Antimicrobial activity in cheese whey as an indicator of antibiotic drug transfer from goat milk

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Abstract

The susceptibility of 18 antibiotics for transfer from goat milk to the resulting cheese whey was evaluated. Raw milk spiked with antibiotics was coagulated by rennet and the whey was separated. The antimicrobial activity of the whey, estimated by using microbial inhibitor tests, was applied as an indicator of antibiotic drug transfer. Antibiotic-free whey (negative whey) spiked with different antibiotics was used as a reference. The antimicrobial activity in whey from milk spiked with most β-lactam drugs was lower (0-40%) to that of the reference whey, suggesting that these antibiotics are mostly released from curd and transferred to the whey. However, for most non-β-lactam drugs, a reduction in the relative antimicrobial activity in whey, ranging 84 to 100% was obtained, indicating the higher susceptibility for retention in curd. The traceability of antibiotics through the cheese-making process will make it possible to determine whether control systems are required to prevent the negative implications of the presence of antibiotic drug residues in cheese and whey products.

1. Introduction

Antibiotic residues in milk and other foodstuffs of animal origin can lead to negative implications for consumer health such as allergic reactions or transient disturbances in the intestinal flora (Demoly & Romano, 2005). Also, the generation of antimicrobial resistance in microbiota is one of the most important public health problems related to the excessive use of antibiotics in the treatment of infectious diseases in livestock (Oliver, Murinda, & Jayarao, 2011; EFSA, 2016).

The processing of contaminated raw milk does not always guarantee the inactivation or elimination of antibiotic residues and consequently, variable amounts of these substances might remain in dairy products and with an increased risk for consumers. Tona and Olusola (2014) indicated the presence of tetracycline residues in soft cheese, yoghurt and butterfat made from contaminated cow milk. Similarly, Adetunji (2011) found benzylpenicillin, streptomycin and tetracycline residues in commercial soft cheese and yoghurt.

Antibiotics could be retained in milk curd to a greater or lesser extent, depending on the physicochemical properties of these substances and their ability to interact with the fat and/or protein fraction of the matrix. Sniegocki, Gbylik-Sikorska, & Posyniak (2015) assessed the potential transfer of chloramphenicol from milk to butter, sour cream, white cheese and whey using the LC-MS/MS method, observing that this antibiotic is mainly retained in higher-fat products, such as butter and sour cream, with lower concentrations in white cheese and whey samples. Berruga, Román, Molina, & Molina (2005), using the Delvotest SP microbial inhibitor test (DSM Food Specialties, The Netherlands), found detectable levels of antibiotic residues in whey from Manchego cheese manufactured from ewe milk spiked with different β-lactams (amoxicillin, ampicillin, benzylpenicillin, cephalexin and ceftiofur) at concentrations close to their respective maximum residue limits (MRLs).

It should be noted that whey is a by-product of the cheese-making process and is used in the manufacture of foodstuffs for human consumption, animal feeding and agricultural applications, among other uses (Carvalho, Prazeres, & Rivas, 2013). Therefore, the presence of antibiotic residues in whey could have negative implications for humans, animals and environmental safety.

Current strategies used to minimize the impact of drug residues reaching the food chain include the routine monitoring of raw milk supplies to detect the presence of these substances above legally established MRLs (EC, 2010). However, the monitoring of drug residues in dairy products such as cheeses and whey is not typically regulated nor have corresponding safety levels been established.

Microbial inhibitor tests are routinely applied in quality control laboratories to screen for antibiotics in raw milk, as they are relatively inexpensive, easy-to use and have a broad spectrum of detection (IDF, 2014). The inhibition of microbial growth is a test in which a positive result is revealed through the use of a dye-type acid-base or redox indicator which produces a change in the colour of the culture medium, allowing visual interpretation of test results. Considering the similarity between both matrices (milk and whey), microbial inhibitor tests could be applied successfully in screening for antibiotics in whey samples.

In the European Union, two million tons of goat milk are annually produced (FAOSTAT, 2016) which are traditionally destined for cheese making, often under quality recognized brands. Studies on the traceability of veterinary drugs during dairy manufacturing processes are rather limited. However, establishment of the traceability of antibiotics is crucial to prevent the negative implications related to presence of such substances in milk and dairy products. Therefore, the objective of this study was to assess the susceptibility of different antibiotics to be transferred from milk to whey during the cheese-making process.

2. Materials and methods

The traceability of antibiotics during an experimental cheese-making process was evaluated using microbial inhibitor tests for screening of antibiotics in goat milk. The antimicrobial activity of the whey derived from goat milk spiked with an antibiotic (Whey from Spiked Milk: WSM) was compared to that of negative goat milk whey (antibiotic-free whey) spiked with an equivalent concentration of the drug (Whey from Negative Milk: WNM). Similar antimicrobial activity exhibited in both types of whey samples (WSM and WNM) will thus indicate that antibiotics added to milk are completely transferred from the curd to the whey. Lower percentages of positive results in WSM compared to WNM will thus indicate greater retention of antibiotic in the curd.

2.1. Whey samples

Whey samples were obtained from a laboratory scale cheese-making process using raw milk obtained from the experimental flock of Murciano-Granadina goats at Universitat Politècnica de Valencia (Valencia, Spain). Animals were in mid-lactation (70-150 days after giving birth), had good health status and did not receive any veterinary drugs, neither before nor during the experimental period.

Raw goat milk (25 mL), with or without antibiotics, was placed in a tube and heated to 33±1ºC in a thermostatically-controlled water bath. Commercial rennet was used for coagulation (chymosin>70%, Suministros Arroyo. Santander, Spain) at 0.06% (v/v). Thirty minutes later, the curd was centrifuged (1026 *g*, 10 min) for separation of whey which was recovered by decanting into a volumetric flask.

2.2. Antibiotics and spiked samples

Eighteen veterinary antibiotics were selected for this study. Antibiotics, i.e. 8 β-lactams (amoxicillin, ampicillin, benzylpenicillin, cloxacillin, cefacetrile, cefquinome, ceftiofur, and cephalexin) and 10 non-β-lactam antibiotics (gentamycin, neomycin, erythromycin, tylosin, ciprofloxacin, enrofloxacin, sulphadiazine, sulphadimethoxine, oxytetracycline and tetracycline), were provided by Sigma-Aldrich Quimica, S.A. (Madrid, Spain), except for cefacetrile, which was not commercially available, and was kindly provided by Fatro S.p.A. (Bologne, Italy).

For use, antibiotics were dissolved in distilled water (1 mg mL^{-1}) at the time when analyses were performed. In some cases the use of small amounts (i.e. 2-4 mL) of a suitable solvent (AcOH 5% for enrofloxacin, EtOH for erythromycin, HCl 0.1N for ciprofloxacin and tetracycline drugs, and NaOH 0.1N for ceftiofur) was necessary before adding water.

Spiked milk samples were prepared following ISO/IDF recommendations (ISO/IDF, 2003). Spiked whey samples (WNM) were made as follows: 25 mL of antibiotic-free whey were spiked with a relatively high antibiotic concentration (C1) and subsequently, seven antibiotic concentrations were obtained by successive dilutions with negative whey (Table 1) in order to establish the reference dose-response curve. Negative whey (no antibiotic) was also included in the analysis as a negative control (C8). The ranges of concentrations for each antibiotic were selected according to the sensitivity of the microbial test to detect the substance in whey samples.

For comparison, the whey resulting from coagulation of 25 mL of antibiotic-free milk spiked with an antibiotic (WSM) was recovered in a volumetric flask after centrifugation and negative whey was added to obtain a volume of 25 ml. Thereafter, the same dilution procedure as described above was followed to obtain the dose-response curves of recovered WSM.

2.3. Microbial inhibitor tests

The antimicrobial activity of whey samples was evaluated using the Eclipse 100 test (Zeulab, Zaragoza, Spain), a microbial inhibitor assay employing *Geobacillus stearothermophilus* var *calidolactis* as a test microorganism and bromocresol purple as acid-base indicator. For the analysis of whey samples containing quinolones, the Equinox test (Zeulab), using *Escherichia coli* bacteria and brilliant black as redox indicator, was utilized. Both tests were carried out according to the manufacturer instructions for milk analysis. The interpretation of test results was carried out independently by three trained technicians visually assessing the colour change of the culture medium after incubation, and classifying the whey samples as either positive (Eclipse: purple and Equinox: blue) or negative (Eclipse: yellow and Equinox: orange brown).

Experimental cheeses were made in triplicate and whey analysis was performed in twelve replicates, resulting in a total of 36 determinations for each antibiotic concentration and type of whey considered.

2.4. Statistical analysis

To evaluate the antimicrobial activity in whey samples, a logistic regression model was applied. Statistical analysis was performed employing the stepwise option of the logistic procedure of SAS software (version 9.2, 2001; SAS Institute, Inc., Cary, NC, USA), using the following model:

$$L_{ijk} = \text{logit}\ [P_{ijk}] = \beta_0 + \beta_1 C_i + \beta_2 W_j + \varepsilon_{ijk} \quad \text{(Eq. 1)}$$

where: L_{ijk}= linear logistic model; $[P_{ijk}]$= [Pp/(1-Pp)]: the probability of a "positive" response/probability of a "negative" response; β_0, β_1 and β_2= coefficients estimated for the logistic regression model; C_i= antimicrobial concentration (i= 8); W_j= whey type as dummy variable (W= 0 for WNM; W= 1 for WSM); ε_{ijk}= residual error. The concordance coefficient (SAS, 2001) was applied as a range correlation between the observed responses and predicted probabilities.

The detection capability (CCβ) of the microbial inhibitor tests was calculated from logistic regression equations as the antibiotic concentration producing 95% positive results (ISO/IDF, 2002) in the reference whey (WNM).

To investigate the susceptibility of antibiotic transfer to the whey, positive outcomes in both types of whey samples were compared at an antibiotic concentration equivalent to the CCβ of the test (Figure 1). Thus, the potential variation in antimicrobial activity (AAV) was calculated by using the following mathematical expression:

$$AAV(\%) = ((95 - PR_j)/95)*100 \qquad \text{(Eq. 2)}$$

where: AAV= Antimicrobial Activity Variation in WSM with respect to the WNM; PR_j= positive results in WSM (j= 18) at an equivalent concentration producing 95% positive results in the WNM.

3. Results and Discussion

Table 2 shows the regression equations used to predict positive results in the microbial inhibitor tests calculated for the 18 antibiotics included in this study. In general, the frequency of positive results was positively related to the drug concentration present in the whey samples ($\beta_1 > 0$). However, positive outcomes decreased in whey samples obtained from goat`s milk spiked with most antibiotics tested ($\beta_2 < 0$). These results indicate that the cheese-making processes of milk coagulation and curd draining significantly affects ($p<0.05$) the antimicrobial activity of the recovered whey, being lower as a consequence of the total or partial retention of these drugs in the cheese curd.

The CCβs of the microbial screening tests calculated in cheese whey are summarized in Table 2. In general, CCβ values were similar to those reported by other authors for the Eclipse 100 test in raw goat milk (Beltrán et al., 2015), and by the manufacturer (Zeulab) for the Equinox test in raw cow milk, suggesting that they could be successfully applied to detect a great variety of substances in this by-product.

Regarding the susceptibility of antibiotics transferred from milk to whey, Figure 2 summarizes the variation of antimicrobial activity (AAV) in WSM with β-lactam antibiotics. No antimicrobial activity reduction (AAV= 0%) was found for amoxicillin and ampicillin, suggesting that almost all of these antibiotics are released from cheese curd during the draining process. For the rest of the penicillins and for cephalosporins, a moderate antimicrobial activity reduction ranging from 16 to 40% was demonstrated, except for cephalexin which presented the highest reduction, around 90%, indicating a greater likelihood of this antibiotic to be retained in the curd.

The high transfer rate of β-lactam antibiotics from milk to whey could be due to the water soluble nature of these substances (Rang, Dale, & Ritter, 2000). However, the lower presence of cephalexin in whey samples could be related to the low solubility in water of the hydrated molecule used in this study (NCBI, 2016) suggesting that almost none of this antibiotic is transferred to the whey.

Results obtained for penicillins and cephalosporins are in agreement with those reported by Berruga, Román, Molina, & Molina (2005) in whey from ewe milk spiked with β-lactam antibiotics. These results could also explain the lower benzylpenicillin concentration (5.4±0.1 μg kg^{-1}) reported by Adetunji (2011) in soft cheeses made from contaminated cows milk (7.0±0.2 μg kg^{-1}).

For most non-β-lactam antibiotics, the frequencies of positive results in microbial tests decreased a great deal in WSM (Figure 2). However, similar antimicrobial activity values were obtained for both types of whey only in the case of sulphadiazine.

As can be appreciated in Figure 2, the quinolone, aminoglycoside and tetracycline families presented the highest relative antimicrobial activity reduction. Thus, for enrofloxacin and ciprofloxacin, AAV were 100%, and ranging from 84 to 100% for aminoglycosides and tetracyclines.

The susceptibility of quinolones and tetracyclines for retention in the curd after whey draining could be related to their high fat-solubility. Moreover, tetracyclines might form metal ion complexes with calcium in casein micelles, which would decrease their solubility in water (Rang, Dale, & Ritter, 2000). Thus, despite the curd draining process of cheese-making procedure, similar amounts of tetracycline residues were reported by Adetunji (2011) in contaminated cow milk (2.7±0.6 $\mu g\ kg^{-1}$) and soft cheeses made from it (2.1±0.1 $\mu g\ kg^{-1}$). In the case of gentamicin and neomycin, although aminoglycosides are soluble in water (Drugbank, 2016), results herein suggest that these substances are largely retained in the curd.

For macrolides and sulphonamides, the AAV calculated for the two substances considered in each antibiotic family was highly variable, while tylosin, hardly soluble in water, was retained in the curd (AAV= 96 %), and erythromycin, much more soluble in water, was mainly released into the whey (AAV below 30 %). Regarding sulphonamides, which have low solubility in water, differences found in AAV in WSM with sulphadiazine (0%) and sulphadimethoxine (100%) could be related to the partition coefficient value being much higher for sulphadimethoxine (log P= 1.63 *vs* log P= 0.25) (Drugbank, 2016), indicating a more lipophilic character and therefore greater propensity for adsorption into fat matrices.

4. Conclusions

In summary, results herein suggest that the manufacture of cheese made from goat milk containing antibiotics will result in drug residues in the cheese and in resulting whey which could compromise the utilization of this by-product, and potentially affect consumer safety. Antibiotics are transferred from milk to whey to a greater or lesser extent depending essentially on their physicochemical properties. Thus, B-lactam antibiotics, except cephalexin, are mostly transferred from goat milk to whey, while aminoglycosides, quinolones and tetracyclines present a higher susceptibility for retention in cheese curd. The transfer rate for antibiotics belonging to macrolides and sulphonamides are highly variable.

Besides the physicochemical properties of antibiotics, other factors such as milk composition and specific steps in the cheese-making process, might also affect the curd retention/whey loss of these substances. Studies on the traceability of veterinary drugs during dairy manufacturing processes are rather limited. Therefore, performing similar studies at pilot-scale using HPLC analysis to quantify antibiotic residues is recommended in order to establish the effects of milk composition and cheese-making process steps on curd retention and whey recovery of antibiotics.

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Figure legend

Fig. 1. Calculation of the antimicrobial activity variation (AAV %) by comparing the dose-response curves (positive results %) obtained for the reference whey (spiked Whey from Negative goat Milk: WNM) and for the Whey from Spiked goat Milk (WSM).

Fig. 2. Antimicrobial activity variation (AAV %) as indicator of the antibiotic drug transfer from goat milk to cheese whey (AAV= 0%, total transfer; AAV= 100%, no transfer)