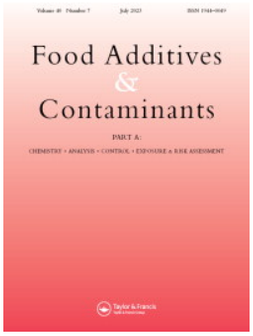

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Transfer of β-lactam and tetracycline antibiotics from spiked bovine milk to Dambo-type cheese, whey, and whey powder

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ABSTRACT

The aim of this study was to investigate the transfer of residues of five β-lactam antibiotics (ampicillin, penicillin G, cloxacillin, dicloxacillin and cephalexin) and two tetracyclines (tetracycline and oxytetracycline) in the processing of cheese and whey powder, evaluating the effect of the processes and the final concentration in each product generated. Raw milk was fortified at two concentration levels with the seven antibiotics. The first concentration level (C1) was chosen according to the maximum residue limit (MRL) of each antibiotic (ampicillin and penicillin G: 4 μg kg^{-1}; cloxacillin and dicloxacillin: 30 μg kg^{-1}; cephalexin, tetracycline and oxytetracycline: 100 μg kg^{-1}). The second concentration level (C2) was spiked as follows according to each antibiotic: 0.5 MRL (cloxacillin, dicloxacillin, cephalexin), 0.1 MRL (tetracycline and oxytetracycline) and 3 MRL (ampicillin and penicillin G). The antibiotics were analyzed by LC-MS/MS. No ampicillin or penicillin G residues were found in cheese or whey powder, although they were detected in whey at concentrations similar to those added to raw milk. Cephalexin was mostly distributed in whey between 82% and 96%, being the antibiotic that presented the highest concentration in whey powder (784 ± 98 μg kg^{-1}) when milk was spiked at the MRL. The whey distribution of cloxacillin and dicloxacillin ranged from 57% to 59% for cloxacillin and from 46% to 48% for dicloxacillin, and both concentrated in whey powder. Tetracyclines were the antibiotics that concentrated in cheese, with retentions between 75% and 80% for oxytetracycline and between 83% and 87% for tetracycline. The distribution of antibiotics in the dissimilar stages of the cheese and whey powder production processes, as well as their concentration in the final products, depend on each type of antibiotic. Knowledge of the transfer of antibiotic residues during the process and final disposal is an input for the risk assessment of their consumption.

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Introduction

Antimicrobials are components of intensive animal production systems. They are classified into the following groups: β-lactams, sulfonamides, carbapenems, aminoglycosides, glycopeptides, lincomycin, macrolides, polypeptides, polyenes, rifamycin, tetracyclines, chloramphenicol, quinolones, and fluoroquinolones (Gothwal and Shashidhar 2015). Antibiotics are used in the treatment of infectious diseases in dairy farms mainly for the control of mastitis, and metritis (Jamali et al. 2018). There are studies reporting that 73% of antimicrobials sold are used in production animals (Tiseo et al. 2020).

The use of β-lactam antibiotics in milking animals is increasing (Sachi et al. 2019). Likewise, for dry cow udder therapy, penicillin G and cephapirin are preferred (Oliver et al. 2011). In Uruguay, β-lactams (penicillin, ampicillin, amoxicillin, cloxacillin, etc.) are one of the most widely used and recommended groups of antimicrobial agents as first-line drugs, due to their low cost, toxicity, and risk of development of antimicrobial resistance (Gianneechini et al. 2005).

Veterinary drug residues pose potential risks to human and animal health, as well as to industrial processes. From a health point of view, in humans, they can cause allergic reactions and

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adverse effects on the intestinal flora and can contribute to the development of antimicrobial resistance (Van Boeckel et al. 2015). From a technological point of view, in the dairy industry, antimicrobials can slow down or inhibit the processes of acidification, curdling and ripening, deteriorating organoleptic properties and even completely inhibiting fermentation in the production of cheeses and yogurts (Grunwald and Petz 2003; Berruga et al. 2008, 2016). There are international regulations that ban the processing of milk with antibiotic residues above the maximum residue limit (MRL) (Codex Alimentarius 2018). Globally, while there are MRLs for milk, there are no MRLs for milk-based foodstuffs which is a limitation for control bodies and possible international trade agreements (Cabizza et al. 2017).

The various technological processes used to produce dairy products affect the transfer, degradation and/or concentration of antibiotic residues from milk. Some authors have studied the effect of heat treatments on β-lactam antibiotics (Zorraquino et al. 2008; Roca et al. 2010; Garzon et al. 2020), and on tetracyclines (Kurjogi et al. 2019; Khaskheli et al. 2021). The distribution of diverse types of antibiotics between the fat and non-fat phases of milk depends on the drug used and the partition between the hydrophobic and hydrophilic phases (Hakk et al. 2016; Ozdemir et al. 2018), affecting the final antibiotic concentration depending on the antibiotic and the composition of the final product.

In recent years, there has been an increasing number of publications describing the distribution and concentration of antibiotic residues in various dairy products for a better understanding of the potential risk of consumption exposure. However, considering the diverse types of antibiotics and milk from various sources, there is still limited information available. In fatty products such as butter, studies have been reported with cloxacillin (Gbylik-Sikorska et al. 2021), cefquinome (Di Rocco et al. 2021) and tetracyclines (Gajda et al. 2018). In yogurts and fermented milks, there are studies conducted on the residues of tetracycline, penicillin, cloxacillin, oxacillin, dicloxacillin and ampicillin (Grunwald and Petz 2003; Adetunji 2011; Gbylik-Sikorska et al. 2021). The effect of cheese and whey processing with oxytetracycline residue was studied in bovine milk (Shappell et al. 2017; Gajda et al. 2018; Hassan et al. 2021), sheep milk (Cabizza et al. 2017, 2018) and goat milk (Quintanilla et al. 2019), while in the case of β-lactams, penicillin G and cloxacillin are the most studied (Adetunji 2011; Shappell et al. 2017; Quintanilla et al. 2019; Gbylik-Sikorska et al. 2021).

There are around 1,500 cheese varieties around the world, being a widely consumed product with a global production of 19,000,000 tons per year. Approximately, 35% of the milk produced worldwide is used for cheese production (Fox et al. 2017). Dambo-type cheese is a semi-hard and mid-moisture product (MERCOSUR1994) made from bovine milk. Whey from cheese manufacturing has become a valuable raw material for the manufacture of whey powder and other derivatives. Knowledge of the distribution and effect of the processes involved in the manufacture of cheese, whey, and whey powder on antibiotic residues at concentrations close to the MRL is important to promote product safety and avoid potential risks associated with consumption. Therefore, considering the most-used antibiotics in dairy farms, the objective of this work was to study the effect of the processing and residue transfer of five antimicrobial β-lactams (ampicillin, penicillin G, cloxacillin, dicloxacillin and cephalexin) and two tetracyclines (tetracycline and oxytetracycline) spiked to bovine milk, in the production of Dambo-type cheese, whey and whey powder.

Materials and methods

Chemicals and reagents

Reference standard: cloxacillin sodium monohydrate (purity 98%), ampicillin trihydrate (purity 99%), cephalexin monohydrate, oxytetracycline, and tetracycline were purchased from LGC GmbH (Luckenwalde, Germany); Penicillin G Sodium (USP, Reference standard); dicloxacillin sodium salt hydrate (Sigma-Aldrich St. Louis, MO, USA). Acetonitrile (ACN), HPLC-MS grade, was from Carlo Erba (Milan, Italy). Formic acid was from Merck. Ammonium formate was from Sigma-Aldrich. Water was deionized (>18 MΩ cm^{-1})

in-house using a Millipore system (Purelab Ultra, Elga). All reagents were at least HPLC grade.

Stock solutions of standards at 0.25 mg mL^{-1}, 2.5 µg mL^{-1} and 0.25 µg mL^{-1} concentrations were prepared according to the solubility characteristics of the given antibiotics: cephalexin in 1:9 water/methanol mixture; cloxacillin, dicloxacillin, ampicillin, penicillin G and tetracyclines in 1:9 acetonitrile/-water. Stock solutions were kept at 5 °C for a week. Working solutions were prepared daily and stored at 5 °C until spiked samples were prepared.

Experimental design

Antibiotic-free bovine raw milk was obtained from the experimental herd of the Veterinary Faculty of the Universidad de la República (San José, Uruguay). The study was carried out over 3 weeks, and each week 180 L were collected from the herd. The chemical composition of the milk used was total solids, 13.28 ± 0.26% (w/w); protein, 3.40 ± 0.12% (w/w); fat, 4.36 ± 0.15% (w/w); and lactose, 4.55 ± 0.03% (w/w). The whole raw milk was divided into three portions of 60 kg each, one of which was considered as a control (not spiked). The second portion was fortified to reach an antibiotic concentration level similar to the Codex Alimentarius MRL (C1) (ampicillin and penicillin G: 4 µg kg^{-1}; cloxacillin and dicloxacillin: 30 µg kg^{-1}; cephalexin, tetracycline and oxytetracycline: 100 µg kg^{-1}). The third portion was spiked to reach antibiotic concentration levels equal to 0.5 MRL (cloxacillin, dicloxacillin, cephalexin), 0.1 MRL (tetracycline and oxytetracycline) and 3 MRL (ampicillin and penicillin G) values of the given antibiotic (C2). The antibiotics used in trials were all supplied by Sigma-Aldrich. The antibiotics were added individually to a portion of milk (~500 mL). The mixture was then poured into the tank totaling 60 kg of milk in order to maintain the concentration levels selected for this study. The spiked milk was then left for 30 min at 38 °C in the tank with agitation to allow the components to equilibrate.

Cheese making process

Cheese manufacture trials were carried out at Latitud - Fundación LATU pilot plant (Montevideo, Uruguay). Raw spiked milk was skimmed at 38 °C with a cream separator (GEA Westfalia Separator model LWA 205-1, Germany), and standardized to 2.96 ± 0.11% (w/w) fat. The Dambo-type cheeses were produced as previously described by Escobar et al. (2014). Briefly, the cheese making process was carried out in a 50-L double-O vat with a double jacket and a controlled mechanical agitation and cutting system. The milk was pasteurized at 72 °C for 15 s; when the milk had cooled to 32 °C, CaCl2 (Promilk®) and mesophilic homofermentative starter culture (CHR HANSEN R 704, Christian Hansen, Denmark) were added. Thirty minutes later, the 100% chymosin coagulant (Maxiren®) was added, and the cultured milk was allowed to set for approximately 30 min. The curd was cut until obtaining a size of "corn grain" and cooked at 42 °C (stirring continuously). After cooking, the whey was drained off, and the curd was distributed on a 1 kg polypropylene cylinder mold and pressed for 3 h (with intermediate rotations). The acidification of the cheese was measured after pressing, and the pH was checked until reaching the final pH of 5.40 ± 0.10 and was kept at 4 °C for 16 h. The cheeses were kept in a ripening chamber at 12 °C for 30 days until analysis. Subsequently, the cheeses were kept in a chamber at 4 ± 2 °C for 24 h, after which they were vacuum-packed. The cheeses were kept in a ripening chamber at 12 °C for 10 days until analysis.

Whey powder process

The whey obtained in the rennet cheese making process was filtered to remove small particles of curd. Fat in drained whey was separated using a separator (GEA Westfalia Separator, model LWA 205-1, Germany). After separation, whey was pasteurized at 72 °C for 15 s. After pasteurization, 30 kg of whey were nanofiltered at 20 ± 1 °C to obtain a nanofiltered retentate at 11% total solids. Nanofiltration was performed using a crossflow filtration pilot plant module 92.101 R4 (Iberlact, Spain) having an active membrane area of 1.87 m^2. During the trial, the transmembrane pressure was between 14.0 and 14.5 bar. The retentate flow was adjusted manually to obtain an average feed flow rate of 80 L/h. A circular nanofiltration membrane (model ATF 2540 - LS05 - S

Membrane; Nanofiltration, Parker, USA) with a molecular weight cutoff of 200 Da was used. The total time was approximately 2.5 h. After that, the nanofiltered retentate was spray dried using a laboratory-scale spray dryer (Buchi, model B-290), and the feed flow rate was 15 mL/min. The inlet and outlet temperatures were maintained at 170 °C and 65 °C, respectively.

Raw milk, cheese, and whey composition

Total solids, fat, protein and lactose in milk and whey were measured using MilkoScan FT2 (Foss, Hillerød, Denmark) according to the ISO 9622:2013 method (ISO 2013). Total solids were measured by gravimetric analysis after drying to constant weight at 102 ± 2 °C according to the ISO 6731:2010 method (ISO 2010). Total solids content according to ISO 5534 (ISO 2004); fat according to ISO 3433/IDF 222 (ISO 2008) and protein according to ISO 8968-1/IDF 20-1 (ISO 2014) were determined in samples of each cheese. Total solids, fat, protein, and lactose in whey powder were measured using INFRAXACT (Foss, Hillerød, Denmark) according to the ISO 21543/IDF 201:2006 method (ISO 2006).

Determination of β-lactam and tetracycline antibiotics in milk and dairy products

Milk, whey, and whey powder

Sample preparation. Milk and whey were kept at 4–8 °C before analysis, and whey powder was stored at 20–30 °C. Solid samples were dissolved in water, using the following procedure: 0.1 g of sample (whey powder) were weighed into a 15 mL centrifuge propylene tube, followed by the addition of 1.0 mL of ultrapure H2O. The samples were then vortexed for 5 min until total homogenization, before the extraction. Then, all dairy products followed the same procedure: 1.0 mL of sample in a 15 mL centrifuge propylene tube was extracted by addition of 1 mL of ACN (HPLC grade) and protein precipitating agent. The mixture was vortexed for 5 min, then mixed on a rotary stirrer for 10 min, followed by ultrasonic application for 10 min and centrifuged at 8500 g for 10 min at 4 °C. After this, 400 µL from the supernatant were passed through a 0.22 µm filter into a UniPrep vial and then analyzed by liquid chromatography coupled with a tandem quadrupole mass spectrometer system.

Analytical conditions of UPLC-MS. A UPLC Agilent 1290 system connected to the triple quadrupole mass spectrometer Agilent 6430 was used for the analysis. Chromatographic separation of β-lactams and tetracyclines was achieved using a Poroshell 120 EC-C18 (2.1×100 mm, 2.7 µm) Agilent column and C18 guard column (4x2 mm), with a mobile phase consisting of 1% formic acid and 0.5 mM ammonium formate in ACN (Solvent A) and 1% formic acid and 0.5 mM ammonium formate in H2O (Solvent B).

Gradient elution at a flow rate of 0.25 mL/min was performed as follows: 0.01–10.0 min (5% A), 10.1–13.0 (50% A), 13.1–16.00 (100% A), 16.1–30.0 (5% A). The column was maintained at 30 °C, the autosampler at 6 °C, and the injection volume of the sample was 10 µL. The detection was performed in the positive electrospray ion mode (ESI+) at an ion spray voltage of 4000 V, a source temperature of 300 °C, an ion source gas of 50 psi and a gas flow rate of 10 L/min.

The following transitions were used: Cephalexin m/z 348.1 > 158 Vfragmentador (Vf) of 62 and Collision Energy (CE) 5 and m/z 348.1 > 106.1 Vf of 62 and CE 29, Dicloxacillin m/z 470 > 160 Vf of 83 and CE 13, m/z 470 > 114.1 Vf of 83 and CE 45 m/z and 470 > 311 Vf of 83 and CE 9, Oxytetracycline m/z 461 > 426.1 Vf of 91 and CE 17, m/z 461 > 201 Vf of 91 and CE 45 and 461 > 443.2 Vf of 91 and CE 9, Tetracycline m/z 445 > 427 Vf of 120 and CE 15 and m/z 445 > 410 Vf of 120 and CE 20, Cloxacillin m/z 436.1 > 160 Vf of 99 and CE 11 and m/z 436.1 > 277 Vf of 99 and CE 9, Ampicillin m/z 350 > 106.1 Vf of 77 and CE 13, m/z 350 > 114 Vf of 77 and CE 33 and 350 > 160 Vf of 77 and CE 9, Penicillin G m/z 335.1 > 176.1 Vf of 67 and CE 9, m/z 335.1 > 160 Vf of 67 and CE 9 and 335.1 > 114 Vf of 67 and CE 33.

Method validation. The method developed for determining β-lactams and tetracyclines in dairy products was evaluated and validated according to European Council Decision 2002/657/EC

(European Commission 2002). The following validation studies were carried out: linearity, repeatability, and reproducibility (CV, %), recovery, decision limit (CCα), detection capability (CCβ), specificity, precision (CV, %, repeatability, and reproducibility).

Linearity was determined from a matrix-matched calibration curve (for each matrix) prepared by fortifying dairy products (control samples obtained during the experiments) at eight concentration levels corresponding to 2–100 µg/kg. Selectivity was investigated by analysis of different blank samples (for each matrix) to test for potential interferences with endogenous substances. Repeatability was determined after fortifying six blank samples at three concentration levels of 0.5, 1.0 and 1.5 MRL for each matrix. Samples were analyzed on the same day with the same instrument and the same operators, and CVs (%) were calculated. Within-laboratory reproducibility was determined after fortifying another two sets of blank samples at the same concentration levels as for repeatability, which were analyzed on three different days with the same instrument and different operators. The overall CVs (%) of the fortified samples were calculated. The average recovery was evaluated in the same experiment as repeatability by comparing the mean measured concentration with the fortified concentration of the samples. The decision limit (CCα) and detection capability (CCβ) were calculated on the chromatograms of 20 different blank samples. The limit of quantification (LOQ) was calculated as the lowest point of the method's matrix-matched calibration curve. Linearity was tested by preparing the matrix-matched calibration curve on eight concentration levels in the range 2 µg kg^{-1} - 100 µg kg^{-1}. The matrix effect (ME, %) was investigated by comparing the slope of the matrix-matched calibration curve with the slope of a standard calibration curve (the solvent is water) at the same concentration. Validation data are summarized in Table 1. Example of chromatograms (cloxacillin and oxytetracycline) obtained during the experiments (blank and spiked milk sample at 10 µg kg^{-1}), are shown in Figure 1.

The matrix-matched calibration curves of all matrices showed good linearity ($R2 > 0.997$) in the wide concentration range of 2 µg kg^{-1}–100 µg kg^{-1}. The average percentage of recovery was in the 75–110% range, depending on the matrix and concentrations. The results of the studies indicate that there is a matrix effect for all the dairy products.

Determination of antibiotic residues in cheese.

The analyses of tetracyclines and β-lactams in the cheeses were performed by Eurofins WEJ Contaminants GmbH, Hamburg, Germany. For the determination of tetracyclines, the internal standard and EDTA-Succinate buffer at pH 4 were added to the weighed sample. Then, the proteins were precipitated with ACN. Subsequently, NaCl was added to separate the phases and ethylene glycol in ethanol was added to an aliquot of the ACN layer. The ethylene glycol was evaporated, and the residue was reconstituted for analysis by LC-MS/MS. For β-lactam analysis, water was added to the weighed sample, and then ACN was added to precipitate the proteins. The sample was centrifuged, and an aliquot was analyzed by LC-MS/MS.

Statistical analysis

The analysis was performed under a mixed-effects modelling framework. Inferential results were obtained through likelihood ratio test (after optimizing the marginal likelihood) and p-values were declared significant at a 0.05 level. All analyses were obtained with R software (R Core Team 2021) using the nlme (to fit mixed-effects models) (Pinheiro et al. 2021) and emmeans (to obtain marginal means) (Lenth 2021) libraries.

Results and discussion

Compositional analyses in raw milk and dairy products

The composition of the raw milk used for cheese making, as well as the composition of the products resulting from the process are presented in Table 2. Fat concentration in raw milk used for the cheese making process was 4.36 ± 0.15%. After centrifugation, the skim milk showed a concentration of 0.72 ± 0.21% and was standardized using Pearson's correlation to a concentration of 2.96 ± 0.11% fat.

Table 1. Validation parameters of the HPLC-MS/MS method used for the determination of the antibiotic's concentration (whole milk, skim milk and whey).

Analyte	Concentration (μg kg^{-1})	Repeatability (%)	Recovery (%)	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
Cephalexin	6.25	12	86–98	0.5	0.9
	12.5	13			
	25	8			
Dicloxacillin	12.5	11	76–102	0.6	1.1
	25	15			
	37.5	8			
Oxytetracycline	6.25	16	66–98	2.8	4.0
	12.5	15			
	25	11			
	37.5	11			
Tetracycline	6.25	24	58–76	3.3	5.2
	12.5	10			
	25	11			
Cloxacillin	6.25	14	79–107	0.7	1.3
	12.5	9			
	25	14			
	37.5	10			
Ampicillin	4	10	91–98	1.9	3.2
	12.5	9			
	25	11			
	37.5	13			
Penicillin G	4	16	95–109	0.2	1.4
	12.5	15			
	25	13			
	37.5	12			

Replicates for each validation level: $n = 18$ (6 replicates of each level and the blank were made on the same day and repeated for three different days). This was done for each matrix (whole milk, skim milk, whey).

No adverse effects on the cheese making process and its by-products (processing times, cheese pH, yield) were observed when using milk spiked with the seven antibiotics at the MRL level. No significant differences ($p < 0.05$) were found between the fat, protein and moisture composition of cheeses spiked with antibiotics and the control, contrary to what was found by other authors who report delays in acidification in the cheese making process (Cabizza et al. 2017; Quintanilla et al. 2019). These results show that there was no inhibition of the starter used by spiking at the MRL with the seven antibiotics.

In order to carry out this study, preliminary trials were carried out in which each antibiotic was spiked to the milk at MRL, and the cheeses were checked for acidification up to a pH of 5.4 at 16 h after the end of pressing. The use of ceftiofur (at MRL) as an antibiotic was also planned for this study; however, it was ruled out because in all the preliminary tests performed, acidification (pH 6.5–6.7) was not achieved after 16 h of pressing the cheese. In studies conducted with ceftiofur in yogurts, it was found to produce a total inhibition of metabolic activity in five selected ferments containing strains of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* (Navrátilova et al. 2022).

Antibiotic residues in the cheese making process

Antibiotic residues were determined at all processing stages, from the spiked raw milk to the final products, cheese, whey, and whey powder.

The measured concentrations of antibiotic residues (ampicillin, penicillin G, cloxacillin, dicloxacillin, cephalexin and oxytetracycline and tetracycline) in whole milk, skim milk, standardized milk, whey, and cheese are presented in Table 3.

Concentration measurements of all antibiotics studied in whole, skim and standardized milk did not differ statistically ($p < 0.05$). These results show that all the antibiotics being studied were mostly distributed in the non-fat phase of milk. Similar results were found by other authors in studies of oxytetracycline and penicillin G. Hakk et al. (2016) found that oxytetracycline and penicillin G in bovine milk are mostly distributed in the aqueous phase. Hassan et al. (2021) studied the effect of skimming on oxytetracycline residue in the processing of white cheese and found no

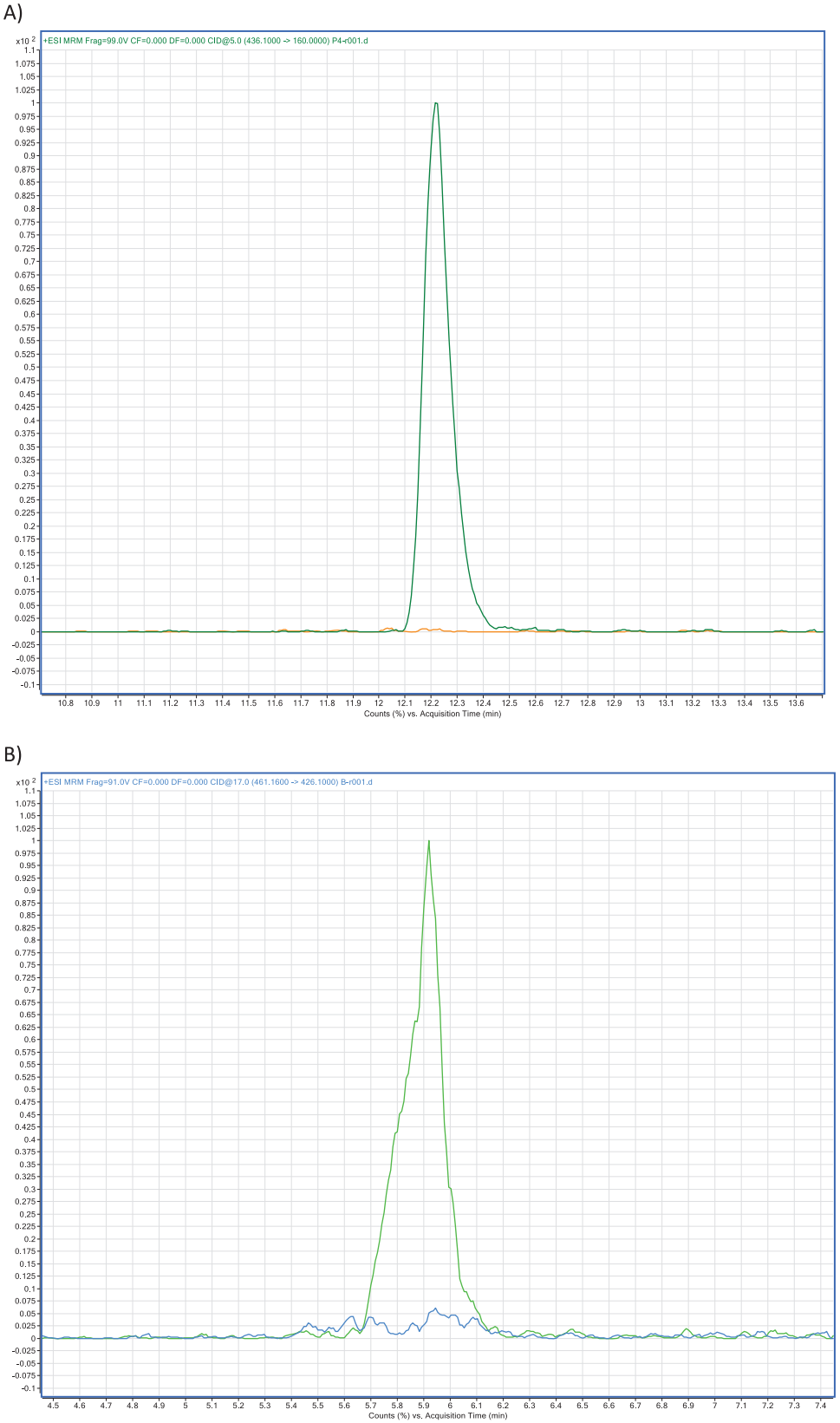

Figure 1. Chromatogram A) Cloxacillin: blank milk sample (orange line) and spiked milk sample at 10 μg kg^{-1} (green line). Chromatogram B) Oxytetracycline: blank milk sample (blue line) and spiked milk sample at 10 μg kg^{-1} (green line).

Table 2. Composition of raw, standardized bovine milk, cheese, whey, permeate, retentate and whey powder according to antibiotic spiked (C1 and C2) levels and control (C0).

Parameter	Antibiotic concentration level	Whole raw milk	Standarized milk	Cheese	Whey	Permeate	Retentate	Whey powder
Fat (%, w/w)	C1	4.36 ± 0.15	2.96 ± 0.11	22.25 ± 0.91	0.13 ± 0.02	<0.01	0.27 ± 0.07	4.46 ± 0.64
	C2		2.95 ± 0.03	22.75 ± 0.66	0.13 ± 0.03	<0.01	0.32 ± 0.04	4.61 ± 0.32
	C0		3.10 ± 0.11	22.75 ± 0.50	–	–	–	–
Protein	C1	3.40 ± 0.12	3.49 ± 0.13	23.12 ± 1.02	0.99 ± 0.02	0.17 ± 0.01	1.83 ± 0.01	20.58 ± 0.78
(%, w/w)	C2		3.49 ± 0.13	23.39 ± 0.70	0.98 ± 0.02	0.18 ± 0.02	2.01 ± 0.01	27.33 ± 1.25
	C0		3.49 ± 0.13	23.25 ± 1.60		0.17 ± 0.01	1.83 ± 0.01	20.58 ± 0.78
Total Solid (%, w/w)	C1	13.28 ± 0.26	11.94 ± 0.20	–	7.28 ± 0.07	–	–	–
	C2		11.96 ± 0.05	–	7.24 ± 0.04	–	–	–
	C0		12.10 ± 0.20	–	–	–	–	–
Moisture (%, w/w)	C1		–	48.25 ± 1.28	–	–	–	5.01 ± 0.92
	C2		–	47.53 ± 1.08	–	–	–	4.83 ± 0.98
	C0		–	47.80 ± 1.89	–	–	–	–
Lactose	C1		–	–	5.02 ± 0.03	3.42 ± 0.04	5.30 ± 0.04	60.30 ± 0.04
(%, w/w)	C2		–	–	5.01 ± 0.04	3.52 ± 0.04	5.10 ± 0.04	59.00 ± 0.04

Values are means ± standard deviation ($n = 6$). No significant differences ($p < 0.05$) were found between control cheese and cheese made from spiked milk. Antibiotic concentration level, C1 level: ampicillin and penicillin G (4 μg kg^{-1}); cloxacillin and dicloxacillin (30 μg kg^{-1}); cephalexin, tetracycline and oxytetracycline (100 μg kg^{-1}). C2 level: cloxacillin, dicloxacillin, cephalexin (50 μg kg^{-1}), tetracycline and oxytetracycline (10 μg kg^{-1} and ampicillin and penicillin G (12 μg kg^{-1}).

Table 3. Antibiotic residue concentration along the different processing stages in Dambo-type cheese making made from bovine milk spiked with antibiotic at C1 and C2 levels.

		Antibiotic concentration (μg kg^{-1})				
Antibiotic	Antibiotic concentration level	Whole milk	Skim milk	Standarized milk	Cheese	Whey
Ampicillin	**C1**	5.0 ± 1.3	5.6 ± 1.9	5.5 ± 2.0	<40*	5.2 ± 1.5
	C2	15.2 ± 1.8	14.6 ± 2.3	14.8 ± 3.3	<40*	12.0 ± 3.2
Penicillin G	**C1**	5.8 ± 1.2	5.7 ± 1.1	5.3 ± 1.2	<10*	4.0 ± 1.1
	C2	13.7 ± 2.5	11.8 ± 1.6	13.6 ± 3.8	<10*	10.0 ± 1.6
Cloxacillin	**C1**	32.8 ± 3.8	32.7 ± 2.7	31.7 ± 3.2	<20*	22.1 ± 1,6
	C2	16.6 ± 2.3	15.4 ± 3.2	15.6 ± 2.2	<20*	10.5 ± 0.7
Dicloxacillin	**C1**	32.4 ± 3.4	32.1 ± 5.0	31.7 ± 5.3	<20*	17.5 ± 0.5
	C2	16.5 ± 3.1	15.6 ± 4.8	15.5 ± 2.4	<20*	8.8 ± 0.2
Cephalexin	**C1**	95 ± 15	102 ± 30	96.5 ± 2,8	<150*	107 ± 11
	C2	56 ± 12	55.3 ± 6.8	53 ± 11	<150*	49.7 ± 7.4
Oxytetracycline	**C1**	96 ± 22	100 ± 26	90 ± 18	580 ± 96	41.3 ± 5.1
	C2	9.5 ± 2.0	9.9 ± 2.2	10.9 ± 2,8	90 ± 35	6.5 ± 0.7
Tetracycline	**C1**	88 ± 25	90 ± 32	87 ± 27	895 ± 87	16.7 ± 0.7
	C2	8.9 ± 4.2	8.5 ± 2.3	7.7 ± 2.8	102 ± 36	<6*

(*) indicates LOQ of each antibiotic. Values are means ± standard deviation ($n = 6$). No significant differences ($p < 0.05$) were found between whole, skim and standardized milk. Antibiotic concentration level, C1 level: ampicillin and penicillin G (4 μg kg^{-1}); cloxacillin and dicloxacillin (30 μg kg^{-1}) cephalexin, tetracycline and oxytetracycline (100 μg kg^{-1}). C2 level: cloxacillin, dicloxacillin, cephalexin (50 μg kg^{-1}), tetracycline and oxytetracycline (10 μg kg^{-1} and ampicillin and penicillin G (12 μg kg^{-1}).

significant difference between whole and skim milk when changing from 3.5 to 0.1% fat.

For all the β-lactam antibiotics studied (ampicillin, penicillin G, cloxacillin, dicloxacillin and cephalexin), the concentration of residues in cheese was lower than the limits of quantification (Table 3), which suggests that these antibiotics are not concentrated in cheese. The antibiotic concentration values found in the whey of cheeses made from milk spiked with ampicillin, penicillin G and cephalexin were similar to those of the initial raw milk, while those made from milk spiked with cloxacillin and dicloxacillin presented lower concentration values than the initial milk, between 33% and 37% less for cloxacillin and between 46% and 47% less for dicloxacillin. Therefore, the concentration of antibiotic residues in cheese whey is lower than the milk MRL for each type of antibiotic studied.

The results show that tetracycline and oxytetracycline remain mostly in the cheese, concentrating in it. In the studies carried out with milk spiked at values around the MRL, it was observed that oxytetracycline present at 100 ± 26 μg kg^{-1} in standardized milk, concentrates 5.8 times in cheese (580 ± 96 μg kg^{-1} in cheese) (Table 3). For tetracycline, the concentration is 10 times, with values of 895 ± 87 μg kg^{-1} in cheese obtained from 90 ± 32 μg kg^{-1} of standardized milk. When raw milk was spiked with tetracyclines

(oxytetracycline and tetracycline) at an initial concentration of MRL/10 ($10\,\mu g\,kg^{-1}$), the concentration of these tetracyclines in cheeses was 10 times higher than the initial values, with concentrations of $90 \pm 35\,\mu g\,kg^{-1}$ and $102 \pm 36\,\mu g\,kg^{-1}$ (Table 3). This indicates that even using values 10 times lower than the MRL in milk spiked with tetracyclines, these antibiotics appear concentrated in the cheese. Studies conducted on cheeses made from goat milk (Quintanilla et al. 2019) and sheep milk (Cabizza et al. 2017) showed similar results as they have found that oxytetracycline concentrates by 4.3 and 3.9 times, respectively, with respect to the initial concentration of that tetracycline in milk (using the MRL, $100\,\mu g\,kg^{-1}$).

Distribution of antibiotics in cheese and whey

The distribution of antibiotics in whey and cheese was studied, and mass balances of the corresponding flows were performed. Of the 49.9 ± 0.2 kg of standardized milk in each vat, 5.9 ± 0.4 kg of cheese and 41.9 ± 0.5 kg of filtered whey were obtained, resulting in 2.1 kg of weight loss between fines and drainage.

Figure 2(A) shows the retention of antibiotics in cheese whey for the two spiked concentration levels. β-lactam antibiotics were found to be mostly retained in whey, cephalexin being the antibiotic with the highest percentage of retention in whey, with $96 \pm 4\%$ and $82 \pm 10\%$ at C1 and C2 concentrations, respectively. Whey retention percentages close to and above 50% were found for ampicillin ($63 \pm 16\%$ and $69 \pm 14\%$), penicillin G ($57 \pm 13\%$ and $65 \pm 16\%$), cloxacillin ($59 \pm 5\%$ and $57 \pm 8\%$) and dicloxacillin ($46 \pm 3\%$ and $48 \pm 7\%$). In studies carried out by Gbylik-Sikorska et al. (2021), a high retention of cloxacillin in whey was also found, although with higher percentages (85%) than in this study (59%). The whey retention in the group of penicillins within the β-lactam antibiotics is lower than the retention of cephalexin, which was the only cephalosporin in the group studied. Tetracycline is the antibiotic with the lowest retention in whey, with only an18% retention. The retention of oxytetracycline found in cheese ranged between 75% and 80%, while that of tetracycline ranged between 83% and 87% (Figure 2(B)).

Quintanilla et al. (2019) further studied the retention of penicillin G and cloxacillin in cheese and obtained a low retention rate in goat cheese. These authors suggest that the low or high transfer of antibiotics from milk to cheese could be related to the solubility characteristics of each antibiotic. The β-lactams are highly soluble in water, which could explain their low retention in cheese. On the contrary, these authors refer to the fact that oxytetracycline shows a moderate affinity for fats, which could favor its retention in the cheese matrix containing fat and protein. On the other hand, other authors suggest that the higher retention of oxytetracycline in cheese is due to an interaction between tetracyclines and casein (Cabizza et al. 2017 and Hassan et al. 2021), in addition to the Ca^{2+} content in cheese. Another factor that could be affecting the distribution of tetracyclines in the products is the mineral balance of milk and cheese, and their high affinity for Ca^{2+} and Mg^{2+} ionic fractions (Arias et al. 2007). Further studies are needed to determine the nature of the different interactions that explain the association of tetracyclines with the main components of cheese.

Table 4 shows the distribution of β-lactams, taking the limit of quantification values of antibiotics in cheese, which allows us to have a maximum possible retention value in cheese. According to values in Table 4 and Figure 2(A), and if the retention in penicillin G, cloxacillin and dicloxacillin presented the maximum calculated values, the sum of antibiotics retained in whey and cheese is less than the antibiotics spiked in milk (100%). These results could be indicating a loss in these antibiotics, which could be found in the whey draining and/or filtration stages. Other potential causes for the losses could be related to temperature degradation during the heat treatments taking place in the manufacturing process. However, some studies have reported that penicillin G is stable at pasteurization temperature (63 °C, 30 min) (Zorraquino et al. 2008 and Roca et al. 2011), and low degradation rates have been reported for cloxacillin, 7% to 17%

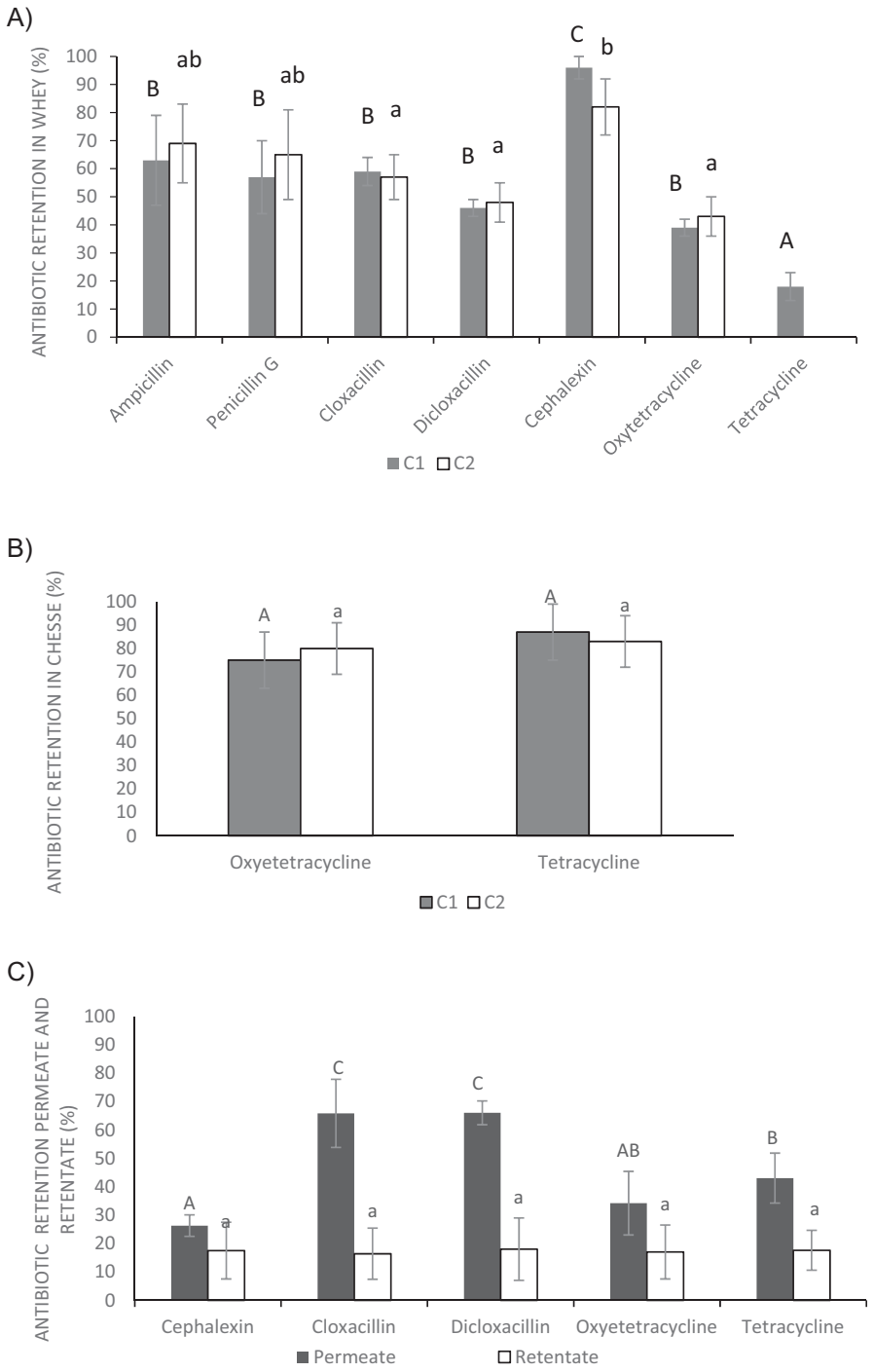

Figure 2. Antibiotic retention (%; mean ± standard deviation, $n = 6$) in: A) cheese whey as by-product of Dambo type cheese making from bovine whole milk spiked with antibiotics at C1 and C2 levels, B) Dambo type cheese from bovine whole milk spiked with oxytetracycline and tetracycline at C1 and C2 levels, C) whey permeate and retentate in the nanofiltration process of milk spiked with antibiotics at MRLs concentration (C1). Mean values bearing the same letter are not significantly different from each other ($p < 0.05$).

(Zorraquino et al. 2008, Roca et al. 2011 and Escobar et al. 2020) and for dicloxacillin, 27% (Escobar et al. 2020). On the other hand, given that cheese is a fermented product, there could be enzymatic interactions that degrade the antibiotic during cheese production. Rey et al. (2019) studied the potential for penicillin G degradation in milk using β-lactamase from *Enterobacter cloacae*, so further studies on milk-derived enzymes could be of interest to support

Table 4. Distribution of β-lactam antibiotic in cheese (calculated considering the limit of quantification values of antibiotics in cheese).

Antibiotic retention (%)						
Product	Antibiotic concentration level	Ampicillin	Pepicillin G	Cloxacillin	Dicloxacillin	Cephalexin
Milk	**C1**	100	100	100	100	100
	C2	100	100	100	100	100
Cheese	**C1**	$<94 \pm 24^*$	$<21 \pm 3^*$	$<8 \pm 1^*$	$<8 \pm 1^*$	$<19 \pm 1^*$
	C2	$<33 \pm 11^*$	$<9 \pm 3^*$	$<15 \pm 3^*$	$<15 \pm 1^*$	$<34 \pm 57^*$

(*) indicates that retention was calculated considering the equivalent value to the limit of quantification in cheese (ampicillin: 40 μg kg^{-1}, penicillin G: 10 μg kg^{-1}, cloxacillin and dicloxacillin: 20 μg $kg^{-1}$and cephalexin: 150 μg kg^{-1}).

Table 5. Antibiotic residue concentration along the different processing stages of whey powder.

Antibiotic concentration (μg kg^{-1})						
Antibiotic		Whey	Pasteurized whey	Permeate	Retentate	Whey powder
Ampicillin	C1	5.2 ± 1.5	4.3 ± 0.7	$<4^*$	5.3 ± 1.5	ND**
	C2	12.0 ± 3.2	10.9 ± 2.9	$<4^*$	6.0 ± 3.4	ND**
Penicillin G	C1	4.0 ± 1.1	4.2 ± 1.0	$<4^*$	$<4^*$	ND**
	C2	10.0 ± 1.6	10.3 ± 1.1	$<4^*$	$<4^*$	ND**
Cloxacillin	C1	22.1 ± 1.6	22.6 ± 1.2	17.4 ± 1.1	23.4 ± 2.9	139 ± 32
	C2	10.5 ± 0.7	11.2 ± 0.7	9.0 ± 1.1	9.7 ± 4.0	65.4 ± 5.5
Dicloxacillin	C1	17.5 ± 0.5	17.0 ± 0.5	13.4 ± 1.9	18.6 ± 2.7	123 ± 21
	C2	9.0 ± 0.2	9.0 ± 0.6	$<9^*$	$<9^*$	66.6 ± 5.8
Cephalexin	C1	107 ± 11	110 ± 15	33.9 ± 3.9	112 ± 16	784 ± 98
	C2	49.7 ± 7.4	53.5 ± 7.7	15.9 ± 2.9	52.3 ± 2.6	406 ± 54
Oxytetracycline	C1	41.3 ± 5.1	42.2 ± 3.7	17.0 ± 5.7	44.6 ± 9.8	520 ± 31
	C2	6.5 ± 0.7	6.5 ± 0.5	$<6^*$	7.3 ± 2.3	ND**
Tetracycline	C1	16.7 ± 0.7	17.6 ± 1.9	8.9 ± 1.5	18.7 ± 2.5	142 ± 19
	C2	$<6^*$	$<6^*$	$<6^*$	$<6^*$	ND**

*Indicates LOQ.
**ND: Not detected (result < limit of detection).
Values are means ± standard deviation ($n = 6$). No significant differences ($p < 0.05$) were found between whey and pasteurized whey. Antibiotic concentration level, C1 level: ampicillin and penicillin G (4 μg kg^{-1}); cloxacillin and dicloxacillin (30 μg kg^{-1}); cephalexin, tetracycline and oxytetracycline (100 μg kg^{-1}). C2 level: cloxacillin, dicloxacillin, cephalexin (50 μg kg^{-1}), tetracycline and oxytetracycline (10 μg kg^{-1} and ampicillin and penicillin G (12 μg kg^{-1}).

or rule out the hypothesis that these enzymes may be degrading antibiotics during the cheese making and ripening process.

Antibiotic residues in permeate, retentate and whey powder

The measured concentrations of antibiotic residues (ampicillin, penicillin G, cloxacillin, dicloxacillin, cephalexin and oxytetracycline and tetracycline) in whey permeate, whey retentate and whey powder are presented in Table 5. The pasteurization process (72 °C for 15 s) performed on whey did not degrade any of the antibiotics in this study ($p < 0.05$).

No residues of ampicillin and penicillin G were found either in the whey powder or in the whey permeate when the milks were spiked to MRLs and three times the MRL. These results could not be attributed to process effects, because the residue concentration values are close to the quantification limits of the technique. Although cloxacillin and dicloxacillin residues were found in the whey permeate and retentate, in both cases they were lower than the milk MRL or MRL/2, according to the amount spiked.

The antibiotic residue concentrations found in the whey powder upon addition to C1 were as follows: 139 ± 32 μg kg^{-1} (cloxacillin), 123 ± 21 μg kg^{-1} (dicloxacillin), 784 ± 98 μg kg^{-1} (cephalexin), 142 ± 19 μg kg^{-1} (tetracycline), 520 ± 31 μg kg^{-1} (oxytetracycline). Table 2 shows that the whey powder concentrates 11.5 times from $8.32 \pm 0.33\%$ total solids of the retentate to $94.99 \pm 0.92\%$ of the powder (Table 2). Only in oxytetracycline, an 11.5-fold concentration of antibiotic residues was observed, ranging from $44.6 \pm 9.8\%$ of retentate to 520 ± 31 μg kg^{-1} in the powder (Table 5). Therefore, it could be assumed that the whey drying process does not degrade the antibiotics. On the other hand, the concentration of antibiotics in the rest of the antibiotics studied ranged from 5.9 to 7.7 times. Thus, it could be considered that the antibiotics were

degraded in the drying process in the cases of ampicillin, penicillin G, cloxacillin, dicloxacillin, cephalexin and tetracycline.

Distribution of antibiotic residues from whey to permeate and retentate

The distribution of antibiotics in the whey permeate and retentate was analyzed, and mass balances of the corresponding flows were performed. Of the 30.0 ± 1.2 kg of pasteurized whey that entered the crossflow filter, 26.6 ± 2.3 kg of permeate and 4.5 ± 2.0 kg of retentate were obtained.

Figure 2(C) shows the retention of antibiotics in the whey permeate and retentate resulting from the nanofiltration process of pasteurized whey obtained from the cheese making process with milk spiked with antibiotics at their MRL (C1). Figure 2(C) does not show the retention of ampicillin and penicillin G since they were found at levels lower than the limit of quantification in the permeate. Among the antibiotics studied, cloxacillin and dicloxacillin are the ones that are mostly found in the permeate, 66 ± 12% and 66 ± 4%, respectively, which could be due to the high water solubility of these antibiotics. On the other hand, of the antibiotics studied, cephalexin is the least distributed in the permeate and shows a similar distribution between permeate and retentate. The distribution percentages of oxytetracycline retained in whey permeate and retentate were 34 ± 11% and 17 ± 9%, respectively, while for tetracycline they were 43 ± 9% and 18 ± 7%, respectively. These percentages show that there was a loss of residues of these antibiotics during the ultrafiltration process, and in the case of cephalexin, the highest loss was found during this process, considering the transfers of 26.3 ± 3.8 and 17.5 ± 9.1 of permeate and retentate, respectively.

Conclusion

The studies carried out show that the distribution of antibiotics in the different stages of the cheese and whey powder production process, as well as their concentration in the final products, depend on each type of antibiotic. Ampicillin and penicillin G were the two antibiotics that remained undetected in cheese and whey powder, even when concentrations three times higher than their respective MRLs were added to the milk. These results suggest that the addition of the starter culture and the heat treatments involved in the processes favor the degradation of these antibiotics, causing them not to be detected in the final products. A similar behavior in the distribution and concentration of antibiotics was found in cloxacillin and dicloxacillin. These were not detected in cheese but were found in whey powder. Cephalexin is mostly transferred to whey, where it is concentrated when transformed into whey powder and was the antibiotic that showed the highest concentration in this product. Tetracyclines were mostly distributed in cheese, and antibiotic concentrations higher than the MRL in milk were found in cheese when Dambo-type cheese was made from milk containing oxytetracycline and tetracycline residues at MRL levels recommended by the Codex Alimentarius. Although these are transferred in smaller proportion to whey, after the nanofiltration and drying process, antibiotic residues were found in whey powder, with the concentration of oxytetracycline ($520 \pm 31\ \mu g\ kg^{-1}$) higher than that of tetracycline ($142 \pm 19\ \mu g\ kg^{-1}$). However, they were not detected in the whey powder when it was made from milk spiked to MRL/10. Considering that cheese is a food for direct consumption, it would be advisable to conduct further studies on the concentration of tetracyclines in different types of cheese to assess the potential risk to public health.

Whey powders are used as raw material in the food industry to produce a wide variety of foods (bakery products, nutritional formulations, etc.), so the potential risk associated with their consumption should be assessed depending on each final product. Knowledge of the transfer of antibiotic residues during the process and final disposal is a key input for the risk assessment of their consumption.

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