{-1}), showing no significant differences among counts of OTC (both MRL and half MRL) and control 60-day cheeses.

4. Conclusions

The thermal treatment performed did not reduce the concentration of OTC, both in half MRL and MRL spiked ovine milk samples, probably because of a protective effect of the higher total solid content of the ovine milk, especially fat, compared to cow milk. No differences on mass balance, yields, chemical composition between experimental and control cheese-making processes, 1 and 60-day cheeses, were observed.

The LC-HRMS based method adopted allowed to obtain an overall recovery of the molecule spiked in milk close to 100%.

The OTC residues in cheese underwent a modest decrease during ripening. Thermisation may have caused a reduction of the redox potential and a selection of the enzymatic pattern, which contributed to the stability of the molecule, resulting in a lower decrease, compared to that observed for raw milk cheese, in a previous work.

Table 2
Physico-chemical parameters of 1-day and 60-day cheese [A].

Parameter	1-day cheese									60-day cheese								
	Control			Half MRL OTC			MRL OTC			Control			Half MRL OTC			MRL OTC		
pH	5.4[a]	±	0.1	5.4[a]	±	0.1	5.4[a]	±	0.1	5.1[a]	±	0.1	5.2[a]	±	0.1	5.2[a]	±	0.1
Moisture (g 100 g^{-1} of cheese)	45.0[a]	±	0.4	45.2[a]	±	0.6	45.0[a]	±	0.9	33.9[a]	±	0.8	33.9[a]	±	0.8	34[a]	±	1
FDM (g 100 g^{-1} DM)	50[a]	±	1	50[a]	±	1	49[a]	±	2	49.8[a]	±	0.5	50.0[a]	±	0.2	49.9[a]	±	0.3
PDM (g 100 g^{-1} DM)	41.7[a]	±	0.7	42.1[a]	±	0.3	42.3[a]	±	0.3	40.5[a]	±	0.4	41[a]	±	1	41[a]	±	1
SN (g 100 g^{-1} TN)	6.9[a]	±	0.8	8[a]	±	1	7[a]	±	2	13.7[a]	±	0.3	15[a]	±	1	15[a]	±	1
TCA-SN (g 100 g^{-1} TN)	2.7[a]	±	0.6	2.6[a]	±	0.6	2.6[a]	±	0.6	8[a]	±	1	8.9[a]	±	0.6	8.6[a]	±	0.9
PTA-SN (g 100 g^{-1} TN)	1.4[a]	±	0.2	1.5[a]	±	0.2	1.5[a]	±	0.2	4.0[a]	±	0.2	3.9[a]	±	0.3	3.8[a]	±	0.3
FFA (meq 100 g^{-1} fat)	1.0[a]	±	0.3	1.3[a]	±	0.2	1.0[a]	±	0.2	1.8[a]	±	0.1	2.1[a]	±	0.8	1.3[a]	±	0.4
Ash (g 100 g^{-1} of cheese)	2.7[a]	±	0.3	3[a]	±	0.2	3.3[a]	±	1	5.2[a]	±	0.4	4.9[a]	±	0.7	5.2[a]	±	0.2
Salt (g 100 g^{-1} of moisture)	–			–			–			6.2[a]	±	0.7	6.4[a]	±	0.7	6.5[a]	±	1.1

[A] Values are means ± standard deviation (n = 3); Abbreviations are: OTC, oxytetracycline; MRL, maximum residue limit; FDM, fat in dry matter; PDM, protein in dry matter; TN, total nitrogen; SN, nitrogen soluble in water; TCA-SN, nitrogen soluble in 12% trichloroacetic acid; PTA-SN, nitrogen soluble in 10% phosphotungstic acid; FFA, free fat acids.

Table 3
Distribution of OTC in milk, wheys and cheese [A].

	Half MRL OTC			MRL OTC		
OTC partition (%)						
Milk	100			100		
1-day cheese before salting	81.4	±	0.8	77	±	8
Sweet whey	23	±	9	20	±	3
Draining whey	2.9	±	0.5	2.2	±	0.4
OTC Concentration ($\mu g\ kg^{-1}$)						
Milk	41.8	±	0.6	90	±	13
1-day cheese before salting	226	±	4	457	±	34
Sweet whey	13	±	5	24	±	6
Draining whey	20	±	2	33	±	3
60-day cheese	222	±	3	470	±	18

[A] Values (%, μg kg^{-1}) are means ± standard deviation (n = 3); OTC partition (%) in component derived from milk, defined as percentage of absolute amount (μg) in 1-day cheese, sweet whey and draining whey on total absolute amount of OTC added in milk (50 μg kg^{-1}, Half MRL OTC; 100 μg kg^{-1}, MRL OTC).

The observed delay in acidification of thermised milk spiked with OTC, compared to an antibiotic free control, proved a dose-dependent influence of low levels OTC residues on the starter culture development and metabolism, which affected lactic acid production. However, analysing 1-day cheese samples no differences in pH and SLAB counts were detected, leading to assume a temporary OTC effect, limited to the early hours of the acidification phase that has to be further investigated.

Acknowledgments

This work was supported by the Italian Health Ministry. [Current Research IZSSA 05/13].

Table 4
Thermised milk, thermised milk + starter, 1-day and 60-day cheese viable counts [A].

Parameter	Thermised milk									Thermised milk + starter								
	Control			Half MRL OTC			MRL OTC			Control			Half MRL OTC			MRL OTC		
Thermophilic cocci	1.1^{a}	±	0.5	1.37^{a}	±	0.05	1.42^{a}	±	0.14	5.6^{a}	±	0.1	5.9^{a}	±	0.1	5.8^{a}	±	0.1
Thermophilic lactobacilli	0.6^{a}	±	0.6	1.0^{a}	±	0.2	1.0^{a}	±	0.1	4.23^{a}	±	0.08	4.49^{a}	±	0.05	4.3^{a}	±	0.1
Mesophilic lactobacilli	0^{a}	±	0	0.1^{a}	±	0.1	0.3^{a}	±	0.4	0.1^{a}	±	0.2	0^{a}	±	0	0^{a}	±	0
Enterococci	0.2^{a}	±	0.3	0^{a}	±	0	0.6^{a}	±	0.6	0^{a}	±	0	1^{a}	±	1	0.4^{a}	±	0.7
Coliform bacteria	0.3^{a}	±	0.5	0.4^{a}	±	0.5	0^{a}	±	0	0.3^{a}	±	0.5	0.3^{a}	±	0.6	0.1^{a}	±	0.2
Parameter	1-day cheese									60-day cheese								
	Control			Half MRL OTC			MRL OTC			Control			Half MRL OTC			MRL OTC		
Thermophilic cocci	8.6^{a}	±	0.4	8.6^{a}	±	0.4	8.8^{a}	±	0.5	7.7^{b}	±	0.3	8.6^{a}	±	0.4	8.6^{a}	±	0.4
Thermophilic lactobacilli	5.3^{a}	±	0.3	5.6^{a}	±	0.2	5.5^{a}	±	0.1	4.6^{a}	±	0.4	4.8^{a}	±	0.4	4.7^{a}	±	0.2
Mesophilic lactobacilli	0.2^{a}	±	0.4	0.1^{a}	±	0.1	0^{a}	±	0	4.7^{a}	±	0.2	4^{a}	±	2	5^{a}	±	2
Enterococci	3.5^{a}	±	0.6	4.4^{a}	±	0.4	4^{a}	±	1	3.6^{a}	±	0.6	4.1^{a}	±	0.5	4^{a}	±	1
Coliform bacteria	5.1^{b}	±	0.2	4.8^{b}	±	0.5	6.1^{a}	±	0.1	1.7^{a}	±	0.5	1^{a}	±	1	2^{a}	±	3

[A] Values (log CFU mL^{-1} for milk and log CFU g^{-1} for cheese) are means ± standard deviation (n = 3). Values within a row not sharing common superscript letters were significantly different ($P < 0.05$).

References

Adetunji, V.O., 2011. Effects of processing on antibiotic residues (streptomycin, penicillin − G and tetracycline) in soft cheese and yoghurt processing lines. Pakistan Journal of Nutrition 10 (8), 792–795.

Awuah, G.B., Ramaswamy, H.S., Economides, A., 2007. Thermal processing and quality: Principles and overview. Chemical Engineering and Processing 46, 584–602.

Berruga, M.I., Battacone, G., Molina, M.P., Román, M., Molina, A., 2008. Influence of β-lactams on manchego cheese manufacture. In: International Dairy Federation (Ed.), Special issue of the international dairy federation 0801/Part 3 (special is, vol. 3). International Dairy Federation, Brussels, Belgium.

Berruga, M.I., Beltrán Martínez, M., Novés, B., Molina, A., Molina, M.P., 2011. Effect of penicillins on the acidification of yogurt made from Ewe's milk during the storage. In: Mendez-Vilas, A. (Ed.), Science and technology against microbial pathogens. World Scientific, Valladolid, Spain, pp. 145–149.

Berruga, M.I., Molina, A., Althaus, R.L., Molina, M.P., 2016. Control and prevention of antibiotic residues and contaminants in sheep and Goat's milk. Small Ruminant Research

Beyene, T., 2016. Veterinary drug residues in food-animal products: Its risk factors and potential effects on public health. Veterinary Science & Technology 7 (1), 1–7.

Broszat, M., Grohmann, E., 2014. Spread of antibiotic resistance in the environment: Impact on human health. In: Malik, A., et al. (Eds.), Environmental deterioration and human health. Springer Science + Business Media, Dordrecht (The Netherlands), pp. 125–162.

Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., et al., 2017. Transfer of oxytetracycline from ovine spiked milk to whey and cheese. In: International dairy journal, 70(special issue from the 5th IDF cheese ripening symposium, dublin, 11-13th april 2016). pp. 12–17.

European Food Safety Authority EFSA, 2017. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. EFSA Journal 15 (2), 4694.

Gripon, J.C., Desmazeaud, M.J., Le Bars, D., Bergere, J.L., 1975. Etude du rôle des micro-organismes et des enzymes au cours de la maturation des fromages. II. - influence de la présure commerciale. Le Lait 55 (548), 502–516.

Hassani, M., Lázaro, R., Pérez, C., Condón, S., Pagán, R., 2008. Thermostability of oxytetracycline, tetracycline, and doxycycline at ultrahigh temperatures. Journal of Agricultural and Food Chemistry 56 (8), 2676–2680.

IDF, 1996. Milk and milk products preparation of samples and dilutions for microbiological examination. IDF Standard 122 C. International Dairy Federation, Brussels, Belgium.

ISO, 1964. Milk and liquid milk products - determination of ash content. ISO 27:1964. International Organisation for Standardisation, Geneva, Switzerland.

ISO, 2004. Cheese and processed cheese - determination of the total solids content (Reference Method ISO 5534/IDF 4:2004). International Organisation for Standardization, Geneva, Switzerland.

ISO, 2006. Cheese and processed cheese products - determination of chloride content - potentiometric titration method. ISO 5943/IDF 88:2006. International Organisation for Standardization, Geneva, Switzerland.

ISO, 2010. Milk, cream and evaporated milk - determination of total solids content (Reference method ISO 6731/IDF 21:2010). International Organisation for Standardization, Geneva, Switzerland.

ISO, 2013. Milk and liquid milk products - guidelines for the application of midinfrared spectrometry ISO 9622:2013/IDF 141:2013. International Organisation for Standardisation, Geneva, Switzerland.

ISO, 2014. Milk and milk products - determination of nitrogen content. Part 1: Kjeldahl principle and crude protein. ISO 8968-1/IDF 20-1:2014. International Organisation for Standardization, Geneva, Switzerland.

Isolini, D., Grand, M., Glaettli, H., 1990. Selective media for the enumeration of obligately and facultatively heterofermentative lactobacilli. Schweizerische Milchwirtschaftliche Forschung 19 (344), 57–59.

Kellnerová, E., Navrátilová, P., Borkovcová, I., 2015. Effect of pasteurization on the residues of tetracyclines in milk. Acta Veterinaria Brno 83 (10), S21–S26.

McSweeney, P., Fox, P.F., 2009. Advanced dairy chemistry volume 3: Lactose, water, salts and minor constituents. Springer New York.

Moats, W.A., 1999. Veterinary residues in foods. In: Jackson, L.S., Knize, M.G., Morgan, J.N. (Eds.), Impact of processing on food safety, 1st ed. Springer US, New York, USA, pp. 233–241.

Nagel, O., Zapata, M., Basílico, J.C., Bertero, J., Molina, M.P., Althaus, R., 2009. Effect of chloramphenicol on a bioassay response for the detection of tetracycline residues in milk. Journal of Food and Drug Analysis 17 (1), 36–42.

Nuñez, M., García-Aser, C., Rodríguez-Martin, M.A., Medina, M., Gaya, P., 1986. The effect of ripening and cooking temperatures on proteolysis and lipolysis in Manchego Cheese. Food Chemistry 21, 115–123.

Park, Y.W., Juárez, M., Ramos, M., Haenlein, G.F.W., 2007. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Research 68 (1–2), , 88–13.

Pirisi, A., Comunian, R., Urgeghe, P.P., Scintu, M.F., 2011. Sheep's and goat's dairy products in Italy: Technological, chemical, microbiological, and sensory aspects. Small Ruminant Research 101 (1–3), 102–112.

Pirisi, A., Pes, M., 2011. Formaggi ovi-caprini. In: Tecniche-Nuove (Ed.), Manuale lattiero caseario. Vol. 1, pp. 1–49, (Milano, Italy).

Roca, M., Castillo, M., Marti, P., Althaus, R.L., Molina, M.P., 2010. Effect of heating on the stability of quinolones in milk. Journal of Agricultural and Food Chemistry 58 (9), 5427–5431.

Roca, M., Villegas, L., Kortabitarte, M.L., Althaus, R.L., Molina, M.P., 2011. Effect of heat treatments on stability of β-lactams in milk. Journal of Dairy Science 94 (3), 1155–1164.

Shahani, K.M., 1958. Factors affecting terramycin activity in milk, broth, buffer, and water. Journal of Dairy Science 41, 382–391.

Shappell, N.W., Shelver, W.L., Lupton, S.J., Fanaselle, W., Van Doren, J.M., Hakk, H., 2017. Distribution of animal drugs among curd, whey, and milk protein fractions in spiked skim milk and whey. Journal of Agricultural and Food Chemistry 65, 938–949.

Soxhlet, F., 1879. Die gewichtsanalytische Bestimmung des Milchfettes. Dinglers Polytechnisches Journal 232, 461–465.

Suhren, G., 1998. Influence of antimicrobials on starter cultures in milk – model trials. In: Gaukel, V., Spieß, W.E.L. (Eds.), 3rd karlsruhe nutrition Symposium: European Research towards safer and better food. Review and transfer congress. Druckerei Grässer, Karlsruhe (Germany), pp. 3–9.

Tian, L., Khalil, S., Bayen, S., 2017. Effect of thermal treatments on the degradation of antibiotic residues in food. Critical Reviews in Food Science and Nutrition 57 (17), 3760–3770.

Zorraquino, M.A., Althaus, R.L., Nagel, O.G., Roca, M., Molina, M.P., 2010. Efecto de los tratamientos térmicos sobre la actividad antimicrobiana de tetraciclinas en leche. Revista FAVE - Ciencias Veterinarias 9 (2), 60–68.

Zorraquino, M.A., Althaus, R.L., Roca, M., Molina, M.P., 2009. Effect of heat treatments on aminoglycosides in milk. Journal of Food Protection 72 (6), 1338–1341.

Zorraquino, M.A., Althaus, R.L., Roca, M., Molina, M.P., 2011. Heat treatment effects on the antimicrobial activity of macrolide and lincosamide antibiotics in milk. Journal of Food Protection 74 (2), 311–315.

Zorraquino, M.A., Roca, M., Castillo, M., Althaus, R.L., Molina, M.P., 2008a. Effect of thermal treatments on the activity of quinolones in milk. Milchwissenschaft 63 (2), 192–195.

Zorraquino, M.A., Roca, M., Fernandez, N., Molina, M.P., Althaus, R.L., 2008b. Heat inactivation of B-lactam antibiotics in milk. Journal of Food Protection 71 (6), 1193–1198.