Journal of Dairy Research (2015) **82** 248–255. doi:10.1017/S0022029915000138
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Microbiological bioassay using *Bacillus pumilus* to detect tetracycline in milk

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Received 29 April 2014; accepted for publication 13 January 2015; first published online 27 February 2015

The tetracyclines (TCs) are widely used in the treatment of several diseases of cattle and their residues may be present in milk. To control these residues it is necessary to have available inexpensive screening methods, user-friendly and capable of analysing a high number of samples. The purpose of this study was to design a bioassay of microbiological inhibition in microtiter plates with spores of *Bacillus pumilus* to detect TCs at concentrations corresponding to the Maximum Residue Limits (MRLs). Several complementary experiments were performed to design the bioassay. In the first study, we determined the concentration of spores that produce a change in the bioassay's relative absorbance in a short time period. Subsequently, we assessed the concentration of chloramphenicol required to decrease the detection limit (DL) of TCs at MRLs levels. Thereafter, specificity, DL and cross-specificity of the bioassay were estimated. The most appropriate microbiological inhibition assay had a *B. pumilus* concentration of $1{\cdot}6 \times 10^9$ spores/ml, fortified with 2500 µg chloramphenicol/l (CAP) in Mueller Hinton culture medium using brilliant black and toluidine blue as redox indicator. This bioassay detected 117 µg chlortetracycline/l, 142 µg oxytetracycline/l and 105 µg tetracycline/l by means of a change in the indicator's colour in a period of 5 h. The method showed good specificity (97·9%) which decreased slightly (93·3%) in milk samples with high somatic cell counts (>250 000 cells/ml). Furthermore, other antimicrobials studied (except neomycin) must be present in milk at high concentrations (from >5 to >100 MRLs) to produce positive results in this assay, indicating a low cross specificity.

Keywords: *Bacillus pumilus*, bioassay, microbial inhibition method, milk, tetracycline.

Tetracyclines (TCs) are broad-spectrum antibiotics (ATBs) with antimicrobial action against a variety of Gram-negative and Gram-positive bacteria (Hlavka & Boothe, 1985; Roberts, 1996). These molecules prevent bacterial growth by inhibiting protein synthesis (Rasmussen et al. 1991). In fact, TCs interfere in the binding between aa-tRNA and the site 30S ribosomal subunit, which inhibits the elongation of the peptide chain (Chopra et al. 1992).

In veterinary medicine, TCs are used in prevention and treatment of several diseases of cattle, pig, poultry and fish (Aga et al. 2003). In dairy cattle, TCs are employed frequently for the treatment of bacterial enteritis, metritis, mastitis and infectious keratoconjunctivitis. An investigation carried out by European Federation of Animal Health regarding the use of ATBs in veterinary medicine indicates that TCs represent 65% of antibacterial agents used for therapeutic or preventive purposes (Bogialli et al. 2006).

The presence of ATBs residuals in milk may cause different diseases or disorders in human consumers (Dewdney et al. 1991; Demoly & Romano, 2005; Wilke et al. 2005). Moreover, these residues can modify or inhibit the fermentation processes during the elaboration of dairy products such as cheese and yogurt (Packham et al. 2001).

For this reason, monitoring ATBs residues is very important for the control of food safety and innocuity. Furthermore, a Maximum Residue Limits (MRL) of 100 µg/l for TCs residues in milk have been established by several control authorities such as the European Union (Council Regulation, 2009) and Codex Alimentarius (2010). Microbiological inhibition methods are successfully used to ensure the minimum levels of antimicrobial residues in milk, because they allow analysis of a high number of samples in a relatively short time and at low cost (Toldra & Reig, 2006; IDF, 2010). Also, receptor-binding tests are used to detect tetracycline in milk, because they provide a response in a short time (IDF, 2010).

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https://doi.org/10.1017/S0022029915000138 Published online by Cambridge University Press

The most commonly used microbiological methods contains spores of *Geobacillus stearothermophilus* subsp. *calidolactis* (Pikkemaat, 2009) due to its fast growth at high temperatures (64 °C) and good sensitivity for detecting beta-lactams in milk, although this bacteriological test exhibits a limited sensitivity for TCs residues (Diserens et al. 2005; Navrátilová, 2008; Nagel et al. 2013).

Due to this limited sensitivity for TCs, Nagel et al. (2011) proposed the use of chemometric techniques for the design of bioassays in microtiter plates with *Bacillus cereus* for detection of these residues in milk. This biossay is easy to implement in laboratories for the analysis of large amounts of samples because it shows a dichotomous response of simple interpretation (positive or negative) in a short time period (5 h). However, the use of this microorganism may present risks, as vegetative cells or spores of *B. cereus* can produce enterotoxin and emetic toxin (Logan & de Vos, 2009) which cause diarrhoea and emetic syndrome (Abee et al. 2011).

Therefore, the objective of this study was to apply experimental design techniques and a logistic regression model to optimise a bioassay in microtiter plates with *Bacillus pumilus* for the detection of TCs residues at levels approximate to MRLs, since it is an innocuous bacterium.

Material and methods

Bioassays elaboration

Mueller Hinton culture medium (38 g/l, Biokar, Francia, Ref. 10272) was fortified with glucose (10 g/l, Anhedra®, Argentina, Ref. 6837), adjusted to pH = 8·0 ± 0·1 and a combination of indicators were added consisting of Brilliant Black (200 mg/l, Sigma Aldrich®, Ref. 211842) and Toluidine Blue (10 mg/l, Anhedra®, Argentina, Ref. 6356). Then, this medium was inoculated with different concentrations of *B. pumilus* CECT 510 spores and CAP (1000 µg/ml, Sigma Aldrich®, Ref. C0378) which are detailed below for each experiment.

Each microplate well was filled with 100 µl culture medium using an electronic dispenser (Eppendorf Research® Pro, Eppendorf, Germany). Subsequently, the microplate was sealed with aluminised bands and preserved refrigerated at 4 °C.

Analysis of dose-response curves

An antimicrobial concentration scale was used (detailed below for each experiment) for studying dose-response curves of the bioassays. For every ATBs, 16 replicates of 12 concentrations were assayed in order to obtain negative results in the first two levels of ABTs and two positive results in the last two concentrations tested. A volume of 50 µl was added into each microplate well. Then, the bioassay was left at room temperature for 1 h to allow the ATB to diffuse into the culture medium. Subsequently, they were washed 3 times with distilled water and incubated in a water floating bath at 40 ± 1 °C until negative samples changed the indicator's colour. Photometric readings were measured with an ELISA reader (Biotek ELx800TM, Biotek Instrument Inc., Winooski, Vernont, USA) using a wavelength of 550 nm. Photometric measurements were transformed by the following equation:

$$A = \frac{(A_x - A_0)}{(A_{100} - A_0)} \quad (1)$$

where: A: relative absorbance, A_x = absorbance of the milk sample with an 'x' ATB concentration; A_0: absorbance of ATB-free milk (negative control) and A_{100}: absorbance of the milk sample that yielded 100% positive results. These relative absorbance values were analysed using the LOGISTIC procedure of the statistical package StatGraphics Plus versión 5.1 Centurión® (2008). Then, for each experiment, Detection Limits were calculated as the concentration of ATBs that produces 45% of relative absorbance (Luitz & Suhren, 1995).

Effect of the spore concentration on the response time of the bioassay

A volume of culture medium was prepared as described above and was divided into five aliquots to evaluate the effect of different B. pumilus spore concentration ($1{\cdot}6 \times 10^9$, 8×10^7, $6{\cdot}4 \times 10^6$, $1{\cdot}2 \times 10^6$ and $2{\cdot}5 \times 10^5$ spores/ml) on the detection limit and response time of the bioassay. These concentrations were obtained by serial dilutions of B. pumilus spore suspension ($1{\cdot}6 \times 10^{11}$ spores/ml) determined by counting with PetrifilmÔ plates (3M, St Paul, MN, USA).

Oxytetracycline (Sigma O-5750) was used to study the detection limit (OTC: 0, 500, 750, 1000, 1250, 1500, 1750, 2000, 2500, 3000, 4000, 5000 µg/l). When the negative samples shifted colour (black to yellow), the incubation time and the relative absorbance was recorded. The following logistic regression model was used:

$$L_{ijk} = \text{Logit}\,[A_{ijk}] = \beta_0 + \beta_1 OTC_i + \beta_2 \log S_j + \beta_3 \log S_j^2 + \varepsilon_{ijk} \quad (2)$$

where L_{ijk} = lineal logistic model; A_{ijk} = relative absorbance (Eq. 1); β_0 = intercept; β_1, β_2, β_3 = estimated parameters for model; OTC_i = Oxytetracycline concentration (i: 1, 2,…12 levels); log S_j = logarithmic transformation of the spore concentration; log S_j^2 = square of the logarithmic transformation of the spore concentration and ε_{ijk} = residual error.

Effect of CAP concentration on the TCs detection limits

Culture medium prepared as described above was inoculated with $1{\cdot}6 \times 10^9$ spores/ml of B. pumilus. Then, it was divided into four aliquots to evaluate different CAP concentrations (CAP: 0, 1500, 2000 and 2500 µg/l). So, the TCs detection limits were assessed using the following

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Table 1. Antimicrobial concentrations used to determine the detection limits of the bioassay using *Bacillus pumilus*

Antimicrobials	Concentrations (µg/l or mg/l*)
Beta-lactams	
Amoxicillin	0, 100, 200, 300, 400, 500, 600, 800, 1000, 2000, 3000, 5000
Ampicillin	0, 25, 50, 100, 250, 500, 1000, 1500, 2000, 2500, 3000, 3500
Cloxacillin	0, 100, 200, 300, 400, 500, 600, 800, 1000, 2000, 3000, 5000
Oxacillin	0, 100, 200, 300, 400, 500, 600, 800, 1000, 2000, 3000, 5000
Penicillin 'G'	0, 50, 100, 200, 300, 400, 500, 600, 800, 1000, 2000, 3000
Cefoperazone	0, 50, 100, 150, 200, 400, 600, 800, 1000, 1500, 2000, 3000
Ceftiofur	0, 50, 100, 150, 200, 400, 600, 800, 1000, 1500, 2000, 3000
Cephalexin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 1500, 2000, 3000
Aminoglucosides	
Kanamycin	0, 100, 150, 200, 300, 400, 600, 800, 1000, 2000, 3000, 4000
Neomycin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 1500, 2000, 3000
Streptomycin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 1500, 2000, 3000
Macrolides	
Erythromycin	0, 40, 80, 120, 160, 200, 300, 400, 600, 800, 1000, 1200
Tylosin	0, 25, 50, 75, 100, 200, 300, 400, 600, 800, 1000, 1500
Tilmicosin	0, 50, 100, 150, 200, 250, 300, 350, 400, 600, 800, 1000
*Sulphamides**	
Sulphadiazine*	0, 0·5, 1·0, 1·5, 2·0, 4·0, 6·0, 8·0, 10, 20, 40, 60
Sulphadimethoxine*	0, 0·5, 1·0, 1·5, 2·0, 4·0, 6·0, 8·0, 10, 20, 40, 60
Sulphamethoxazole*	0, 0·5, 1·0, 1·5, 2·0, 4·0, 6·0, 8·0, 10, 20, 40, 60
Sulphathiazole*	0, 0·5, 1·0, 1·5, 2·0, 4·0, 6·0, 8·0, 10, 20, 40, 60
Quinolones	
Ciprofloxacin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 2000, 3000, 4000
Enrofloxacin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 2000, 3000, 4000
Marbofloxacin	0, 50, 100, 150, 200, 400, 600, 800, 1000, 2000, 3000, 4000

concentration scales: oxytetracycline (Sigma O-5750, OTC: 0, 25, 50, 75, 100, 150, 200, 250, 300, 400, 600, 800 µg/l), tetracycline (Sigma T-3258, TC: 0, 50, 75, 100, 150, 200, 300, 400, 500, 600, 800, 1000 µg/l) and clortetracycline (Sigma C-4881, CTC: 0, 50, 75, 100, 150, 200, 300, 400, 500, 800, 1000, 1500 µg/l), Since, the graph of dose-response curve should include at least two negative and two positive concentrations (Codex Alimentarius, 2010). The regression logistic model was used:

$$L_{ijk} = \text{Logit}\,[A_{ijk}] = \beta_0 + \beta_1[\text{TCs}]_i + \beta_2[CAP]_j + \beta_{12}([\text{TCs}]*[\text{CAP}])_{\text{ij}} + \varepsilon_{\text{ijk}} \quad (3)$$

where: L_{ijk} = linear logistic model; $[A_{ijk}]$ = relative absorbance (Eq. 1); $[\text{TCs}]_i$ = effect of tetracycline concentration ($i = 1, 2, \ldots 12$ levels); $[\text{CAP}]_j$ = effect of CAP concentrations ($j = 0$, 1500, 2000 or 2500 µg/l); $([\text{TCs}] \times [\text{CAP}])_{ij}$ = effect of interaction between tetracycline and CAP concentrations; β_0, β_1, β_2, and β_{12} = coefficients estimated for the model and ε_{ijk} = residual error.

Specificity of the bioassay

The milk samples used in this study came from untreated Holstein Friesian animals. In addition, the values of pH, chemical composition, somatic cell counts (SCC < 400 000 cells/ml) and bacterial count (CFU < 100 000 cfu/ml) were acceptable for dairy cattle. The chemical composition, SCC and CFU were determined with Milko Scan FT-120 (Foss Somatic, USA), Fossomatic 90 (Foss Electric, USA and BactoScan 8.000S (Foss Electric, USA), respectively.

Milk samples were assayed in duplicate using bioassays without CAP and with 2500 µg CAP/l. Visual Interpretations were carried out by three qualified scientists that categorised the response as positive or negative. Only responses with at least two (out of three) coincidences were considered. Specificity was calculated using the following equation:

$$\text{Specificity} = (\text{negative/total samples}) \times 100 \quad (4)$$

Cross-specificity: detection limits for other ATBs

Bioassays were developed using a culture medium (see above) inoculated with $1{\cdot}6 \times 10^9$ spores/ml of B. pumilus and 2500 µg CAP/l. Detection limits were studied for 8 Beta-lactams (amoxicillin, ampicillin, cloxacillin, oxacillin, penicillin 'G', cefoperazone, ceftiofur and cephalexin), 3 Aminoglycosides (kanamycin, neomycin and streptomycin), 3 Macrolides (erythromycin, tylosin and tilmycosin), 4 Sulphonamides (sulphadiazine, sulphadimethoxine, sulphamethoxazole, and sulphathiazole) and 3 Quinolones (ciprofloxacin, enrofloxacin and marbofloxacin). For each ATB, concentrations described in Table 1 were tested. For statistical analysis the following logistic regression model

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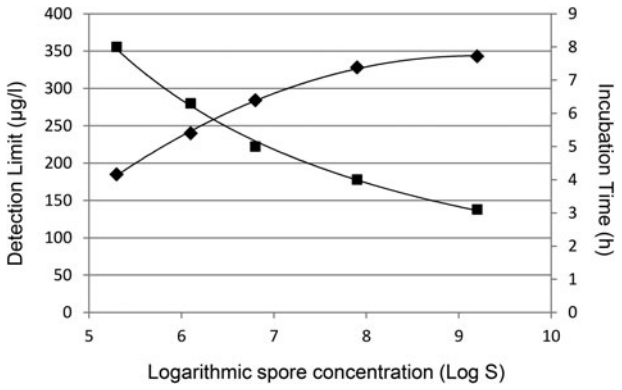


Fig. 1. Effect of spore concentration on incubation time (■) and detection limits (◆) of the bioassay.

was used:

$$L_{ij} = \text{Logit}\,[A_{ij}] = \beta_0 + \beta_1[\text{ATB}]_i + \varepsilon_{ij} \quad (5)$$

where: L_{ij} = logistic linear model; $[A_{ij}]$ = relative absorbance; $[\text{ATB}]_i$ = effect of antibiotic concentration ($i = 1,\ 2,\ldots 12$ levels, Table 1); β_0 and β_1 = estimated coefficients and ε_{ij} = residual error.

Results and discussion

Spore concentration effect on the response time of the bioassay

The spore concentration effect on the dose-response curve of OTC in milk for the bioassay using *B. pumilus* indicates significant effects for the linear and quadratic terms (log S, $\chi^2 = 4{\cdot}795$; $\log^2 S$; $\chi^2 = 3{\cdot}090$, respectively) according to the following equation:

$$\text{Logit}\,P = 8.353 + 0.014\ OTC - 2.919\ \log S + 0.162 \log^2 S \quad (C = 90.95\%)$$

Figure 1 visualises incubation time and detection limit obtained for different *B. pumilus* levels. An increase in spore concentration produces a rise of OTC detection limit accompanied by a decrease in the response time due to increment in the acidification caused by *B. pumilus*, which is observed as a faster change in the colour indicator.

Effect of CAP concentration on TCs detection limits

Table 2 shows χ^2 and *P*-values for the factors included in the model (Eq. 3). The TCs and CAP effects were significant ($P < 0{\cdot}0001$) while their interactions were not significant ($P > 0{\cdot}05$). For the three TCs assayed, χ^2 coefficient of CAP effect was high ($\chi^2_{OTC} = 8{\cdot}696$, $\chi^2_{TC} = 5{\cdot}416$, $\chi^2_{CTC} = 4{\cdot}560$) indicates the importance of CAP incorporation to improve the bioassay sensitivity for this antimicrobials group.

The interaction of both terms (TCs × CAP) was not significant for all three TCs. This indicates that there was not a synergistic effect between these antimicrobials. Therefore, we can determine that an additive model holds for (Eq. 3). This absence of synergism was observed by Nagel et al. (2009, 2011), who added CAP in the culture medium to improve the sensitivity to TCs of *G. stearothermophilus* and *Bacillus cereus*, respectively.

Equations calculated by logistic regression models for factors that significantly affect the response of the bioassay ([TCs] and [CAP]) are summarised in Table 3. All models showed adequate concordance coefficients (OTC = 89·9%; TC = 87·6% and CTC = 87·6%). The 'β_1' coefficients indicate increases the percentage absorbance relative to the extent that rise concentrations of TCs in milk. These coefficients showed that *B. pumilus* had greater sensitivity to detect residues of OTC ($\beta_1 = 0{\cdot}0115$) than TC ($\beta_1 = 0{\cdot}0090$) and CTC ($\beta_1 = 0{\cdot}0070$).

The 'β_2' coefficients indicate the effect of CAP on the relative absorbance of the bioassay. The CAP addition produces greater effect on the detection of CTC (β_2: 0·0013) than OTC (β_2: 0·0009) and TC (β_2: 0·0009). Therefore, addition of CAP will produce a greater reduction of detection limits for CTC than for OTC and TC. Conversely, Nagel et al. (2011) obtained higher values of 'β_2' for OTC (β_2: 0·0051) and TC (β_2: 0·0057) compared with CTC (β_2: 0·0023) in the optimisation of a bioassay employing *B. cereus*. Figure 2 depicts the effects of [CAP] and [TCs] on the bioassay's relative absorbance. For the three TCs assessed, the percentage of relative absorbance increases as concentrations in milk increase. This addition of CAP produces a displacement of dose-response curves to lower detection levels, which is more marked for CTC than OTC and TC (Fig. 2) due to its higher 'β_2' coefficient (Table 3). The dose-response curves constructed using the logistic model overestimate the relative absorbances at low TCs concentrations. Therefore, the logistic curves estimated by the model showed absorbances for antibiotic-free samples (TCs concentration = 0 µg/l).

The bioassay's detection limits (Table 4) were calculated for the three TCs in milk with different CAP levels using the criterion of 45% of relative absorbance from the equations in Table 3. Also, Table 4 shows MRLs for the three TCs established by the current legislation (100 µg/l).

The incorporation of CAP in the culture medium (from 0 to 2500 µg/l) causes a decrease in the detection limits for OTC (from 346 to 142 µg/l), TC (from 380 to 105 µg/l) and CTC (from 575 to 117 µg/l). The levels detected by bioassay fortified with 2500 µg CAP/l are appropriate and slightly higher than the MRL established by the legislation (100 µg/l). The detection level of TC was similar to the value reported by Nagel et al. (2009) when 400 µg CAP/l were incorporated into a bioassay using *G. stearothermophilus* (DL_{TC}:158 µg/l). However, *B. pumilus* detected lower concentrations of OTC and CTC (Table 4) than *G. stearothermophilus* (DL_{OTC}: 273 µg/l, DL_{CTC}: 316 µg/l) reason that its use may be recommended for a more efficient control of TCs residues in milk.

In another study, Nagel et al. (2011) detected 100 µg OTC/l and 109 µg TC/l in milk when optimising a bioassay

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Table 2. χ^2 values and estimated probability for different factors affecting the response of bioassays with addition of CAP for the detection of TCs

	[TCs]		[CAP]		[TCs] × [CAP]	
Tetracyclines	Value 'χ^2'	Value '*P*'	Value 'χ^2'	Value '*P*'	Value 'χ^2'	Value '*P*'
Chlortetracycline	58·3029	0·0001	8·6958	0·0032	3·8612	0·0514
Oxytetracycline	55·1469	0·0001	4·0308	0·0447	1·7093	0·1911
Tetracycline	49·2518	0·0001	5·4158	0·0200	2·2306	0·1353

TCs, Tetracyclines; CAP, Chloramphenicol

Table 3. Logistic regression models representing the effects of concentration of each TCs and different levels of CAP on the response of the bioassay

TCs	$L = \text{logit}\,[P] = \beta_0 + \beta_1\,[\text{TCs}] + \beta_2\,[\text{CAP}]$	C%
Clortetracycline	$L = -4{\cdot}1921 + 0{\cdot}0070 \times [\text{CTC}] + 0{\cdot}0013 \times [\text{CAP}]$	87·6
Oxytetracycline	$L = -4{\cdot}1875 + 0{\cdot}0115 \times [\text{OTC}] + 0{\cdot}0009 \times [\text{CAP}]$	89·9
Tetracycline	$L = -3{\cdot}7999 + 0{\cdot}0090 \times [\text{TC}] + 0{\cdot}0009 \times [\text{CAP}]$	87·6

TCs, Tetracyclines; CAP, chloranphenicol; CTC, Clortetracycline; OTC, Oxytetracycline; TC, Tetracycline; C%, concordance coefficient

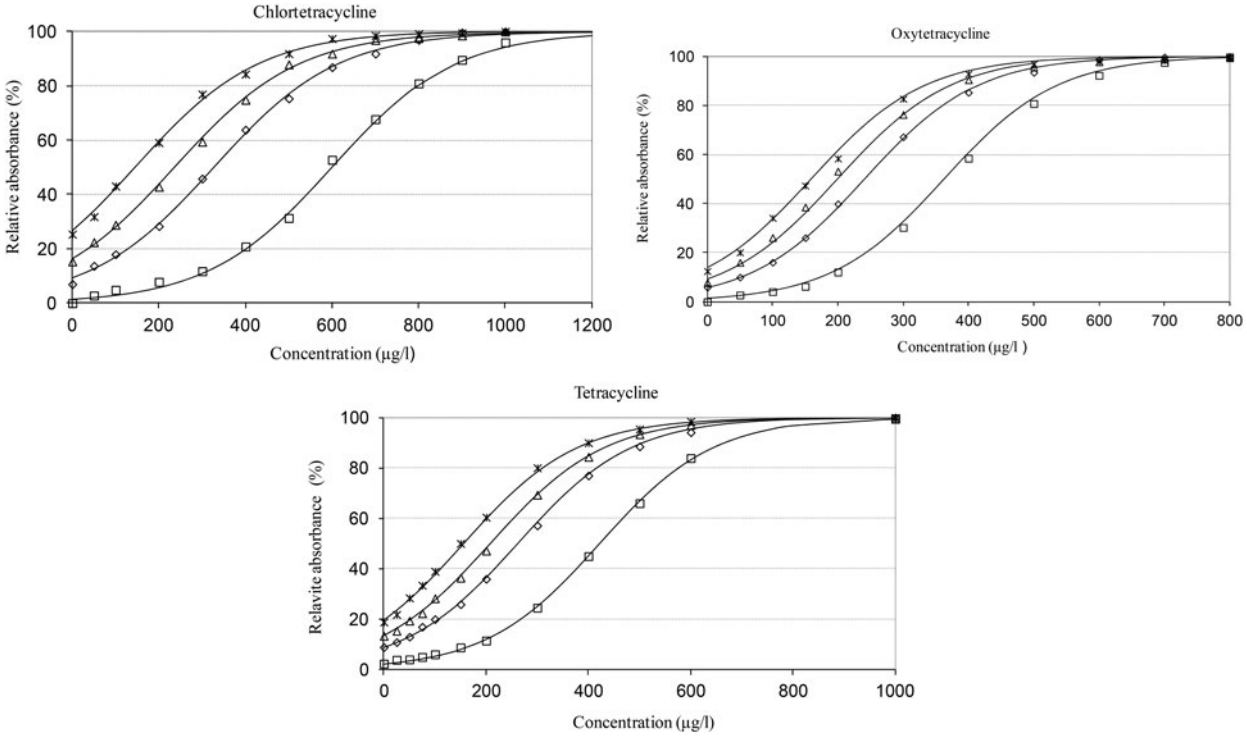


Fig. 2. TCs dose-response curves for different chloramphenicol concentrations (□ CAP: 0 µg/l; ◊ CAP: 1500 µg/l; Δ CAP: 2000 µg/l; × CAP: 2500 µg/l).

containing spores of *B. cereus* (Log S. 5·12) and CAP (470 µg/l), although they did not obtain the levels of sensitivity for CTC detection in milk achieved here with *B. pumilus* (117 µg/l compared with 250–300 µg/l for *B. cereus*). Even though the levels of detection of *B. cereus* are satisfactory, the use of this bacterium could be questioned because it can cause digestive disturbances (Abee et al. 2011), conversely *B. pumilus* is a safe microorganism.

There are no previous studies using bioassay which contains *B. pumilus* for the detection of ATBs residues in milk.

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Table 4. Effect of the concentration of CAP on the detection limits of TCs in milk samples

	Concentration CAP (μg/l)				
TCs	0	1500	2000	2500	MRLs (μg/l)
Clortetracycline	575	299	208	117	100
Oxytetracycline	346	223	183	142	100
Tetracycline	380	330	175	105	100

TCs, Tetracyclines; CAP, chloramphenicol; MRLs, Maximum residue limits

Table 5. Coefficients of the logistic regression model for the bioassay's dose-response curves

Antimicrobial	Logit $[P] = \beta_0 + \beta_1$ [Antimicrobial]	C%
Beta-lactams		
Amoxycillin	Logit $[P] = -2{\cdot}0027 + 0{\cdot}0043 \times$ [AMO]	95·4
Ampicillin	Logit $[P] = -1{\cdot}5405 + 0{\cdot}0024 \times$ [AMP]	95·9
Cloxacillin	Logit $[P] = -1{\cdot}5146 + 0{\cdot}0055 \times$ [CLOXA]	94·1
Oxacillin	Logit $[P] = -2{\cdot}6700 + 0{\cdot}0047 \times$ [OXA]	96·3
Penicillin 'G'	Logit $[P] = -2{\cdot}0807 + 0{\cdot}0058 \times$ PEN]	93·8
Cefoperazone	Logit $[P] = -2{\cdot}8758 + 0{\cdot}0022 \times$ [CFPR]	97·6
Ceftiofur®	Logit $[P] = -3{\cdot}6330 + 0{\cdot}0025 \times$ [CFR]	97·8
Cephalexin	Logit $[P] = -2{\cdot}6175 + 0{\cdot}0036 \times$ CLX]	97·8
Aminoglycosides		
Kanamycin	Logit $[P] = -3{\cdot}9729 + 0{\cdot}0016 \times$ [KANA]	97·6
Neomycin	Logit $[P] = -2{\cdot}2009 + 0{\cdot}0014 \times$ NEO]	90·5
Streptomycin	Logit $[P] = -2{\cdot}3299 + 0{\cdot}0019 \times$ [STR]	97·5
Macrolides		
Erythromycin	Logit $[P] = -2{\cdot}0929 + 0{\cdot}0062 \times$ [ERI]	95·4
Tylosin	Logit $[P] = -3{\cdot}4374 + 0{\cdot}0052 \times$ [TYLO]	96·9
Tilmicosin	Logit $[P] = -5{\cdot}1718 + 0{\cdot}0083 \times$ [TILMY]	98·9
Spiramycin	Logit $[P] = -4{\cdot}6285 + 0{\cdot}0013 \times$ [SPI]	97·8
Sulphamides		
Sulphadiazine	Logit $[P] = -1{\cdot}8324 + 0{\cdot}0001 \times$ [SDA]	88·2
Sulphadimethoxine	Logit $[P] = -1{\cdot}2455 + 0{\cdot}0002 \times$ [SDM]	89·8
Sulphamethoxazole	Logit $[P] = -1{\cdot}1616 + 0{\cdot}0001 \times$ [SMX]	88·8
Sulphathiazole	Logit $[P] = -2{\cdot}0206 + 0{\cdot}0001 \times$ [STZ]	89·4
Quinolones		
Ciprofloxacin	Logit $[P] = -2{\cdot}7605 + 0{\cdot}00304 \times$ [CIPRO]	95·6
Enrofloxacin	Logit $[P] = -3{\cdot}6242 + 0{\cdot}0011 \times$ [ENRO]	96·2
Marbofloxacin	Logit $[P] = -3{\cdot}4711 + 0{\cdot}0024 \times$ [MAR]	97·2

C%, concordance coefficient

However, Pikkemaat et al. (2008) detected 1000 μg OTC/l, 700 μg TC/l and 400 μg CTC/l in renal pelvis fluid when using a method in Petri plates using *B. pumilus* spores fortified with 7 μg trimethoprim/l in culture medium after incubating for a period of 18 h at 37 °C, values that are higher than calculated in this work for diffusion bioassay in microtubes (40 °C – 5 h).

Microbiological inhibition bioassays commercially available using *G. stearothermophilus var. calidolactis* does not have adequate sensitivity to detect residues of TCs in milk. For example, Delvotest® detects 600 μg CTC/l and 500 μg OTC/l in milk while the BRT® AiM detects 750 μg OTC/l and 400 μg TC/l (Diserens et al. 2005). Therefore, these microbiological methods do not guarantee the absence of TCs residues at levels similar to MRLs (100 μg/l).

Bioassay specificity

The absolute frequency of non-compliance results was increased from 2 (specificity: 98·9%) to 4 (specificity: 97·9%) when bioassays were prepared with 0 μg CAP/l and 2500 μg CAP/l, respectively. The specificity calculated for the bioassay with 2500 μg CAP/l was similar to 98% reported by Sischo & Burns (1993) and 95% indicated by Charm & Zomer (1995) when evaluating the Delvotest® method with ATB-free milk samples.

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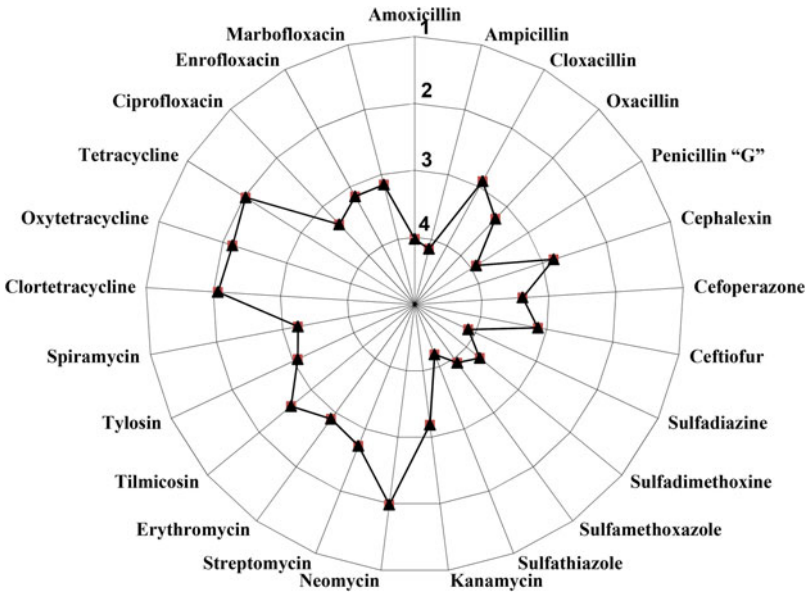


Fig. 3. Detection pattern (DL/MRL) of the bioassay. Polygon 1 = 0·1 MRL, Polygon 2 = MRL, Polygon 3 = 10 MRL and Polygon 4 = 100 MRL.

Bioassay cross-specificity: detection limits for other ATBs

Logistic regression model equations for the detection of others ATBs groups in milk are shown in Table 5. It was observed that the concordance coefficients were high, ranging from 88·2% (sulphadiazine) to 98·9% (tylosine), indicating a good fit with the logistic model. The 'β_1' coefficients values calculated using (Eq. 5) for Beta-lactams: (0·0022–0·0058), Aminoglycosides (0·0014–0·0019), Macrolides (0·0013–0·0083), Sulphamides (0·0001–0·0002) and Quinolones (0·0011–0·0030) were lower than determined for TCs ($\beta_{1,\ OTC} = 0{\cdot}0115$; $\beta_{1,\ TC} = 0{\cdot}0090$ and $\beta_{1,\ CTC} = 0{\cdot}0070$) pointing out lower sensitivity for detection of other ATBs. Figure 3 shows the DL relative to the corresponding MRLs for all ATB tested. The polygonal figure uses a standardised logarithmic scale (DL/MRLs) proposed by Suhren et al. (1996) for these sensitivity studies. The outside polygon (1) represents concentrations equivalent to 0·1 times the respective MRLs, the (2) polygon corresponds to levels equivalent to MRL, while (3) polygon corresponds to milk samples that contain 10 times MRLs and inside polygon (4) concentrations equal to 100 times MRLs. This figure visualised that this bioassay properly detected TCs and neomycin residues, but did not detect the other ATBs. For this reason, except neomycin (with DL similar to MRL), the rest of ATBs had high levels of detection, between 5 (tilmicosin) and 150 (sulphathiazole) times their respective MRLs. From this, it can be established that the *B. pumilus* bioassay has low inconveniences of cross-specificity (except neomycin). This screening method can be used to classify TCs in milk by Microbiological System in microtiter plates (SMMP) and replace the *B. cereus* bioassay proposed by Nagel et al. (2013), because *B. cereus* could present certain risks to the health of laboratory workers.

Conclusions

A microbiological inhibition bioassay containing $1{\cdot}6 \times 10^9$ spores/ml of *B. pumilus* fortified with 2500 µg CAP/l allows adequate levels of detection for TCs residues in milk with a response within 5 h. This microbiological method has good specificity (97·9%), with low cross-specificity problems (except neomycin), offering simple interpretation of results and easy implementation in quality control laboratories. In addition, this bioassay may be incorporated into the multi-residue microbiological microplate systems (SMMP) for previous classification of TCs in milk samples before its quantitation by high pressure liquid chromatography (HPLC).

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https://doi.org/10.1017/S0022029915000138 Published online by Cambridge University Press