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Detection of antibiotics in goat's milk: effect of detergents on the response of microbial inhibitor tests

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The aim of the study was to evaluate the interference of acid and alkaline detergents employed in the cleaning of milking equipment of caprine dairy farms on the performance of microbial tests used in antibiotic control (BRT MRL, Delvotest MCS, and Eclipse 100). Eight concentrations of commercial detergents, five acid (0–0·25%) and five alkaline (0–1%) were add to antimicrobial-free goat's milk to evaluate the detergent effect on the response of microbial inhibitor tests. To evaluate the effect of detergents on the detection capability of microbial tests two detergents at 0·5 ml/l (one acid and one basic) and eight concentrations of four β-lactam antibiotics (ampicillin, amoxicillin, cloxacillin and benzylpenicillin) were used. Milk without detergents was used as control. The spiked samples were analysed twelve times by three microbial tests. The results showed that the presence of acid detergents did not affect the response of microbial tests for any of the concentrations tested. However, at concentrations equal to or greater than 2 ml/l alkaline detergents positive results were found in microbial tests (16·7–100%). The detection limits of the screening tests for penicillins were not modified substantially by the presence of detergents. In general, the presence of acid and alkaline detergents in goat's milk did not produce a great interference in the microbial tests, only high concentrations of detergents could cause non-compliant results, but these concentrations are difficult to find in practice if proper cleaning procedures are applied in goat dairy farms.

Keywords: Detergents, inhibitors, screening methods, goat's milk.

Veterinary drug residues in milk are a growing concern among consumers, because of the risk they might pose for health, i.e. generating allergies, toxic reactions or drug resistance (Alanis, 2005; Demoly & Romano, 2005; Sanders et al. 2011), and technological implications in the manufacture of dairy products (Packham et al. 2001; Adetunji, 2011). Therefore, Maximum Residue Limits (MRLs) of drugs in different foodstuffs of animal origin, including milk, have been defined by Regulation (EC) 470/2009 (European Union, 2009) and established by Commission Regulation (EU) 37/2010 (European Union, 2010).

Currently, there are numerous screening tests commercially available to detect antimicrobial residues in milk (International Dairy Federation (IDF), 2010). In control laboratories, microbial inhibitor tests are widely used thanks to their simplicity, low cost and wide range of detection. Microbial inhibitor tests are based on the inhibition of spore outgrowth of the microorganism-test, the most commonly applied being *Geobacillus stearothermophilus* var. *calidolactis*; a thermophilic bacterium highly sensitive to β-lactam antibiotics. Screening microbial tests are non-specific methods and may be affected by different substances capable of inhibiting microorganism-test growth, causing positive results in antibiotic-free milk samples, such us: natural inhibitors of milk (Andrew, 2001), and preservatives (Molina et al. 2003), among others.

Detergents and disinfectants used in the cleaning of milking parlours and milk tanks are a possible source of residues in milk and have occasionally been associated with the positive results obtained in microbial tests (Fabre et al. 1995).

The hygienic production of milk implies the use of cleaning products to prevent the proliferation of microorganisms on surfaces that come into direct contact with milk, such as milking machines and milk storage tanks (Pontefract, 1991). Following good cleaning practices, the residues of detergents in milk should be minimal ($\leqslant$ 2 ppm; Reybroeck, 1997), although owing to errors in the washing

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temperature, dosage, and inadequate post-rinse the concentration of these cleaning products can be higher, which may alter the organoleptic characteristics of milk (Dunsmore et al. 1985; Merin et al. 1985) and interfere in the activity of starter cultures in the industry (Guirguis & Hickey, 1987; Petrova & Dimitrov, 1993).

Moreover, only few studies in cow milk have evaluated the effect of detergents on the presence of positive results in microbial inhibitor tests, showing controversial results. Some authors (Zvirdauskiene & Salomskiene, 2007; Salomskiene et al. 2013) only found false-positive results at very high concentrations of alkaline detergents, equal or superior to the dose recommended by the manufacturers. However, Schiffmann et al. (1992) obtained non-compliant results at lower concentrations (0·01%), whereas Merin et al. (1985) for these concentrations did not obtain any positive results, although they employed a limited number of cleaning products and microbial methods. Furthermore, these studies focus on positive outcomes; there is no information about the effect of detergents on the detection capability of microbial methods.

Therefore, the goal of this study was to analyse the effect of detergents used in the cleaning of milking equipment on the performance of microbial tests for screening antibiotics in goat's milk.

Material and methods

Microbial inhibitor tests

The microbial inhibitor tests were: Brilliant Black Reduction Test MRL (BRT MRL) (AiM Analytik in MilchProduktions- und Vertriebs-GmbH, Munich, Germany), Delvotest MCS (DSM Food Specialties, Delft, the Netherlands) and Eclipse 100 (Zeu-Inmunotec, Zaragoza, Spain). The tests were used according to each manufacturer's instructions. A negative control (antimicrobial-free milk) and a positive control (antimicrobial-free milk spiked with 4 µg/kg of benzylpenicillin) were included in each test. Visual interpretation of the test results was carried out independently by three trained technicians and was evaluated as 'negative' (yellow) and 'positive' (blue or purple).

Goat's milk samples

Antimicrobial-free milk samples to be used as 'negative milk' were obtained according to the requirements established by the In IDF (ISO13969/IDF183:2003). Therefore, mixed milk of 10 Murciano-Granadina goats in mid-lactation (more than 90 d and below 150 d postpartum) from the experimental flock of Universitat Politècnica de València (Valencia, Spain) was used. Animals had a good health status and did not receive any veterinary drugs before or during the experimental period. Moreover, goats were fed diets formulated and produced in the experimental feed processing plant of Universitat Politècnica de València using first-class raw materials without added antibiotics.

All milk samples were analysed to check the physicochemical and hygienic quality parameters using MilkoScan 6000 (Foss, Hillerød, Denmark) to determine gross composition (fat, protein and total solids); somatic cell count (SCC) was obtained employing Fossomatic 5000 (Foss, Hillerød, Denmark); bacterial count (BC) was determined using Bactoscan FC (Foss, Hillerød, Denmark) and the pH value was measured by a conventional pHmeter (Crison, Barcelona, Spain).

Spiked milk samples: detergents and antibiotics

The detergents used for the study of their presence on the microbial test response were commercial detergents of the acid and alkaline type, which were added to the antibiotic-free goat's milk at concentrations of: 0, 0·25, 0·5, 0·75, 1, 1·5, 2, 2·5 ml/l for acid and 0, 0·5, 1, 2, 4, 6, 8, 10 ml/l for alkaline (Table 1). Concentrations tested for acid detergents were lower than those selected for the alkaline detergents, as higher concentrations produce milk coagulation. Each concentration was tested twelve times by microbial methods (BRT MRL, Delvotest MCS, and Eclipse 100).

To evaluate the effect of detergents on the detection capability of microbial screening methods for penicillins, the detection limits (DLs) for ampicillin, amoxicillin, cloxacillin and benzylpenicillin were calculated according to ISO13969/IDF183:2003 specifications. To do so, two detergents were chosen, an acid one (Circoaction SF, Westfalia Surge Ibérica SL, Spain) and a basic one (Circoaction AF, Westfalia Surge, Ibérica SL, Spain), which were then added to antibiotic-free goat's milk at the maximum detergent concentration, not showing interferences in the response of the microbial tests, nor significantly altering the pH of milk (0·5 ml/l). Furthermore, goat's milk samples without detergents were used as control.

The goat's milk samples, with or without detergents, were spiked with eight different antibiotic concentrations (Table 2), prepared following the recommendations of the IDF (2003). The antibiotics selected for this study were supplied by Sigma-Aldrich (Madrid, Spain): amoxicillin (31 586), ampicillin (A-9518), cloxacillin (C-9393), and benzylpenicillin (PENNA). All the antibiotic standard solutions were prepared daily, and twelve repetitions of milk were analysed within four hours after spiking.

Statistical analysis

To evaluate the effects of the acid (D_{AC}) or alkaline (D_{AK}) detergent on the response of the microbial inhibitor tests, the logistic regression model was used:

$$L_{ij} = \text{Logit}[P_{ij}] = \beta_0 + \beta_1[\text{atb}]_i + \beta_2 D_{AC} + \beta_3 D_{AK} + \varepsilon_{ij}$$

where: L_{ij} = Logit model; $[P_{ij}]$ = probability for the response category (positive or negative); β_0 = intercept; β_1, β_2, β_3 = parameters estimated for the model; $[\text{atb}]_i$ = effect of

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Table 1. Brand name, composition and recommended dose of acid and alkaline detergents

Detergent	Brand name	Composition (%)	Recommended dose (%)
Acid	Cid†	Phosphoric acid/sulphuric acid (15–30/5–15%)	0·5–1
	105 Nifos‡	Phosphoric acid/sulphuric acid (25/5%)	0·5–1
	Grupacid§	Phosphoric acid/sulphuric acid (25/5%)	0·5–2
	Manocid¶	Ortophosphoric acid/nitric acid (25/10%)	0·5–1
	Circoaction SF††	Phosphoric acid/sulphuric acid (20–30/5–10%)	0·5–1
Alkali	Basix†	Sodium hydroxide/sodium hypochlorite (5–15/5–15%)	0·5–1
	Circoaction AF††	Sodium hydroxide/sodium hypochlorite (<20/<10%)	0·5–1
	Clor FW‡	Sodium hydroxide/sodium hypochlorite (5/5%)	0·5–1
	Grupaclor§	Sodium hydroxide/sodium hypochlorite (5/10%)	0·5–1
	Manobactyl¶	Sodium hydroxide/sodium hypochlorite (5–10/5–10%)	0·5–1

† DeLaval International A.B., (Tumba, Sweden)
‡ OXA Chemical Specialties, CYGYC S.A., (Barcelona, Spain)
§ Grupanor-Cercampo S.A., (Madrid, Spain)
¶ Manovac S.L., (Valencia, Spain)
†† GEA Farm Technologies Ibérica, S.L., (Barcelona, Spain)

Table 2. Antibiotic concentrations used for the detection limit calculation of microbial inhibitor tests in goat's milk

Antibiotic	Test	Concentrations (μg/kg)							
Ampicillin	BRT MRL	0	1·0	1·3	1·6	1·9	2·2	2·5	2·8
	Delvotest MCS	0	1·3	1·6	1·9	2·2	2·5	2·8	3·1
	Eclipse 100	0	3·0	3·4	3·8	4·2	4·6	5·0	5·4
Amoxicillin	BRT MRL	0	0·7	1·0	1·3	1·6	1·9	2·2	2·5
	Delvotest MCS	0	0·7	1·0	1·3	1·6	1·9	2·2	2·5
	Eclipse 100	0	1·3	1·6	1·9	2·2	2·8	2·8	3·1
Cloxacillin	BRT MRL	0	5·0	7·0	9·0	11·0	13·0	15·0	17·0
	Delvotest MCS	0	5·0	7·0	9·0	11·0	13·0	15·0	17·0
	Eclipse 100	0	13·0	16·0	19·0	22·0	25·0	28·0	31·0
Benzylpenicillin	BRT MRL	0	0·2	0·4	0·6	0·8	1·0	1·2	1·4
	Delvotest MCS	0	0·4	0·6	0·8	1·0	1·2	1·4	1·6
	Eclipse 100	0	1·0	1·2	1·4	1·6	1·8	2·0	2·2

antibiotic concentration ($n=8$); D_{AC} = effect of the acid detergent; D_{AK} = effect of the alkaline detergent on the dummy variable (without detergent: $D_{AC}=0$, $D_{AK}=0$; acid detergent: $D_{AC}=1$, $D_{AK}=0$; alkaline detergent: $D_{AC}=0$, $D_{AK}=1$), ε_{ij} = residual error of model.

The detection limits (DLs) were calculated as an antibiotic concentration producing 95% positive results (ISO13969/IDF183:2003).

Statistical analysis was performed using Statgraphics Centurion XVI 5.1 (Statpoint Technologies, Inc, Warrenton, VA).

Results and discussion

Effect of detergents in goat's milk on false-positive results of microbial screening tests

The goat's milk samples presented an adequate physico-chemical quality (fat: 4·36 ± 0·12%, protein: 3·61 ± 0·07%, dry matter: 14·24 ± 0·28%, and pH value: 6·66 ± 0·02) and hygienic-sanitary parameters (SCC: $889 \pm 79 \times 10^3$ cell/ml, and BC: $331 \pm 51 \times 10^3$ cfu/ml) to be used as antimicrobial-free milk.

The presence of the acid detergent in goat's milk did not affect the response of the microbial inhibitor tests employed, the results were always negative. Moreover, the acid detergent addition decreased the pH of milk samples, reaching pH between 5·72 and 5·98 for the highest concentration (2·5 ml/l) of the five detergents, being lower compared with the average of pH cited for goat's milk (6·5–6·8; Park et al. 2007). These very low pH values can simulate the effect of acid generation, which is produced as a consequence of the metabolism of the microorganism, favouring the change in colour of the indicator present in the medium test.

The alkaline detergent addition in goat's milk produced positive results at concentrations ⩾ 2 ml/l (Table 3). Besides, at these concentrations, the pH of the milk samples was high, reaching pH of 9·82–10·80 for the highest concentrations

Table 3. Effect of the alkaline detergent concentrations in goat's milk on the positive results of microbial screening tests

			Positive results (%)							
			Concentration ml/l							
Alkaline detergent	Test		0	0·5	1	2	4	6	8	10
Basix		*pH*	*6·75*	*6·87*	*6·95*	*7·19*	*7·90*	*8·95*	*9·67*	*10·11*
	BRT MRL		0	0	0	0	100	100	100	100
	Delvotest MCS		0	0	0	0	100	100	100	100
	Eclipse 100		0	0	0	0	100	100	100	100
Circoaction AF		*pH*	*6·67*	*6·79*	*6·85*	*7·14*	*7·69*	*8·31*	*9·38*	*9·82*
	BRT MRL		0	0	0	0	100	100	100	100
	Delvotest MCS		0	0	8·3	16·7	25	100	100	100
	Eclipse 100		0	0	0	0	100	100	100	100
Clor FW		*pH*	*6·74*	*6·82*	*6·96*	*7·23*	*8·09*	*9·12*	*9·77*	*10·24*
	BRT MRL		0	0	8·3	100	100	100	100	100
	Delvotest MCS		0	0	0	0	100	100	100	100
	Eclipse 100		0	0	0	0	100	100	100	100
Grupaclor		*pH*	*6·76*	*6·87*	*7·03*	*7·41*	*8·94*	*9·86*	*10·41*	*10·80*
	BRT MRL		0	0	0	0	100	100	100	100
	Delvotest MCS		0	0	8·3	100	100	100	100	100
	Eclipse 100		0	0	16·7	100	100	100	100	100
Manobactyl		*pH*	*6·76*	*6·87*	*7·00*	*7·41*	*8·38*	*9·53*	*10·13*	*10·37*
	BRT MRL		0	0	0	0	100	100	100	100
	Delvotest MCS		0	0	0	0	100	100	100	100
	Eclipse 100		0	0	8·3	16·7	100	100	100	100

Table 4. Effect of the detergents in goat's milk on the penicillin detection capability of microbial inhibitor tests

		$L = \text{Logit}[P] = \beta_0 + \beta_1[\text{atb}] + \beta_2 D_{AC} + \beta_3 D_{Ak}$				Goodness-of-fit test		Detection Limit (DL) µg/l		
Antibiotic	Microbial Test	β_0	β_1	β_2	β_3	χ^2-value	*P*-value	DL_{DF}	DL_{AC}	DL_{AK}
Amoxicillin	BRT MRL	−7·6680	4·4169	—	—	3·3615	0·3391	2·26	2·26	2·26
(MRL: 4 µg/kg)	Delvotest MCS	−11·7066	7·4322	—	1·8621	6·9116	0·0747	2·01	2·01	1·59
	Eclipse 100	−14·1769	6·0313	2·4226	2·1191	5·8541	0·1189	2·98	2·36	2·39
Ampicillin	BRT MRL	−8·0068	4·6448	−1·1797	0·9588	2·5763	0·4616	2·25	2·71	2·13
(MRL: 4 µg/kg)	Delvotest MCS	−15·7129	6·2192	0·6260	3·7479	1·4916	0·6841	3·07	2·92	2·26
	Eclipse 100	−24·4051	6·8450	—	−2·0537	0·8155	0·8457	4	4	4·3
Cloxacillin	BRT MRL	−9·8484	1·0550	−1·0595	0·5283	0·7530	0·8606	12·26	13·1	11·48
(MRL: 30 µg/kg)	Delvotest MCS	−12·4914	1·3079	—	—	0·7361	0·8646	12·11	12·11	12·11
	Eclipse 100	−19·4416	0·8936	2·2427	3·3505	1·5334	0·6746	23·67	22·12	23·4
Benzylpenicillin	BRT MRL	−5·7424	11·1144	−0·7414	1·2998	0·9408	0·8155	0·78	0·84	0·66
(MRL: 4 µg/kg)	Delvotest MCS	−11·6532	10·4337	4·0091	5·0684	0·3876	0·9427	1·52	0·93	0·8
	Eclipse 100	−8·1915	5·9682	—	—	3·3534	0·0589	1·9	1·9	1·9

L = ln (Probability (+)/1 − Probability (+)); [atb]: antibiotic concentration; D_{AC} = effect of the acid detergent; D_{AK} = effect of the alkaline detergent on the dummy variable (detergent-free: $D_{AC} = 0$, $D_{AK} = 0$; acid detergent: $D_{AC} = 1$, $D_{AK} = 0$; alkaline detergent: $D_{AC} = 0$, $D_{AK} = 1$); DL_{DF}: detergent-free detection limit, DL_{AC}: acid detergent detection limit, DL_{AK}: alkaline detergent detection limit; MRL: Maximum residue limits; —: no significant differences $P > 0.05$

tested, which could also inhibit the growth of the micro-organism and thus, prevent the colour change of the test indicator system.

These results agree with Zvirdauskiene & Salomskiene (2007) and Salomskiene et al. (2013) who studied the effects of various commercial detergents on the microbial test response in cow milk, and found positive results at alkaline detergent concentrations equivalent to the dose recommended by the manufactures and above. Although, it should be noted that these high concentrations are very unlikely to be found in practice, even with poor cleaning routines. Also, these authors did not find interferences due to the presence of acid detergents in milk. At lower alkaline detergent concentrations (⩽ 1000 mg/l), Merin et al. (1985)

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and Salomskiene et al. (2013) did not find any positive results for the Delvotest microbial test; similar results to those obtained for most alkaline detergents tested in goat's milk (Table 3). However, Schiffmann et al. (1992) observed doubtful and positive results at very low concentrations (0·01 mg/ml) using one acid detergent (Calgonit S) and a basic one (Calgonit D) in different versions of the Brilliant Black Reduction Test (BRT).

In conclusion, only the presence of alkaline detergents in goat's milk at concentrations ⩾ 2 ml/l, can produce positive results in microbial inhibitor tests. However, these amounts are not reached if the rinsing of the milking equipment is carried out in an effective manner after cleaning (Reybroeck, 1997).

Effect of detergents in goat's milk on the penicillin detection capability of microbial screening tests

Table 4 shows the equations resulting from statistical analysis used to predict the positive results for the penicillins and the detection limits (DL) of the microbial inhibitor tests. The goodness-of-fit test shows that the experimental values are similar to those estimated by the logistic model, suggesting a suitable adjustment of this model. The DLs calculated for microbial tests in detergent-free milk were lower than those indicated by Sierra et al. (2009) in goat's milk, which in most cases were closer to MRLs than those calculated in the present study. These differences could be related to modifications carried out by manufacturers to improve the sensitivity of these screening tests.

The presence of acid detergents in goat's milk did not affect or slightly increased the sensitivity of the Delvotest MCS and Eclipse 100, showing lower DLs than those calculated for detergent-free milk (Table 4). However, in the case of BRT MRL, the acid detergent decreased the sensitivity to detect most penicillins in goat's milk (Table 4). For the alkaline detergent, the DLs calculated for penicillins were below or equal to those obtained for detergent-free milk (Table 4), except for ampicillin in the Eclipse 100, which was slightly higher (4·3 *v.* 4 µg/l).

In spite of the statistical significant effect of the presence of detergents in goat's milk on the detection capability of the microbial tests, the DLs calculated were generally below MRLs established for each antimicrobial substance (Regulation EC 37/2010). Therefore, the presence of acid or basic detergent at concentrations equivalents to 0·05% does apparently not have any influence on the detection of raw milk containing penicillins above the safety levels when microbial screening tests are applied in the goat's milk quality control programmes.

However, it is not known if a higher concentration of detergents in milk could have a serious effect on the detection capability of the methods employed to detect antibiotics in milk. No reference concerning the effect of detergents on the sensitivity of inhibitor tests for the penicillins or other antimicrobial agents was found; therefore the comparison of the results with other authors is not possible.

Conclusions

The response of microbial screening tests in goat's milk can be affected by the presence of alkaline detergents at high concentrations equal or greater than 2 ml/l. However, acid detergents did not produce any interference. Small amounts of acid and alkaline detergents in goats' milk (⩽ 0·05%) do not influence the sensitivity of the BRT MRL, Delvotest MCS and Eclipse 100 methods to detect penicillins. To avoid alterations in the milk quality and interferences in microbial screening tests employed in control programmes, the implementation of proper cleaning procedures to minimise the presence of detergent residues in milk is crucial.

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