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ORIGINAL ARTICLE

Five-assay microbiological system for the screening of antibiotic residues

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Abstract A novel microbiological system in microtiter plates consisting of five bioassays is presented for the detection and classification of antibiotic residues in milk. The bioassays were optimized for the detection of beta-lactams (Bioassay B: *Geobacillus stearothermophilus*), macrolides (Bioassay M: *Bacillus megaterium* with fusidic acid), tetracyclines (Bioassay T: *B. megaterium* with chloramphenicol), quinolones (Bioassay Q: *Bacillus licheniformis*) and sulfamides (Bioassay QS: *B. licheniformis* with trimethoprim) at levels near the maximum residue limits (MRL). The response of each bioassay was interpreted visually (positive or negative) after 4–5.5 h of incubation. The system detects and classifies beta-lactams (5 μg/l of amoxicillin, 4 μg/l of ampicillin, 36 μg/l of cloxacillin, 22 μg/l of amoxicillin, 3 μg/l of penicillin, 114 μg/l of cephalexin, 89 μg/l of cefoperazone and 116 μg/l of ceftiofur), tetracyclines (98 μg/l of chlortetracycline, 92 μg/l of oxytetracycline and 88 μg/l of tetracycline), macrolides (33 μg/l of erythromycin, 44 μg/l of tilmicosin and 50 μg/l of tylosin), sulfonamides (76 μg/l of sulfadiazine, 85 μg/l of sulfadimethoxine, 77 μg/l of sulfamethoxazole and 87 μg/l of sulfathiazole) and quinolones (94 μg/l of ciprofloxacin, 98 μg/l of enrofloxacin and 79 μg/l marbofloxacin). In addition, the specificity values were high for B, T, Q (99.4%), M (98.8%) and QS (98.1%) bioassays. The control of antibiotics through this system can contribute to improving the quality and safety of dairy products.

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PALABRAS CLAVE
Leche;
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Sistema microbiológico

Sistema microbiológico de cinco bioensayos para la detección de residuos de antibióticos

Resumen Se presenta un novedoso sistema microbiológico en placas de microtitulación compuesto por 5 bioensayos para la detección y clasificación de residuos de antibióticos en leche. Los bioensayos fueron optimizados para la detección de betalactámicos (bioensayo B: *Geobacillus stearothermophilus*), macrólidos (bioensayo M: *Bacillus megaterium* con ácido fusídico), tetraciclinas (bioensayo T: *Bacillus megaterium* con cloranfenicol), quinolonas (bioensayo Q: *Bacillus licheniformis*) y sulfamidas (bioensayo QS: *Bacillus licheniformis* con trimetoprima), a niveles cercanos a los límites máximos de residuos (LMR). La respuesta de cada bioensayo se interpretó visualmente (positiva o negativa) después de 4 a 5,5 h de incubación. El sistema detecta y clasifica betalactámicos (5 μg/l de amoxicilina, 4 μg/l de ampicilina, 36 μg/l de cloxacilina, 22 μg/l de amoxicilina, 3 μg/l de penicilina, 114 μg/l de cefalexina, 89 μg/l de cefoperazona y 116 μg/l de ceftiofur), tetraciclinas (98 μg/l de clortetraciclina, 92 μg/l de oxitetraciclina y 88 μg/l de tetraciclina), macrólidos (33 μg/l de eritromicina, 44 μg/l de tilmicosina y 50 μg/l de tilosina), sulfamidas (76 μg/l de sulfadiacina, 85 μg/l de sulfadimetoxina, 77 μg/l de sulfametoxazol y 87 μg/l de sulfatiazol) y quinolonas (94 μg/l de ciprofloxacina, 98 μg/l de enrofloxacina y 79 μg/l de marbofloxacina). Además, los valores de especificidad fueron altos para los bioensayos B, T, Q (99,4%), M (98,8%) y QS (98,1%). El control de residuos de antibióticos mediante este sistema puede contribuir a mejorar la calidad e inocuidad de los productos lácteos.

Introduction

Various pathologies of milk-producing cattle, such as mastitis, metritis, pneumonia, enteritis and lameness[26], are treated with penicillins[6], ceftiofur[16], oxytetracycline[5,24], tylosin and enrofloxacin[15], respectively.

Residues of these molecules could remain in the milk and cause adverse effects on consumer health, such as hypersensitivity reactions, allergy and resistance development[17,22,8,1]. In addition, the presence of these substances can delay acidification of fermented products, inhibit starter cultures and alter some properties involved in cheese ripening[3,4].

In order to prevent the risks of antibiotic residues in milk, the European Community has established the maximum residue limits (MRL) and the procedures for their determination by Regulation EC 470/2009[10] and Regulation EC 37/2010[11]. Furthermore, in the USA, the Food and Drug Administration has determined the levels of tolerance of antibiotic residues in milk for consumer protection[12].

Several screening tests have been developed for the control of antibiotic residues at permitted levels. In surveillance programs of veterinary drug residues, microbiological methods are widely used[23], because they are inexpensive, allow large number of samples and do not require microbiology-trained staff. Moreover, these methods have detection limits similar to the MRL for beta-lactams, tetracyclines and sulfamides in milk; however, they do not detect residues of other antibiotics such as macrolides and quinolones[14,2].

Thus, Nagel et al.[20] proposed bioassays that use several bacteria-tests with specific sensitivity for different groups of antibiotics used in the disease treatment of dairy cattle. These bioassays integrate a Microbiological System with dichotomous responses (positive – negative) of easy interpretation in a maximum time of 6 h. This detection metodology detects and classifies residues in four antibiotic groups (beta-lactams, tetracyclines, sulfonamides and quinolones), but with cross-specificity to some macrolides (tylosin, tilmicosin, erythromycin) and aminoglycosides (neomycin).

Therefore, the aim of this study was to evaluate a Microbiological System in microtiter plates (SMmp) to detect and classify antibiotic residues in five groups (beta-lactams, tetracyclines, sulfonamides, quinolones and macrolides) to provide a dichotomous response (positive or negative) in less than 6 h, to be used routinely by milk quality control laboratories.

Materials and methods

Design of a microbiological system in microtiter plates (MSmp)

With the purpose of detecting and classifying antibiotic residues in milk, a microbiological system consisting of five bioassays was designed. This System is constituted by Bioassay B (beta-lactams), Bioassay T (tetracyclines), Bioassay M (macrolides), Bioassay Q (quinolones) and Bioassay QS (quinolones and sulfonamides). Table 1 shows culture media, concentration of sensitivity-enhancing agents, spore concentration of bacteria-tests, indicators, pH, incubation conditions for each bioassay that integrates the MSmp.

Table 1 Composition of bioassays that integrate the microbiological system evaluated

Bioassays	Bacteria test	Agar medium	Incubation	Color response	
				Negative	Positive
B	*G. stearothermophilus* subsp. calidolactis C 953 (2×10^6 spores/ml)	Brilliant black (100 mg/l) toluidine blue (270 mg/l), pH 8.2	64 °C, 4 h	Yellow	Black
T	*B. megaterium* ATCC 9885 (2.8×10^8 spores/ml)	Brilliant black (200 mg/l) toluidine blue (10 mg/l), pH 8.5, chloramphenicol (2000 μg/l)	45 °C, 5 h	Yellow	Black
M	*B. megaterium* ATCC 9885 ($2.8. \times 10^8$ spores/ml)	Brillant black (200 mg/l) toluidine blue (10 mg/l), pH 8.0, fusidic acid (200 μg/l)	45 °C, 5 h	Yellow	Black
Q	*B. licheniformis* ATCC 14580 (4.2×10^{10} spores/ml)	Triphenyl tetrazolium (200 mg/l) toluidine blue (10 mg/l), pH 7.0	45 °C, 5.5 h	Pink	Blue
QS	*B. licheniformis* ATCC 14580 (4.2×10^{10} spores/ml)	Triphenyl tetrazolium (200 mg/l) toluidine blue (10 mg/l), pH 7.0, trimethoprim (100 μg/l)	45 °C, 5.5 h	Pink	Blue

B: beta-lactams; T: tetracyclines; M: macrolides; Q: quinolones; QS: quinolones-sulfamides.

Preparation of bioassays

Mueller Hinton Agar (38 g/l, Biokar®, Ref. 10272, France) fortified with glucose (10 g/l, Sigma Aldrich®, Ref. G8270, USA) was used and sterilized at 121 °C for 15 min. Then, each bioassay was added with redox indicators, spores of specific microorganisms, and sensitivity-improving substances (chloramphenicol, fusidic acid or trimethoprim), as described in Table 1. Subsequently, 100 μl of medium was dispensed into each of the 96 wells of the microtiter plate with an electronic dispenser (Eppendorf Research® Pro, Hamburg, Germany) and then, the plates were heat-sealed with aluminum foil coated with polypropylene and stored in a refrigerator (4 °C) until use.

Antibiotic solutions and fortified samples

For the preparation of antimicrobial solutions, standard antibiotics from Sigma Chemical Co. (St. Louis, MO, USA) were used. Aqueous solutions for each antibiotic were prepared in 10 ml volumetric flasks at a concentration of 1000 mg/l at the time of analysis to avoid possible alteration of molecules and/or their properties.

Dose–response curves

The dose–response curves were constructed for eight beta-lactams (amoxicillin, ampicillin, cloxacillin, oxacillin, penicillin 'G', cefoperazone, ceftiofur and cephalexin), three tetracyclines (chlortetracycline, oxytetracycline and tetracycline), three macrolides (erythromycin, tylosin and tilmicosin), three quinolones (ciprofloxacin, enrofloxacin and marbofloxacin), four sulfonamides (sulfadiazine, sulfadimethoxine, sulfamethoxazole and sulfathiazole) and three aminoglycosides (kanamycin, neomycin and streptomycin), according to the Codex Alimentarius guidelines[9].

For this purpose, 16 replicas of 12 concentrations were tested to obtain negative results in the first two concentrations and positive results in the last two concentrations tested. Then, 50 μl milk samples fortified with antibiotics were added to each bioassay well. The microplates were sealed with adhesive film and incubated under conditions of time and temperature to allow the color change of the control samples (Table 1).

Interpretation of results

Bioassay responses were visually interpreted by three specialists independently. Each analysis was classified in terms of ''positive'' or ''negative'' when there were at least two coincident results.

Milk samples not containing or containing antibiotics at concentrations lower than the method detection limit will allow the growth of the test bacterium, accompanied by a change in the potential redox of the culture medium, manifested by a change in the coloration of the redox indicators. Thus, Bioassays B, M and T, which contained brilliant black and toluidine blue, changed to yellow (negative result), whereas Bioassays Q and QS, which contained triphenyltetrazolium and toluidine blue, changed to pink (negative result) after incubation (Table 1).

Conversely, when a milk sample contains antibiotic residues at levels above the detection limit of each molecule for a bioassay, it will not allow the growth of the microorganism, and thus there will be no change in the redox potential and the original color of the bioassay will remain. Thus, the persistence of the black color in Bioassays B, T and M indicated the presence of antibiotics in milk, whereas the

persistence of the blue color in Bioassays Q and QS also indicated the presence of antibiotics in milk.

Calculation of detection limits

The frequencies of positive results were analyzed according to the following logistic regression model (StatGraphics Centurion X, 2007)[25]:

$$L_{ij} = \text{Logit}\left[P_{ij}\right] = \beta_0 + \beta_1[ATB]_i + \varepsilon_{ij} \quad (1)$$

where: L_{ij} = logistic linear model; $[P_{ij}]$ = logit [Pp/(1 − Pp)]: (probability of a ''positive'' response/probability of a ''negative'' response), β_0 and β_1 = parameters calculated by the logistic model; $[ATB]_i$ = antibiotic concentration (i = 1, 2,..., 12 levels); and ε_{ij} = residual error.

The detection limits were calculated as the concentration producing 95% of positive frequencies[7,9].

Specificity of each bioassay

A total of 160 milk samples from Holstein Friesian cows 60–90 days postpartum with normal values of chemical composition (protein: 2.10–5.75 g/100 ml, fats: 2.38–6.03 g/100 ml, lactose: 3.63–5.17 g/100 ml, total solids: 9.31–12.86 g/100 ml), Bacterial Colony Count (BCC: 13.000–99.000/ml) and Somatic Cell Count (SCC: 2.000–392.000/ml) were used according to the Codex Alimentarius[9]. The chemical composition, SCC and BCC were determined using Milko Scan FT-120 (Foss Somatic, USA), Fossomatic 90 (Foss Electric, USA) and BactoScan 8.000S (Foss Electric, USA), respectively. Milk samples were analyzed with the five bioassays (B, T, M, Q and QS) in triplicate. For this purpose, 50 μl of milk samples was added in each single well of each bioassay and then incubated in a water floating bath at a temperature-time for each bioassay (Table 1). Samples were analyzed simultaneously by the Delvotest® SP method (DSM, Delft, the Netherlands), which is considered a reference method. Then, specificity (%) was calculated using the following mathematical equation:

$$\text{Specificity} = \frac{\text{negative}}{\text{total samples}} \times 100 \quad (2)$$

Results and discussion

Detection limits of the bioassays

Table 2 shows the results obtained by applying the logistic regression model for the 24 antibiotics tested with each of the five bioassays (B, T, M, Q and QS). The adjustments obtained by the logistic regression model were good, since the concordance coefficients were between 98.7% and 99.4%.

Coefficient β_1 indicates the slope of the dose–response curve. Higher values of this coefficient indicate good sensitivity of the bacteria-test to detect antibiotics in milk. Bioassay B had a higher coefficient β_1 for beta-lactam antibiotics (between 0.0616 and 5.5301) in addition to tylosin (0.1458) and neomycin (0.0099). Bioassay T showed high values of β_1 for the three tetracyclines studied ($\beta_{1\text{Chlortetracycline}} = 0.1637$; $\beta_{1\text{Oxitetracycline}} = 0.1258$; $\beta_{1\text{Tetracycline}} = 0.0809$). Bioassay M had high values of this coefficient for macrolides ($\beta_{1\text{Erythromycin}} = 0.2181$; $\beta_{1\text{Tilmicosin}} = 0.1425$; $\beta_{1\text{Tylosin}} = 0.1198$). Bioassay Q showed similar coefficients β_1 for the three quinolones (between 0.1060 and 0.1106), and bioassay QS (with trimethoprim) also exhibited high coefficients β_1 for sulfonamides (between 0.2052 and 0.2707).

The detection limits (DL) calculated for each molecule by the logistic regression model and the respective MRL[9] are summarized in Table 3. For beta-lactams, Bioassay B showed detection limits (DL) similar to their MRL (amoxicillin: 5 μg/l, ampicillin: 4 μg/l, cloxacillin 36 μg/l, oxacillin: 22 μg/l, penicillin 'G' 3 μg/l, cephalexin 114 μg/l, cefoperazone: 89 μg/l, ceftiofur: 116 μg/l). By contrast, in Bioassays M, T, Q and QS, beta-lactams had to be present in milk at levels above 5 MRL to produce positive results.

The detection limits for beta-lactams calculated in Bioassay B (Table 3) were lower than those reported by Nagel et al.[20] for a bioassay in microtiter plates using *Geobacillus stearothermophilus* (4 μg/l of penicillin, 14 μg/l of amoxicillin, 8 μg/l of ampicillin, 49 μg/l of cloxacillin, 25 μg/l of oxacillin, 190 μg/l of ceftiofur, 190 μg/l of cephalexin and 140 μg/l of cefoperazone). For assays in Petri dishes using *G. stearothermophilus*, Nouws et al.[21] reported lower detection levels (2 μg/l of penicillin, 3 μg/l of amoxicillin, 2 μg/l of ampicillin, 15 μg/l of cloxacillin, 20 μg/l of oxacillin, 30 μg/l of ceftiofur, 45 μg/l of cephalexin, 30 μg/l of cefoperazone). Similarly, Gaudin et al.[13] reported DL that were similar to those obtained in this work (Table 3) in Petri dishes (12–16 μg/l of penicillin ''G'', 6–5 μg/l of ampicillin, 16 μg/l of amoxicillin, 50 μg/l of cloxacillin, 30 μg/l of oxacillin, 125 μg/l of ceftiofur, 80 μg/l of cefoperazone and 30 μg/l of cephalexin).

With respect to Bioassay T, it should be noted that the DL obtained were similar to the MRL (100 μg/l) for chlortetracycline (98 μg/l) and oxytetracycline (92 μg/l) and slightly higher for tetracyclines (128 μg/l) due to the improvement in sensitivity of *B. megaterium* with chloramphenicol[29]. However, Bioassay M using the same microorganism without the addition of chloramphenicol showed positive results when tetracyclines were present at greater levels than 2 times the MRL (Table 3).

The detection limits of tetracyclines for Bioassay T (Table 3) were slightly lower than those calculated by Nagel et al.[18,20] when using a bioassay with *B. cereus* spores in microtiter plates (chlortetracycline: 330 μg/l, oxytetracycline: 110 μg/l, tetracycline: 110 μg/l). In previous studies, Tumini et al.[28] found detection limits that were similar to those obtained in this work for a bioassay containing *B. pumilus* (chlortetracycline 117 μg/l, oxytetracycline: 142 μg/l, tetracycline: 105 μg/l).

For assays in Petri dishes, Nouws et al.[21] obtained values lower than those calculated in this work when using *B. cereus* after 18 h incubation (10 μg/l of chlortetracycline, 30 μg/l of oxytetracycline and 30 μg/l of tetracycline). Tsai and Kondo[27] and Gaudin et al.[13] reported higher levels than those calculated in this study (Table 3) when using assays in Petri dishes (chlortetracycline: 100–200 μg/l, oxytetracycline: 200–250 μg/l, tetracycline: 200–250 μg/l).

With regard to macrolides, Bioassay M presented adequate detection limits for erythromycin (33 μg/l), tilmicosin (49 μg/l) and tylosin (50 μg/l). The sensitivity to

Table 2 Coefficients calculated by the Logistic regression model for the dose–response curve

Antibiotics	Bioassay B		Bioassay T		Bioassay M		Bioassay Q		Bioassay QS	
	β_0	β_1	β_0	β_1	β_0	β_1	β_0	β_1	β_0	β_1
Beta-lactams										
Amoxycillin	−6.3773	**1.9392**	−4.9124	0.0545	−4.9057	0.0537	−9.1554	0.0219	−8.7600	0.0261
Ampicillin	−8.8622	**3.0737**	−6.1354	0.0937	−6.1772	0.0924	−4.7193	0.0384	−4.4617	0.0377
Cloxacillin	−11.836	**0.4084**	−5.1654	0.0335	−5.1424	0.0389	−9.1554	0.0219	−8.3468	0.0206
Oxacillin	−16.313	**0.8706**	−4.8035	0.0339	−4.8032	0.0325	−7.2449	0.0267	−7.2674	0.0259
Penicillin ''G''	−15.299	**5.5301**	−10.916	0.0752	−10.955	0.0724	−5.0872	0.1476	−5.0768	0.1456
Cephalexin	−9.7361	**0.1112**	−4.8526	0.0120	−4.8602	0.0117	−10.237	0.0151	−10.364	0.0153
Cefoperazone	−5.9080	**0.0995**	−4.8658	0.0033	−4.8596	0.0032	−6.3529	0.0129	−6.3398	0.0127
Ceftiofur®	−4.2313	**0.0616**	−11.861	0.0239	−11.859	0.0233	−7.1114	0.0128	−7.1498	0.0127
Aminoglycosides										
Kanamycin	−18.733	0.0026	−15.398	0.0246	−15.332	0.0259	−7.3300	0.0017	−7.8288	0.0017
Neomycin	−10.903	0.0099	−2.9801	0.0061	−2.9754	0.0064	−8.8972	0.0002	−8.9971	0.0002
Streptomycin	−12.197	0.0045	−13.685	0.0276	−13.537	0.0262	−11.057	0.0002	−10.906	0.0002
Macrolides										
Erythromycin	−6.5414	0.0337	−5.6254	0.0907	−4.1801	**0.2181**	−5.5534	0.0079	−5.5484	0.0080
Tilmicosin	−5.7655	0.0262	−6.6341	0.0901	−4.0196	**0.1428**	−10.822	0.0146	−10.819	0.0141
Tylosin	−4.6664	0.1454	−7.4681	0.0998	−3.0152	**0.1198**	−8.7288	0.0205	−8.7291	0.0209
Sulfamides										
Sulfadiazine	−10.884	0.0007	−14.268	0.0060	−14.188	0.0059	−12.282	0.0102	−16.282	**0.2123**
Sulfadimethoxine	−16.567	0.0023	−18.394	0.0103	−18.321	0.0102	−11.982	0.0096	−21.825	**0.2541**
Sulfamethoxazole	−15.294	0.0044	−14.895	0.0074	−14.961	0.0075	−13.912	0.0120	−15.912	**0.2052**
Sulfathiazole	−19.432	0.0051	−13.701	0.0054	−13.698	0.0055	−11.709	0.0119	−23.709	**0.2707**
Tetracyclines										
Chlortetracycline	−4.6026	0.0012	−13.119	**0.1637**	−9.3559	0.0576	−11.647	0.0137	−11.723	0.0134
Oxytetracycline	−10.811	0.0072	−8.6151	**0.1258**	−10.999	0.0691	−7.3439	0.0121	−7.3774	0.0123
Tetracycline	−18.384	0.0124	−7.4310	**0.0809**	−10.174	0.0591	−7.1065	0.0088	−7.1163	0.0085
Quinolones										
Ciprofloxacin	−6.3695	0.0337	−15.356	0.0521	−15.329	0.0518	−7.0710	**0.1060**	−7.0712	**0.1097**
Enrofloxacin	−12.297	0.0037	−3.9754	0.0064	−3.9535	0.0067	−7.9332	**0.1106**	−7.9877	**0.1112**
Marbofloxacin	−7.3085	0.0015	−3.9744	0.0066	−3.9986	0.0067	−6.5049	**0.1091**	−6.5235	**0.1035**

B: beta-lactams; T: tetracyclines; M: macrolides; Q: quinolones; QS: quinolones-sulfonamides. β_0 and β_1: parameters calculated by the logistic model.
β_1 coefficient values in bold for the same group of antibiotics indicate greater bioassay sensitivity compared to other bioassays of MSmp.

these compounds can be attributed to the incorporation of fusidic acid in the culture medium containing *B. megaterium* spores[30]. Conversely, macrolides must be present in milk at concentrations between 2 and 3 times the MRL to produce positive results in bioassay T (Table 3).

The detection limits of macrolides obtained by using Bioassay M (Table 3) were higher than those reported by Nouws et al.[21] for erythromycin (10 μg/l) and tilmicosin (6 μg/l) but lower than those reported for tylosin (100 μg/l) when using *Kocuria rhizophila* in Petri dishes. Moreover, Gaudin et al.[13] estimated similar DL for erythromycin (20–30 μg/l) and tilmicosin (25–50 μg/l) and higher ones for tylosin (150–200 μg/l) in a method in Petri dishes containing the same microorganism. However, both Nouws et al.[21] and Gaudin et al.[13] obtained a response after 18–24 h of incubation.

The residues of quinolones in milk were detected by Bioassays Q and QS, with DL similar to the MRL for ciprofloxacin (Q: 94 μg/l QS: 91 μg/l), enrofloxacin (Q and QS: 98 μg/l) and marbofloxacin (Q: 87 μg/l QS: 91 μg/l) due to the good sensitivity of *B. licheniformis* for quinolones[31].

For quinolone residues, the DL of bioassays Q and QS (Table 3) were lower than those reported by Nagel et al.[20] when using a bioassay in microtiter plates containing *B. subtilis* spores (150 μg/l of ciprofloxacin, 160 μg/l of enrofloxacin and 160 μg/l of marbofloxacin). By using bioassays in Petri dishes containing *Escherichia coli* cells and after 24 h of incubation, Nouws et al.[21] (4 μg/l of enrofloxacin and 5 μg/l marbofloxacin) and Gaudin et al.[13] detected low concentrations of quinolones in milk (10 μg/l of ciprofloxacin, 20 μg/l of enrofloxacin and 30 μg/l of marbofloxacin).

For sulfonamides, the addition of trimethoprim into the culture medium containing *B. licheniformis* spores (Bioassay QS) significantly improved the detection of sulfadiazine (76 μg/l), sulfadimethoxine (85 μg/l), sulfamethoxazole (77 μg/l) and sulfathiazole (87 μg/l) with respect to bioassay Q without trimethoprim (Table 3).

Table 3 Detection limits (μg/l) of microbiological system in microplates

Antibiotics	MRL[a]	Bioassays				
		B	TC	MC	Q	QS
Beta-lactams						
Amoxycillin	4	**5**	150	140	550	540
Ampicillin	4	**4**	97	100	200	190
Cloxacillin	30	**36**	242	208	550	550
Oxacillin	30	**22**	229	250	380	390
Penicillin ''G''	4	**3**	184	200	54	55
Cephalexin	100	**114**	650	670	870	870
Cefoperazone	50	**89**	2360	2500	720	730
Ceftiofur®	100	**116**	500	500	780	795
Aminoglycosides						
Kanamycin	100	8300	620	590	5900	6020
Neomycin	1500	**1400**	**460**	**450**	59 300	59 500
Streptomycin	200	3400	490	500	70 100	69 200
Macrolides						
Erythromycin	40	280	94	**33**	1070	1100
Tilmicosin	50	350	106	**44**	700	760
Tylosin	50	**52**	104	**50**	450	400
Sulfamides						
Sulfadiazine	100	18 100	2400	2400	1500	**76**
Sulfadimethoxine	100	8200	1800	1800	1500	**85**
Sulfamethoxazole	100	4100	1900	1900	1400	**77**
Sulfathiazole	100	4400	2500	2500	1200	**87**
Tetracyclines						
Chlortetracycline	100	5900	**98**	214	1060	1100
Oxytetracycline	100	1800	**92**	202	850	840
Tetracycline	100	1700	**88**	222	1200	1100
Quinolones						
Ciprofloxacin	100	4100	290	300	**94**	**91**
Enrofloxacin	100	4000	590	560	**98**	**98**
Marbofloxacin	75	6800	1050	1100	**79**	**77**

B: beta-lactams; TC: tetracyclines; MC: macrolides; Q: quinolones; QS: quinolones-sulfonamides.
[a] MRL: maximum residue limit (μg/l).
Detection limit values highlighted in bold for each bioassay indicate levels close to their respective MRL.

The detection limits for sulfonamides in milk calculated when using Bioassay QS (Table 3) were lower than those reported by Nagel et al.[20] for sulfadiazine (160 μg/l), sulfadimethoxine (260 μg/l), sulfamethoxazole (120 μg/l) and sulfathiazole (110 μg/l) when using a bioassay with *B. subtilis* and trimethoprim in the culture medium. However, in an assay in Petri dish with *B. subtilis* spores and requiring 18–24 h of incubation, Nouws et al.[21] obtained lower levels for residues of sulfadiazine (30 μg/l) and sulfadimethoxine (20 μg/l) than those reported in Table 3, while Gaudin et al.[13] obtained higher detection limits for sulfadiazine (100 μg/l), sulfadimethoxine (175 μg/l), sulfamethoxazole (100 μg/l) and sulfathiazole (1000 μg/l).

Table 4 Specificity of the microbiological system

Bioassays	Milk samples	Positive	Negative	Specificity (%)
B	160	1	159	99.4
T	160	1	159	99.4
M	160	2	158	98.8
Q	160	1	159	99.4
QS	160	3	157	98.1
Delvotest® SP	160	1	159	99.4

Specificity (%): (negative/total samples) × 100.

Specificity of the bioassays

The results of specificity for each bioassay are detailed in Table 4. It can be observed that bioassays B, T and Q had high specificity (99.4%), as determined by the Delvotest® SP method, and that the specificity of bioassays M (98.8%) and QS (98.1%) was slightly lower than that of the other methods. It should be noted that Nagel et al.[18,19] and Tumini et al.[28] reported specificities

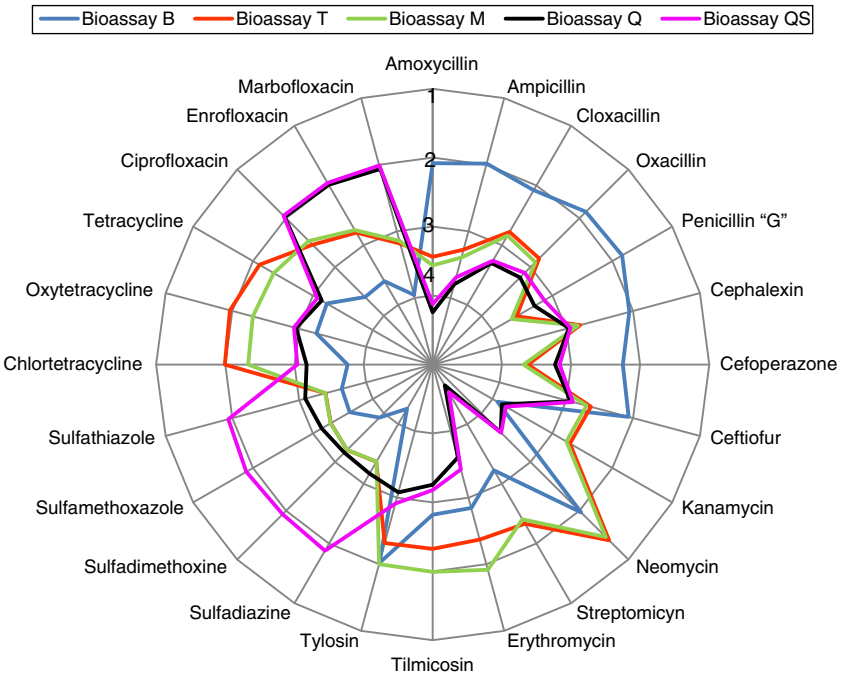

Figure 1 Detection pattern (DL/MRL) for microbiological five-bioassay system. Circle 1 = 0.1 MRL, Circle 2 = MRL, Circle 3 = 10 MRL and Circle 4 = 100 MRL.

between 99.0–99.5% (*B. cereus*), 95.8–98.9% (*B. subtilis*) and 97.9–98.9% (*B. pumilus*) respectively when optimizing microbiological methods in microtiter plates.

Cross-specificity of the five-bioassay system

In order to simultaneously visualize the detection profiles of the five bioassays for the 24 antibiotics studied, we constructed Figure 1. The radial diagram was constructed in logarithmic scale due to the large amplitude in the range of the detection system (Table 3) since the DL were between 3 μg/l (penicillin with bioassay B) and 70 100 μg/l (streptomycin with bioassay Q). In addition, a logarithmic relative scale was chosen in terms of the MRL for each molecule (Log DL/MRL) because it allows to visualize the similarity or discrepancy between the DL and MRL of each antibiotic. In this diagram, circle 1 corresponds to Log DL/MRL = 0.1 values (the bioassay detects antibiotics below MRL levels), circle 2 corresponds to Log DL/MRL = 1 (limits equivalent to LMR detection), circle 3 indicates Log DL/MRL = 10 MRL and circle 4 indicates Log DL/MRL = 100 MRL (the bioassay does not detect the antibiotic).

It can be observed that Bioassay B (blue line) detected the eight beta-lactam antibiotics, as well as tylosin and neomycin; Bioassay T (red line) detected the three tetracyclines and neomycin; Bioassay M (green line) detected the three macrolides and neomycin; Bioassay Q (black line) detected only the three quinolones, and Bioassay QS (purple line) detected the four sulfonamides and the three quinolones. It is also noted that kanamycin and streptomycin were not detected by any of the five bioassays.

In summary, a simple visual analysis of the color of each bioassay easily allows to classify an antibiotic residue into five categories. The simultaneous detection of quinolones (two bioassays), tylosin (two bioassays) and neomycin (three bioassays) provide a tool to ensure the presence of residues, thereby improving food security.

Table 5 Interpretation of results of the microbiological system in microplates

Antibiotics	Bioassays				
	B	T	M	Q	QS
Free antibiotics	—	—	—	—	—
Beta-lactams	+[a]	—	—	—	—
Tetracyclines	—	+[b]	—	—	—
Macrolides	—	—	+[b]	—	—
Quinolones	—	—	—	+	+
Sulfonamides	—	—	—	—	+

B: beta-lactams; T: tetracyclines; M: macrolides; Q: quinolones; QS: quinolones-sulfonamides.
[a] Neomycin or tylosin.
[b] Neomycin; + and —: positive and negative responses, respectively.

Antibiotic classification by the five-bioassay system

Table 5 summarizes the visual interpretation of the results obtained by the simultaneous analysis of the five bioassays. Responses are visually observed by the color change in each method. For Bioassays B, T and M, the yellow color indicates a negative result, whereas the black color indicates a positive result. For bioassays Q and QS, the pink color indicates a negative result, whereas blue indicates a positive result.

The presence of beta-lactams in milk was visualized by the persistence of the original black color in Bioassay B. Tetracycline residues were observed by the persistence of the black color in bioassay T, whereas macrolide residues were observed by the persistence of the black color in bioassay M. Sulfonamides were observed by the persistence of the original blue color in bioassay QS, while quinolone residues were evidenced by the persistence of the original blue color in bioassays Q and QS.

Table 5 shows that neomycin residues were evidenced by the persistence of the original colors of bioassays B, T and M, while tylosin residues were evidenced by the persistence of the original colors of bioassays B and M (Table 3). Finally, it should be noted that the milk samples that produced color change in the five bioassays were classified as negative samples either because they had no antibiotic or the antibiotics were below the detection limits of each bioassay.

Conclusion

The microbiological system in microtiter plates consisting of Bioassays B (*G. stearothermophilus*), Bioassays T (*B. megaterium*), Bioassays M (*B. megaterium* and fucsidic acid), Bioassays Q (*B. licheniformis*) and Bioassays QS (*B. licheniformis* and trimetoprim) can detect a large amount of antibiotic residues in milk and allows to classify residues in beta-lactams, tetracyclines, macrolides, quinolones and sulfonamides. This system provides a simple dichotomous colorimetric response with visual interpretation and allows to analyze a large number of samples in a short period of incubation (4–5.5 h).

The simultaneous use of five bioassays represents a more efficient and rigorous control mechanism than the use of a single-bioassay microbiological system. Indeed, the routine implementation of this system in quality control microbiological laboratories may contribute to improving food security. Furthermore, in contrast to microbiological systems in Petri dishes, which should be used immediately after preparation, this microbiological system designed in a microtiter plate format heat-sealed with aluminized paper and containing sporulated bacteria allows the marketing, refrigerated transport and storage in a refrigerator at 4 °C in the laboratory before being used. In the future, new bioassays using microorganisms with specific sensitivity to aminoglycosides should be developed to improve the detection profile and classification of antibiotic residues in milk.

Conflict of interest

The authors declare that they have no conflicts of interest

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