Utilization of *B. cereus* and *B. subtilis* strains in plate diffusion methods for the detection of tetracycline residues in milk

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Summary

The reference method for the detection of residues of antimicrobial substances in raw materials and foods of animal origin is the plate diffusion method both in the Czech Republic and the European Union (EU). The aim of the study was to verify the suitability of the plate diffusion method with strain *B. cereus* CCM 869 (= ATCC 11778) that is recommended by EU guidelines (the STAR protocol) for the detection of residues of tetracycline antibiotics (chlortetracycline, tetracycline and oxytetracycline) in milk, and to compare its sensitivity with that of strain *B. subtilis* CCM 4062 (= BGA) that is recommended for the detection of antibiotics according to the guidelines of the Czech National Reference Laboratory. Chlortetracycline was the only substance reliably detected by the methods tested at levels equal to the maximum residue limit (MRL) of 0.1 mg·kg^{-1}. The sensitivity of the assay with *B. cereus* for tetracycline and oxytetracycline were six and eight times the MRL, respectively. The sensitivity for tetracycline was higher when using the discs of a diameter of 12.7 mm. The assay with *B. cereus* facilitated detection of tetracycline at a concentration of 0.5 mg·l^{-1} and oxytetracycline at a concentration of 0.7 mg·l^{-1}. Neither of the methods reached the required sensitivities for tetracycline and oxytetracycline, respectively.

Keywords

antibiotic; residue; agar diffusion; tetracycline

One of important roles of the food chain surveillance is to protect the public from harmful effects of exogenous substances and their residues that constitute health hazard for man. Residues of medical drugs and their metabolites in raw materials of animal origin may occur whenever drugs are administered to food-producing animals [1]. In order to protect consumers and assure safety along the food chain, limits have been established for drug residues in foods in the form of tolerances or maximum residue limits (MRL). Within the European Union, each member state is obliged to monitor food-producing animals and their products for residues of legally and illegally used veterinary drugs [1, 2]. In 1991, the International Dairy Federation (IDF) proposed a strategy to control residues of veterinary drugs in milk. The 3-stage system recommends that qualitative (screening) methods be used in the first step to identify positive samples containing residues of inhibitory substances. In the second step, post-screening methods are to be applied that make it possible to categorize the substance into a specific group of drugs and, in the third step, a confirmation method is to be used to identify and quantify the substance in question [3, 4].

Microbiological inhibition methods are widely used for the detection of residues of antimicrobial substances in raw materials and foods of animal origin [3, 5, 6]. They are the most suitable screening methods thanks to their ability to detect a broad range of antibacterial substances of different chemical structures [1]. These methods use liquid or solid media inoculated with a standard culture of test microorganisms, e.g. *Geobacillus stearothermophilus* var. *calidolactis* C 953, *Bacillus subtilis*, *Bacillus cereus, Micrococcus luteus, Escherichia coli, Bacillus megatherium,* or *Streptococcus ther-*

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mophilus [1, 6]. IDF recognizes two major groups of microbiological inhibition methods, i.e. microbial inhibitor screening tests and microbial inhibitor tentative confirmation tests (plate diffusion methods) [6]. The technique of milk sample application differs depending on the method used – in microbiological screening tests, samples are applied directly, while in plate diffusion tests, samples are applied by means of paper discs, stainless steel cylinders, or to wells in the medium. During incubation, the sample diffuses into the medium (principle of agar diffusion) and if it contains residues of inhibitory substances to which the test organism is sensitive, the growth of the test organism will be inhibited. Depending on the method, the presence of inhibitory substances in the test sample is indicated by either a growth inhibition zone or a change in the colour of the medium [5, 6, 7]. The advantage of plate methods is that they allow detection of the antimicrobial substance present in the sample, its group identification and, based on the size of the inhibition zone, its approximate quantification [1].

Microbiological inhibition methods published and recommended by IDF for the detection of inhibitory substance residues in milk include methods that use *B. subtilis* and *B. cereus* strains as test microorganisms [6]. Botsoglou and Fletouris [1] published a survey of contemporary microbiological inhibition methods used in the screening for antibacterial substance residues in milk. Strain *B. subtilis* ATTC 6633 is used as the test microorganism not only in microbiological screening tests such as Arla microtest (Arla Foods, Stockholm, Sweden) or *B. subtilis* field test (Association of Official Analytical Chemists, Gaithersburg, Maryland, USA), but also in plate diffusion methods (three-plate test; six-plate test). *B. cereus* as a test microorganism is used mainly in plate diffusion methods (six-plate test).

In both the Czech Republic and the European Union (EU), the reference method for the detection of residues of antimicrobial substances in raw materials and foods of animal origin is the plate diffusion method [8, 9]. In EU countries and in the rest of the world, many different versions of plate diffusion methods are used. The recommended method for reference laboratories in the European Community (EC) is the STAR method [9].

Tetracyclines constitute an important group of substances with a broad clinical utility and are among the most frequently used antimicrobial substances in food-producing animals [1]. According to the statistics on the use of antibiotics in veterinary medicine in the Czech Republic between 2003 and 2008, tetracycline antibiotics were the unchallenged number one [10]. Together with beta-lactam antibiotics, aminoglycosides, quinolones and polymyxins, tetracyclines rank among the most frequently administered antimicrobial substances for the treatment of mastitis [11].

The aim of the present study was to verify the suitability of the plate diffusion method with the *B. cereus* CCM 869 (= ATCC 11778) strain that is recommended by EC guidelines [9] for the detection of residual tetracycline antibiotics (chlortetracycline, tetracycline and oxytetracycline) in milk, and to compare its sensitivity with that of strain *B. subtilis* CCM 4062 (= BGA) that is recommended and used in the plate diffusion method for the detection of this group of antibiotics by the guidelines of the Czech National Reference Laboratory (NRL, State Veterinary Institute, Jihlava, Czech Republic) [8].

MATERIAL AND METHODS

Bacterial strains

The test strains of *B. subtilis* CCM 4062 (= BGA) and *B. cereus* CCM 869 (= ATCC 11778) for plate diffusion methods were obtained from the Czech Collection of Microorganisms (Faculty of Natural Sciences, Masaryk University, Brno, Czech Republic).

Standard solutions of antibiotisc

For the preparation of standard solutions of antibiotics, chlortetracycline hydrochloride (C 4881; Sigma Aldrich, St. Louis, Missouri, USA), tetracycline hydrochloride (T 3383; Sigma Aldrich) and oxytetracycline hydrochloride (O 5875; Sigma Aldrich) were used. Stock solutions of antibiotics were also prepared at 0.1 $mg \cdot ml^{-1}$ active substance concentration by dissolving an adequate amount of the substance in distilled water. Working solutions at active substance concentrations of 5, 10 and 15 $mg \cdot l^{-1}$ were obtained by appropriately diluting the stock solutions. If the plate diffusion method showed no sensitivity to the antibiotic tested at the antibiotic concentration equal to MRL, 1.5 times the MRL or 0.5 times the MRL in milk samples, working solutions with a higher concentration of antibiotics were prepared (up to 80 $mg \cdot l^{-1}$). Stock solution of penicillin G at a concentration of 100 $IU \cdot ml^{-1}$ was obtained by diluting penicillin G (P 3032; Sigma Aldrich) in distilled water.

Preparation of artificially contaminated milk samples

Fresh milk of 15 $g \cdot l^{-1}$ fat content was added 100 μl of the antibitiotic solution to make a total

of 10 ml. The antibiotic concentration in milk samples was equal to the defined MRL, 0.5 times the MRL value and 1.5 times the MRL value. If no sensitivity to these concentrations was observed, samples with concentrations of up to 8 times the MRL value (oxytetracycline) were prepared. At the same time, a milk sample with no added antibiotics was tested for the presence of antimicrobial substances as a negative control.

Verification of the methods' sensitivity – control discs

Two standard solutions containing reference antibiotics were prepared to verify the sensitivity of the methods tested. To verify the sensitivity of the method with *B. cereus* as a test strain, standard chlortetracycline solution with an active substance concentration of 200 $\mu g \cdot l^{-1}$ is required by EC guidelines [9]. The solution was prepared by diluting the stock chlortetracycline solution. A volume of 30 μl of the control solution was applied onto a disc of 9 mm in diameter (Antibiotics test discs, Fioroni, Ingré, France) and placed on the test agar. The threshold size of the inhibition zone (IZ) was 6 mm ± 1.5 mm.

To verify the sensitivity of the plate diffusion method using the *B. subtilis* strain, penicillin G solution of 1 $IU \cdot ml^{-1}$ concentration is specified by the Czech NRL guidelines [8]. The control solution was prepared by diluting the stock penicillin G solution. The method's sensitivity was tested using sterile paper discs of 6 mm in diameter (Blank cartridges; Oxoid, Basingstoke, United Kingdom) to which 10 μl of control penicillin G solution was applied. The threshold size of the inhibition zone was 6 mm ± 1.5 mm.

Plate methods with the *B. cereus* strain

Culture and solutions

In this method, the media used for cultivation included the Tryptone soya agar (TSA; HiMedia, Mumbai, India), medium for sporulation and test agar of pH 6 (Antimicrobial inhibitor test agar pH 6; HiMedia). Cultivation media were prepared according to the manufacturer's recommendations, and the sporulation medium in accordance with the EU guidelines [9]. Sterile physiological saline solution contained 8.5 $g \cdot l^{-1}$ NaCl.

Preparation of test plates with B. cereus

Gel discs with *B. cereus* CCM 869 were reconstituted according to the manufacturer's recommendations. The spore suspension of *B. cereus* was prepared in accordance with EU guidelines [9]. The initial spore concentration in the suspension was set to 10^8 $CFU \cdot ml^{-1}$. The pH 6 test agar was heated to 55 °C and inoculated with *B. cereus* spore suspension to approximately 10^4–10^5 $CFU \cdot ml^{-1}$. The agar with the test strain was pipetted at 5 ml doses to sterile glass Petri dishes of 90 mm in diameter.

The plate method with *B. subtilis*

Cultivation media and solutions

In order to prepare test plates with *B. subtilis*, test agar pH 6 was used. Further, sporulation medium was used (containing, in 500 ml, proteose peptone 1.725 g (HiMedia), casein enzyme hydrolysate 1.725 g (HiMedia), NaCl 2.55 g; Agar No. 1 6.5 g (Oxoid); potassium dihydrogen phosphate 0.5 g (KH_2PO_4, Merck, Darmstadt, Germany) pH 7, sterilized at 121 °C for 15 min.

Preparation of B. subtilis spore suspension

The *B. subtilis* spore suspension was prepared in accordance with the method of Bogaerts and Wolf [12]. Gel discs with *B. subtilis* CCM 4062 were reconstituted according to the manufacturer's recommendations.

Preparation of test plates with B. subtilis CCM 4062

The pH 6 test agar was heated to 55 °C and inoculated with *B. subtilis* spore suspension to approximately 10^4 $CFU \cdot ml^{-1}$. The agar with the test strain was pipetted at 4 ml doses to pre-heated sterile glass Petri dishes of 90 mm in diameter.

Tests of artificially contaminated milk samples

Sensitivity tests of the plate method with *B. cereus* were conducted in accordance with the STAR method [9] with discs of 9 mm in diameter (Antibiotics test discs, Fioroni) and, at the same time, according to methodological guidelines of the Czech NRL [8] using discs of 12.7 mm in diameter (Blanc paper discs, Albet LabScience, Barcelona, Spain). In accordance with the methodological guidelines, only 12.7 mm diameter discs were used to test milk samples in the plate method with *B. subtilis*. Milk samples with a standard concentration of the antibiotic were mixed and the paper disc was put into the sample soak. Excess liquid was removed by wiping the disc against the inner surface of the sample container. Each sample with a standard concentration of the antibiotic and the *B. cereus* strain was tested 20 times and each of samples with the *B. subtilis* strain at least 10 times.

Results assessment

At the end of the incubation, the total growth inhibition of the test strain was assessed around the disc (inhibition zone), and its size in mm was

recorded. The inhibition zone width is defined as the distance of the inhibition zone border from the disc edge. The zone diameter was measured using the HiAntibiotic Zone Scale (HiMedia). A regular inhibition zone around the disc ≥ 2 mm in size was interpreted as a positive test result. If the inhibition was biphasic, the distance of zone's inner edge was measured. Only complete growth inhibition of the test strain was evaluated. Cases of partial growth inhibition without clear inhibition zone delimitation were not evaluated.

Interpretation of results

Relationship between the percentage of positive (IZ ≥ 2 mm) and negative (IZ < 2 mm) results and the analyte concentration was calculated, from which the calculations were made of the concentration threshold value at which the tests become unreliable and, at the same time, a concentration to which the given strain is sensitive. Strains were evaluated as sufficiently sensitive if all discs (100%) returned a positive result when the sample was tested at a standard concentration of the antibiotic.

Statistical evaluation of results

Basic statistical evaluation of results was performed using Unistat 5.1 statistical software (Unistat, London, United Kingdom). The statistical comparison of inhibition zone sizes of milk sample tests employing the *B. cereus* strain and identical tetracycline residue concentrations but different disc sizes (12.7 mm and 9 mm) was performed by Student's *t*-test. Student's *t*-test was also used to compare the sensitivity of the method with the *B. cereus* strain to oxytetracycline when samples were tested using discs of different sizes.

RESULTS AND DISCUSSION

In accordance with methodological guidelines of the Czech National Reference Laboratory for Residues of Veterinary Drugs [8], the minimum basic range stipulated in the Czech Republic to test for the presence of inhibitory substance residues in milk for the purpose of the state veterinary surveillance comprises the plate diffusion method and the microbiological screening test Eclipse 50 (Zeu-Inmunotec, Zaragoza, Spain). For the detection of tetracycline antibiotics in milk by the diffusion plate method, different test strains are used in the Czech Republic and in the EU.

Similar to other screening methods, plate diffusion methods should provide reliable and accurate information regarding the presence of the substance at hazardous or prohibited concentrations [1]. Plate diffusion methods employing sensitive test strains should therefore facilitate detection of antibiotics at least at concentrations set as MRL. MRL for tetracycline (TTC), oxytetracycline (OTC) and chlortetracycline (CTC) in raw cows' milk is 0.1 mg·kg^{-1}, as set by EC legislation [13].

Tab. 1. Sensitivities of plate diffusion methods utilizing *B. cereus* and *B. subtilis* test strains to tetracycline antibiotics.

Antimicrobial drug	Sensitivity [mg·l^{-1}]	
	B. cereus	*B. subtilis*
tetracycline	0.6 [a]; 0.5 [b]	0.5 [b]
chlortetracycline	0.1 [a]; 0.1 [b]	0.1 [b]
oxytetracycline	0.8 [a; b]	0.7 [b]

MRL for all antibiotics was 0.1 mg·kg^{-1}.
a – using discs of 9 mm in diameter, b – using discs of 12.7 mm in diameter.

A summary of sensitivity results of the tested plate methods with *B. cereus* CCM 869 and *B. subtilis* CCM 4062 to CTC, TTC and OTC residues in milk is presented in Tab. 1. Of the tetracycline antibiotics tested, the plate diffusion method with *B. cereus* was sensitive only to CTC at a concentration close to MLR when discs of 9 mm as well as of 12.7 mm in diameter were used (Tab. 1 and 2). Of the tetracyclines tested, the plate diffusion method with *B. subtilis* was, at a concentration equal to MLR, sensitive only to CTC with discs of 12.7 mm in diameter (Tab. 1 and 3).

It is apparent from Tab. 1 that TTC and OTC were not detected at concentrations lower than or equal to MRL, and that they were only detected at concentrations several times higher. The plate diffusion method with *B. subtilis* proved more sensitive to OTC than the method using *B. cereus* (Tab. 1).

The two methods and the test strains of microorganisms showed equal sensitivities to TTC when discs of 12.7 mm in diameter were used (Tab. 1). A difference in sensitivity to TTC was observed with *B. cereus* when discs of 9 mm in diameter compared to discs of 12.7 mm in diameter (Tab. 1 and 4). The plate diffusion method using *B. cereus* was found more sensitive if samples were tested using the discs of 12.7 mm in diameter. When the prescribed method was used (discs of 12.7 mm in diameter), *B. subtilis* CCM 4062 was sensitive to TTC in milk at a concentration of ≥ 0.5 mg·ml^{-1} (Tab. 5). Previously published data on the sensitivity of plate methods utilizing *B. cereus* and *B. subtilis* for the detection of tetracycline residues showed

Tab. 2. Results of the analysis of milk samples artificially contaminated with chlortetracycline (CTC) by the plate diffusion method with *B. cereus.*

	CTC concentration			
	0.1 mg·l^{-1} (= MRL)		0.15 mg·l^{-1} (= 1.5 MRL)	
Disc diameter [mm]	9.0	12.7	9.0	12.7
Number of samples	20	20	20	20
Mean inhibition zone [mm]	2.28	3.03	3.26	4.53
*S*tandard deviation [mm]	0.36	0.54	0.30	0.49
Minimum inhibition zone [mm]	2.00	2.00	3.00	5.00
Maximum inhibition zone [mm]	3.00	4.00	4.00	5.00

Tab. 3. Results of the analysis of milk samples artificially contaminated with chlortetracycline (CTC) by the plate diffusion method with *B. subtilis.*

	CTC concentration		
	0.05 mg·l^{-1} (= 0.5 MRL)	0.1 mg·l^{-1} (= MRL)	0.15 mg·l^{-1} (= 1.5 MRL)
Disc diameter [mm]	12.7	12.7	12.7
Number of samples	20	20	20
Mean inhibition zone [mm]	0.58	2.28	2.81
*S*tandard deviation [mm]	0.25	0.25	0.46
Minimum inhibition zone [mm]	0.00	2,00	2.00
Maximum inhibition zone [mm]	1.50	2.25	3.50

Tab. 4. Results of the analysis of milk samples artificially contaminated with oxytetracycline (OTC) and tetracycline (TTC) and by the plate diffusion method with *B. cereus.*

	Antibiotic, concentration			
	OTC 0.8 mg·l^{-1}		TTC 0.6 mg·l^{-1}	TTC 0.5 mg·l^{-1}
Disc diameter [mm]	9.0 *	12.7 *	9.0	12.7
Number of samples	20	20	20	20
Mean inhibition zone [mm]	2.64	3.13	2.32	2.99
*S*tandard deviation [mm]	0.21	0.31	0.29	0.48
Minimum inhibition zone [mm]	2.25	2.50	2.00	2.50
Maximum inhibition zone [mm]	3.00	3.75	3.00	3.75

* – the differences between arithmetic means of inhibition zones for 9.0 mm and 12.7 mm discs as calculated by the Student's *t*-test are significant ($P = 0.01$).

Tab. 5. Results of the analysis of milk samples artificially contaminated with oxytetracycline (OTC) and tetracycline (TTC) by the plate diffusion method with *B. subtilis.*

	Antibiotic, concentration	
	OTC 0.7 mg·l^{-1}	TTC 0.5 mg·l^{-1}
Disc diameter [mm]	12.7	12.7
Number of samples	20	20
Mean inhibition zone [mm]	2.53	2.58
*S*tandard deviation [mm]	0.30	0.44
Minimum inhibition zone [mm]	2.00	2.00
Maximum inhibition zone [mm]	2.75	3.00

significant differences. HESCHEN and BLÜTHGEN [4] published a survey of microbiological inhibition methods recommended by IDF for the detection of residues of antimicrobial substances in milk, and a comparison of sensitivity values of those methods to antimicrobial substances. At that time, the sensitivity to tetracycline residues of the 3-plate method (utilizing strains *Bacillus stearothermophilus* var. *calidolactis* C 953, *Bacillus subtilis* and *Bacillus megatherium*) and of the 6-plate method (with the test strains of *Bacillus cereus, Bacillus subtilis, Sarcina lutea, Escherichia coli* and *Bacillus stearothermophilus* var. *Calidolactis* C 953) was reported at concentrations of 0.4–5 $\mu g \cdot ml^{-1}$ and 1.5 $\mu g \cdot ml^{-1}$, respectively. In another survey, detection limits for tetracyclines of 0.4 $mg \cdot kg^{-1}$ and 0.4–0.5 $mg \cdot kg^{-1}$ for the 3-plate method and the 6-plate method, respectively, were published [1]. We found a comparable sensitivity to tetracycline using the the *B. subtilis* strain (Tab. 1), but, in contrast to the originally recommended procedure, we used discs of 12.7 mm in diameter in this case as well as when we used the *B. subtilis* strain. If we were to assess sensitivity of 3-plate and 6-plate methods based on the above literary sources, we would conclude that tetracycline detection limits did not coincide with concentrations set as MRL, and for that reason these methods are not suitable for the detection of tetracycline residues in milk.

According to another literary source, plate diffusion methods are only suitable for the detection of CTC in milk. GAUDIN et al. [14] determined sensitivities to 66 antimicrobial substances when validating the STAR method. According to these results, which also correspond to our conclusions, the method with the *B. cereus* strain is able to detect only CTC of the group of tetracycline antibiotics at levels equal to or lower than MRL. With respect to the other antibiotics of that group, i.e. TTC and OTC, the authors found sensitivity to concentrations higher than MLR and lower than 4 times the MLR value (200–250 $\mu g \cdot l^{-1}$). In our study, the sensitivity to TTC using the variant with *B. cereus* was 6 times the MLR value, and to OTC 8 times the MLR value. A difference in the evaluation of results could be one of possible reasons for the difference in the determined sensitivity. When validating the STAR method, GAUDIN et al. [14] tested each of the milk samples artificially contaminated with an antimicrobial substance in 5 Petri dishes, with each of the dishes containing 4 discs (i.e. they used 20 discs for each sample). When evaluating results in individual Petri dishes, they measured the diameter of the inhibition zone (IZ) of all 4 discs there, and then they determined the mean diameter of IZ in mm. A sample was considered as positive when the mean diameter of IZ thus calculated was ≥ 2 mm. The method's sensitivity to an antimicrobial substance was defined as the lowest concentration at which a positive result was obtained with at least four discs, i.e. in one Petri dish. In our study, however, a method was considered as sufficiently sensitive at a given antibiotic concentration when a positive result, i.e. IZ ≥ 2 mm, was obtained with all the discs (i.e. in 100% of cases, $n = 20$ discs).

A number of factors may influence sensitivity results of agar diffusion methods besides the type of the test organism. It has been reported that the results might be affected by, e.g., the composition of the sample tested, agar thickness, concentration of the test strain spores in the medium, technique of sample application, agar pH, evaluation of results, etc.

Tetracyclines are generally characterized by their low lipophilicity and they do not occur at high concentrations in milk fat. Tetracyclines in milk are, however, bound to proteins, in particular to casein [1]. For that reason, the activity of the antimicrobial substance in milk samples artificially contaminated with antibiotics may be reduced due to differences in milk composition and the binding of antibiotics to milk components. Certain components naturally occurring in raw milk display antibacterial action and may produce false-positive results in microbiological inhibition methods [15, 16].

The two methods tested differed not only in the type of test strain sensitive to tetracycline residues, but also in the size of discs used in milk tests and the thickness of the agar layer in the Petri dish. The thickness of the agar layer affects the method's sensitivity and repeatability. Thinner agar layers are more sensitive than thicker ones [17]. The method employing the *B. subtilis* strain uses a thinner agar layer (4 ml agar per Petri dish) than the STAR method utilizing the *B. cereus* strain (5 ml agar per Petri dish). For that reason, the plate method with *B. cereus* may have a disadvantage compared to the method using *B. subtilis*.

Some studies, however, rank plate diffusion methods among techniques with a sufficient or even high sensitivity to the residues of tetracycline antibiotics [18–20]. Those methods, however, differ from methods tested in our study.

KIRBIŠ [20] used this technique (cylinders 8 mm in diameter) to hold samples when determining detection limits of a 5-plate method. Using the method with *B. cereus*, this author found detection limits for tetracycline and chlortetracycline in milk at 20 $\mu g \cdot kg^{-1}$. Those detection limits were significantly lower than in our study (Tab. 1). The

study, however, did not mention the volume of the sample.

The influence of the application of different sample quantities on the method's sensitivity was also observed in our study when samples were tested using discs of different sizes with different absorption capacities. The method using *B. cereus* strain was more sensitive to tetracycline (Tab. 1) when larger discs (12.7 mm in diameter) were used. A statistical comparison employing Student's *t*-test of inhibition zone diameters in tests of milk samples containing TTC at 0.5 $mg \cdot l^{-1}$ (5 times the MRL value) using samples with discs of different sizes (12.7 and 9 mm in diameter) and the method utilizing the *B. cereus* strain showed statistically highly significant differences ($p = 0.01$). Student's *t*- test also showed statistically highly significant differences ($p = 0.01$) in tests of the sensitivity of the method utilizing *B. cereus* to OTC residues (c = 8 MRL) when tests with discs of different sizes were compared.

CONCLUSIONS

Of the tetracycline antibiotics tested, plate diffusion methods recommended for the detection of tetracycline antibiotics in milk by European Union guidelines (STAR Protocol) and methodological guidelines of the Czech National Reference Laboratory that utilize *B. cereus* CCM 869 (= ATCC 11778) and *B. subtilis* CCM 4062 (= BGA) as test strains were able to reliably detect only chlortetracycline at a maximum residue limit (MRL) of 0.1 $mg \cdot kg^{-1}$. The sensitivity of the tested plate diffusion methods to TTC and OTC was low. This might have depended on several factors. The *B. cereus* plate method (the STAR protocol) was compromised in sensitivity when compared to *B. subtilis* plate method (according to guidelines of the Czech National Reference Laboratory) in the thickness of the agar and the size of the discs used. It may be concluded that plate diffusion methods utilizing *B. cereus* CCM 869 and *B. subtilis* CCM 4062 as test strains are not sufficiently sensitive to all tetracycline antibiotics with regard to MRL, and therefore do not meet the requirements for screening methods. It is therefore legitimate that sample testing, based on the recommendation of the Czech National Reference Laboratory, is extended to include another screening microbiological method with a greater sensitivity to tetracycline antibiotics.

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