Detection of antibiotic residues in food – pitfalls and optimization of agar diffusion tests in comparison with commercial test kits

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The presence of antibiotic residues in food and feed can cause serious problems for consumers (e.g. penicillin allergy) or by giving rise to the development and spread of antibiotic resistances. To safeguard food quality and consumer safety, the EU implemented regulations governing maximum residue levels in certain food products of animal origin such as milk. To detect the presence of antibiotic residues in food, regulatory authorities depend on quick screening methods. Bacterial growth inhibition tests are therefore routinely employed to screen for the presence of antibacterial residues, with some of these tests being available as commercial test kits. Different microbiological screening methods, including commercial systems such as the Delvotest or a lab based disk based agar diffusion method in combination with different *Bacillus* species, were evaluated for their applicability to detect selected antibiotics (chloramphenicol, tetracycline, penicillin G) in food or food related products. The two commercially available systems tested, Delvotest®SP Mini-NT and BR-Test®AS Brilliant, demonstrated to be useful for the detection of specific antibiotics. However, for the lab based agar diffusion test, a variety of adjustable parameters such as pH, agar concentration, disk diameter or the *Bacillus* species used influenced the detection of target antibiotics. Therefore, an optimized and statistically verified agar diffusion assay proved to be a useful addition to the commercial tests as it enabled a higher sensitivity for the detection of tetracycline.

Keywords: Antibiotic residues; food materials; agar diffusion test; Delvotest; BR-Test

1. Introduction

Veterinary drugs, especially antimicrobial substances, are widely used in animal production to prevent (prophylactic use) and treat (therapeutic use) diseases as well as to promote growth [1, 2]. As a result of their extensive use, antibiotic residues are frequently detected in animal products as well as in the environment [3, 4]. In 2012 alone, more than 50 notifications reporting the detection of veterinary medicinal products - including antibiotics - in food or feed in the EU were published via the European Rapid Alert System for Food and Feed (RASFF) [5]. Between 2006 and 2012, 7 notifications by the RASFF specifically reported the detection of antibiotics including β-lactams (penicillin-like) and chloramphenicol in milk and milk products [5].

Potential health risks due to the presence of antibiotic residues in food and the environment have been discussed for a long time [3, 6-10], frequently with a focus on the increasing occurrence of antibiotic resistant bacterial strains [10-12]. More recently, the treatment of Danish farm land with pig manure slurry was linked to the occurrence of tetracycline resistant bacteria in the farm soil [13]. Shipp and Dickson [14] reported that the introduction of cattle farming led to the appearance of multi-drug resistant species of the family *Enterobacteriaceae*. Studies by Grote *et al*. [15, 16] showed in model farming experiments that even plants can take up antibiotics from manure present in soil. This raised concern as antibiotic residues might be transferred into plants in amounts that could pose a health risk for consumers [17, 18]. In addition, the dairy industry might encounter problems due to the inhibition of microorganisms involved in fermentation and ripening processes of milk products caused by the presence of antibiotic residues [19].

To prevent these problems and safeguard consumers' health, maximum residue levels (MRLs) for antibiotics and for veterinary drugs were implemented by the European Union and similarly suggested by other international institutions [20-22]. For certain veterinary drugs such as for chloramphenicol, the use is prohibited in the EU for animals which are directly involved in the production of food. For chloramphenicol no MRL is specified by the EU and residues in food are not permitted. Therefore, methods with low detection limits are necessary to ensure the absence of such compounds in food materials.

The use of screening tests for the detection of antibiotic residues in food is therefore very important. This includes a large variety of detection methods, ranging from physico-chemical analysis (e.g. LC/MS) or immunological detection (e.g. ELISA) to microbiological methods (e.g. growth inhibition tests) [23, 24], with some commercially available as test kits. To detect specific antibiotics present in very low quantities, physico-chemical analytical techniques such as LC/MS are considered to be the most precise. However, as these sophisticated methods need expensive technical apparatus and often specific sample preparations, they are costly and laborious. While the screening for a broad variety of antibiotics with one single preparation might be compromised due to the different extraction behaviour of individual antibiotics, the verification of the presence or absence and the quantification of specific antibiotics can be done at method detection limits which are typically in the ng to μg per litre or kg range for dairy products or other food [25-27].

The use of microorganisms for the detection of growth inhibiting compounds is well known and has been in use for a long time [28-34]. It is a screening method which is fast, inexpensive as it does not require sophisticated equipment and easy to perform. Some agar diffusion methods are even implemented into official national standard methods such as the three-plate-test which is using *Bacillus subtilis* BGA [35] or the brilliant black reduction test (BRT) using *Geobacillus stearothermophilus* [36]. Most of the commercially available rapid detection test kits were specifically developed for the dairy sector to detect antibiotics in milk [23, 37-39]. Among these are growth inhibition tests (for example Delvotest®SP Mini-NT and BR-Test®AS Brilliant) which again use *Geobacillus stearothermophilus*.

The aim of this study was to compare two commercially available test kits with lab based agar diffusion tests by evaluating an alternative test organism (*Bacillus pumilus*) and by analysing the influence of certain test parameters for the detection of three selected antibiotics.

2. Methods

2.1 Bacterial strains and commercial test kits

Bacillus subtilis BGA (DSM 618), *Bacillus pumilus* (ATCC 27142) and *Geobacillus stearothermophilus* (spore suspension, Merck no. 1.11499, Germany) were used as test strains in this study. As commercially available test kits the Delvotest®SP Mini-NT and the BR-Test®AS Brilliant (DSM Food Specialities, Netherlands) were used; both of which use *Geobacillus stearothermophilus* var. *calidolactis* as test strain.

2.2 Media

The sporulation medium used for the production of spores contained 8.0 g Nutrient Broth (Difco) per litre, sterilized at 121°C for 20 minutes. After sterilization 5 ml metal mix (1.03 g $CaCl_2$ x $2H_2O$; 0.10 g $MnCl_2$ x $4H_2O$ and 0.95 g $MgCl_2$ x $2H_2O$ dissolved in 50 ml demineralized water, sterile filtrated) and 0.5 ml iron solution (30 mg $FeCl_3$ x $6H_2O$ dissolved in 50 ml demineralized water, sterile filtrated) were added.

For the agar based diffusion tests, Kundrat-agar (test-agar for the residue test acc. to Kundrat, Merck no. 1.10662) was prepared according to the instructions of the manufacturer. However, the pH was adjusted to 8.0.

2.3 Spore production

For the production of spores, cultures of *Bacillus subtilis* and *Bacillus pumilus* were incubated in sporulation medium for 72 h at 37°C and 150 rpm. Spores were harvested by centrifugation (9500 x g) for 20 minutes, washed five times with sterile demineralized water, suspended in sterile demineralized water and adjusted to a concentration of about $5x10^{10}$ spores per ml by microscopy using a Helber type bacterial counting chamber. Aliquots of these spore suspensions were stored at -18°C until usage. Before adding spore suspension to the sterilized test medium (final concentration of about 4 x 10^7 spores per ml test medium), the spores of *Bacillus subtilis* and *Bacillus pumilus* were heat activated at 75°C for 30 minutes. The spore suspension of *Geobacillus stearothermophilus* was used according to the manufacturer, resulting in a final spore concentration of about 10^6 spores per ml test medium.

2.4 Antibiotics

All stock solutions were freshly prepared on the day of analysis in 100 ml volumetric flasks to a concentration of 100 mg per 100 ml each. Tetracycline-HCl (Carl Roth GmbH, Germany) and penicillin G sodium salt (Fluka, Germany) were dissolved in sterile demineralized water, while chloramphenicol (Carl Roth GmbH, Germany) was first dissolved in 5 ml pure ethanol and then adjusted to 100 ml with sterile demineralized water. Subsequent dilutions were prepared in sterile demineralized water. Additionally, similar solutions of the three antibiotics were prepared by using UHT milk (1.5 % and 3.5 % fat content). Demineralized water and UHT milk were used as negative controls. All solutions were kept on ice during the analysis.

2.5 Agar diffusion tests

2.5.1 Disk based agar diffusion test

For the disk based diffusion test, 5 ml of Kundrat-agar (pH 8.0) containing the spore suspension of individual test strains was added to sterile Petri dishes (90 mm diameter). Cellulose disks (Whatman Type 2668, 12.7 mm diameter) were sterilized at 121°C for 10 minutes in glass Petri dishes and dried over night at 65°C. The disks were soaked in test solutions and gently pressed onto the agar surface. All plates were incubated at 37°C overnight. The zones of inhibition were determined with a digital calliper (Top Craft; DMV-SL05), measuring the diameter between the edge of the antibiotic test disk and the outer edge of the growth inhibition zone. Analyses were done in triplicate with 5 single measurements (= 5 disks) for every antibiotic concentration tested.

2.5.2 Modified agar diffusion test

For the modified agar diffusion test, 25 ml of Kundrat-agar (pH 8.0, with added spores) were poured into sterile Petri dishes (90 mm diameter) and holes of about 6-8 mm diameter were punched out after solidification. Holes were filled with 60 µl of test solutions. All plates were incubated at 37°C overnight. The inhibition zones were determined as specified above. Analyses were done in triplicate with 5 single measurements (= 5 holes) for every antibiotic concentration tested.

2.5.3 Commercial test kits

The Delvotest®SP Mini-NT and BR-Test®AS Brilliant were performed according to the instructions of the manufacturer. Analyses were done in triplicate for every antibiotic concentration tested.

2.6 Influence of disk diameter, agar concentration and pH

The influence of disk diameter, agar concentration and pH on the detection of antibiotic residues was analysed by using the disk based agar diffusion test as mentioned under 2.5.1.
Disk diameter: In addition to 12.7 mm disks, cellulose disks (Whatman Type 2668) with a diameter of 6 and 9 mm were tested. Analyses were done in triplicate with 5 single measurements (= 5 disks of 9 mm) and 7 single measurements (= 7 disks of 6 mm) for every antibiotic concentration tested.
Agar concentration: Kundrat-agar was prepared manually according to Merck no. 1.10662 but with a varying agar concentration of 6.0; 8.0; 10.0; 12.0 and 14.0 g/l. Antibiotic concentrations of 1 mg/l (penicillin G and tetracycline) and 10 mg/l (penicillin G, tetracycline and chloramphenicol) were tested with 5 single measurements each.
pH: In addition to pH 8.0, Kundrat-agar (Merck no. 1.10662) was adjusted to pH 5.0; 6.0 and 7.2, respectively. However, only *Bacillus pumilus* was used as test strain. Antibiotic concentrations of 10 mg/l were tested with 4 single measurements each.

2.7 Chemicals

If not stated otherwise, all chemicals used were of the highest purity commercially available.

2.8 Statistics

The data were analysed using SPSS (Release 13.0) to determine the minimum antibiotic concentration that was detectable (growth inhibition zone ≥ 1 mm or colour change of the indicator). The resulting detection limits were calculated at 95 % confidence level.

3. Results and Discussion

3.1 Use of agar diffusion tests with different test strains and comparison to Delvotest®SP Mini-NT and BR-Test®AS Brilliant

Kundrat-agar contains bromocresol purple as pH indicator which turns from purple to yellow due to the formation of acid from glucose concomitant with bacterial metabolism. Hence, growth inhibition is not only indicated by a clear zone but also by a lack of change in colour from purple to yellow. However, only *Geobacillus stearothermophilus* was able to bring about the change in colour of the pH indicator present in the medium from purple to yellow. Nevertheless, a clear inhibition zone was detectable when using the two other test strains.

Bacillus pumilus proved to be a useful addition as this strain was more susceptible to tetracycline than *Geobacillus stearothermophilus*, *Bacillus subtilis* BGA and the two commercial test systems Delvotest®SP Mini-NT and BR-Test®AS Brilliant (Table 1). Considering that for *Bacillus pumilus* the detection limit for tetracycline was 19 µg/l (water), 31 µg/l (milk, 1.5% fat) and 38 µg/l (milk, 3.5% fat) with the disk based agar diffusion method, of the strains tested in this study only *Bacillus pumilus* would be able to detect tetracycline at a concentration below the MRL of 100 µg/kg specified by the EU for milk [21]. A positive result obtained when using one of the other test systems would therefore be due to tetracycline concentrations exceeding the EU MRL for tetracycline in milk. The results for tetracycline detection using *Bacillus subtilis* were similar to those reported by Navrátilová *et al*. [34] for *Bacillus* strains. However, using a *Bacillus cereus* strain Nouws *et al*. [40] determined lower detection limits for tetracycline that were in the same range as those established for *Bacillus pumilus* in this study. In contrast to this, the MRL for penicillin G (benzylpenicillin) in milk is specified by the EU [21] as 4 µg/kg; a level that could only be detected by the two commercial test kits. Obviously, the agar diffusion tests were not sensitive enough. However, all test systems used in this study showed a quite high detection limit for chloramphenicol (Table 1). This is worrying as the presence of this substance in food is prohibited in the EU [21] while the test systems detected chloramphenicol only at concentrations exceeding 3 mg/l.

Overall, the detection limits established by using the two commercial test kits (Table 1) were in the same range as those specified by the manufacturer, albeit a detection limit for chloramphenicol is not specified for the BR-Test®AS Brilliant. Similar results using a Delvotest®SP were reported for tetracycline, penicillin G [39, 41] and chloramphenicol [41].

The detection limits established using the modified agar diffusion test were always higher than those obtained using the disk based agar diffusion test (Table 1). This is not surprising as the holes of the modified agar used in this test were filled with 60 µl of the test solutions while the 12.7 mm disks carried an average test solution volume of 101 µl. Of all three test strains, *Bacillus subtilis* seemed to be the least sensitive with the exception of the detection of chloramphenicol, in which case *Bacillus pumilus* was the least susceptible. Although Delvotest®SP Mini-NT and BR-Test®AS Brilliant are also using *Geobacillus stearothermophilus*, the results for the agar diffusion tests with *Geobacillus stearothermophilus* showed higher detection limits. A reason for this might be due to the fact that the agar diffusion tests were incubated at a temperature of 37°C over night in this study while the commercial tests prescribe an incubation for 2-3 h at a temperature of 64°C which is closer to the optimum temperature for *Geobacillus stearothermophilus*.

Table 1 Detection limits for selected antibiotics with different test organisms and test systems (all data in µg/l).

	Penicillin G in water / milk 1.5 % fat / milk 3.5 % fat	**Tetracycline** in water / milk 1.5 % fat / milk 3.5 % fat	**Chloramphenicol** in water / milk 1.5 % fat / milk 3.5 % fat
Disk based agar diffusion test			
B. subtilis	113 / 122 / 126	380 / 534 / 561	11200 / 11800 / 12200
B. pumilus	81 / 92 / 95	19 / 31 / 38	23500 / 28200 / 32900
G. stearothermophilus	12 / 15 / 16	125 / 194 / 205	9600 / 9900 / 10500
Modified agar diffusion test			
B. subtilis	161 / 206 / 214	432 / 854 / 911	13400 / 19000 / 20600
B. pumilus	96 / 114 / 123	25 / 77 / 84	27800 / 38100 / 40500
G. stearothermophilus	13 / 17 / 19	136 / 288 / 324	11400 / 15600 / 16100
Commercial test kits			
Delvotest®SP Mini-NT	2 / 3 / 3	106 / 122 / 128	3400 / 5600 / 5700
BR-Test®AS Brilliant	4 / 4 / 5	165 / 201 / 215	5800 / 7500 / 8200

The results shown in Table 1 indicate that the presence of milk somewhat reduced the sensitivity of the test systems. However, this effect of milk seemed to differ among bacterial test species and test systems used and depended on the antibiotic. The observed increase of the detection limit in the presence of milk was most apparent for the modified agar diffusion test with tetracycline. This could be due to the formation of a visible protein/fat layer inside the holes, which might have caused a reduced diffusion of the test antibiotics into the agar matrix thus rendering this test system less sensitive.

Even in the presence of milk, the two commercial test kits showed the lowest detection limit for penicillin G with the Delvotest®SP Mini-NT still meeting the EU MRL of 4 µg/kg for penicillin in milk. In the agar diffusion tests the presence of milk appeared to exhibit a lesser influence on the detection of penicillin G than on the detection of tetracycline. Tetracycline is known to form complexes with divalent cations such as Ca^{2+} and Mg^{2+} ions which are present in milk thereby providing a possible explanation for this effect [42-44]. To overcome this problem, the use of chelating agents was suggested [44-45].

Despite the fact that the Delvotest®SP Mini-NT showed the lowest detection limits for chloramphenicol, the presence of milk appeared to interfere to a higher degree with the detection of chloramphenicol than observed especially in the disk based agar diffusion method using *Bacillus subtilis* and *Geobacillus stearothermophilus*. Because of its physico-chemical properties, chloramphenicol is the most lipophilic of the three selected antibiotics and will probably tend to stay in the lipid phase of the milk. This in turn might result in a limited bioavailability and diffusion of this compound into the aqueous phase. Shakila *et al*. [46] therefore used a solvent extraction prior to a growth inhibition assay performed with *Photobacterium leiognathi* for the detection of chloramphenicol in shrimps. However, the need for solvent extraction and the use of solvent extracts somewhat complicates a simple and easy screening test.

Overall the commercial test kits Delvotest®SP Mini-NT and BR-Test®AS Brilliant are clearly suitable for the detection of penicillin G and showed the lowest detection limits for chloramphenicol albeit this antibiotic was detected only at concentrations exceeding 3 mg/l. However, these two commercial test kits appear to be less suited for the detection of tetracycline as the detection limits established in this study were higher than the EU MRL value of 100 µg

per kg of milk. Still, the Delvotest®SP Mini-NT was more sensitive than the BR-Test®AS Brilliant for tetracycline. Nevertheless, as results are obtained after only 3 h incubation for these two commercial test systems, they are much faster than the agar diffusion tests employed in this study which were incubated for about 18-24 h. To ensure the detection of tetracycline at or below the EU MRL of 100 µg/kg for milk, *Bacillus pumilus* proved to be a promising addition to the agar diffusion tests. However, it appeared that the use of *Bacillus subtilis* did not improve the detection limits for any antibiotic tested in this study.

3.2 Influence of disk diameter, agar concentration and pH on antibiotic detection

The impact of disk diameter upon the detection limit for selected antibiotics when using *Bacillus pumilus* as test species is shown in Table 2. As expected, the larger the disk diameter the lower the detection limit, which was similar for the other two test strains used (data not shown). Evidently, the determined detection limits for tetracycline with *Bacillus pumilus* using 9 mm disks were still lower than the specified EU MRL of 100 µg/kg. Rolinson & Russell [47] reported that 90% of penicillin G present (when tested in a range from 1-100 µg) on a 10 mm square of Whatman No. 1 paper had diffused into the underlying agar within 110 minutes, while this took about 230 minutes for about 80% of tetracycline (when tested at 10 and 100 µg). A shorter incubation time as it is normally used with *Geobacillus stearothermophilus* at higher temperature might lead to an incomplete diffusion of some antibiotics into the agar and might therefore reduce the degree of growth inhibition.

When using 90 mm Petri dishes, 5 disks with a diameter of 12.7 mm can be easily placed on the surface without interfering with each other. This disk diameter is recommended in some official guideline methods [34, 48], while other instructions still recommend the use of 6 mm disks [49]. Again, the presence of milk increased the detection limit for all three antibiotics tested [Table 2].

Table 2 Detection limits for selected antibiotics with different disk diameters and *Bacillus pumilus* as test strain (all data in µg/l).

	Penicillin G in water / milk 1.5 % fat / milk 3.5 % fat	**Tetracycline** in water / milk 1.5 % fat / milk 3.5 % fat	**Chloramphenicol** in water / milk 1.5 % fat / milk 3.5 % fat
Disk diameter			
6 mm	91 / 98 / 103	237 / 443 / 561	31800 / 39300 / 42400
9 mm	88 / 95 / 100	42 / 67 / 95	27000 / 35200 / 39700
12.7 mm	81 / 92 / 95	19 / 31 / 38	23500 / 28200 / 32900

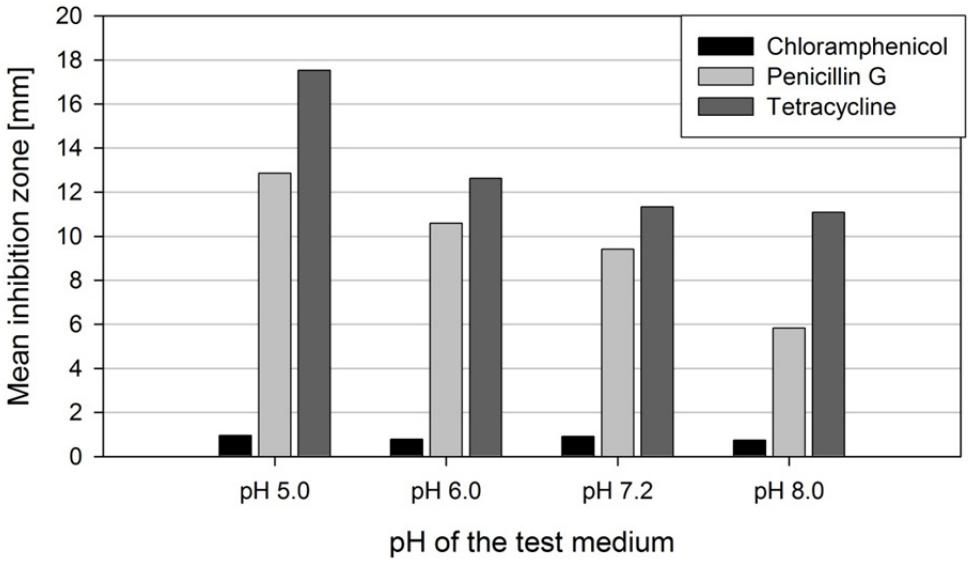

Fig. 1 Influence of pH of the test medium (Kundrat-agar) on the mean inhibition zone measured for three selected antibiotics (each at 10 mg/l) with the disk based agar diffusion method (12.7 mm disks) and *Bacillus pumilus* as test strain.

There was no evident effect of pH of the test medium (Fig. 1) on the detection of chloramphenicol based on the observed mean inhibition zones for *Bacillus pumilus*, which is probably due to the fact that this strain is not sufficiently sensitive to chloramphenicol at 10 mg/l (Table 2). Both for penicillin G and tetracycline, a lower pH resulted in larger mean inhibition zones which will lead to improved detection limits. Other authors showed a similar effect of pH on the detection of or sensitivity to antibiotics with different microorganisms [43, 50, 51] and that the pH of an assay system

can have variable effects on the stability and activity of the assayed substances [29]. It is therefore sensible to implement several pH values in agar diffusion assays as prescribed for example in the three-plate-test for meat [35], as this can increase the sensitivity of the screening method for certain target antibiotics.

When varying the agar concentration of the medium (Fig. 2), it was apparent that a higher agar content somewhat decreased the mean inhibition zone and therefore increased the detection limit for all antibiotics tested in this study. Again, this effect was evident for all three test strains used (data not shown). The agar content of a medium has a general effect on the diffusion which is governed by the resulting pore size of the agar matrix. For practical reasons, a content of 10 g agar per litre appears to be a suitable agar concentration; lower concentrations led to mechanical instability of the agar and therefore precluded the punching of holes which is required for the modified agar diffusion assay.

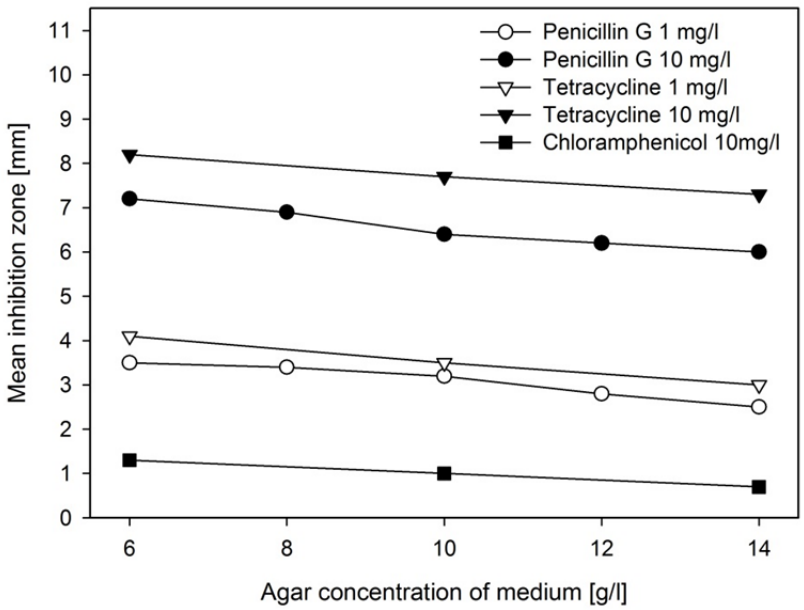

Fig. 2 Effect of agar concentration on the mean inhibition zone measured for three antibiotics with the disk based agar diffusion test (12.7 mm disks) and *Bacillus subtilis* as test strain.

These results demonstrate that the variation in media characteristics (pH, agar concentration) and test strains can greatly influence the detection of antibiotics. It is not only important to consider that different microorganisms prefer different types of media which in turn might alter their susceptibility towards different antibiotics, but at the same time the impact of target antibiotics can be influenced by factors such as pH or agar concentration. Gavin critically reviewed [29, 52] the agar diffusion test approach in view of physico-chemical and microbiological variables that need to be considered when employing and optimizing this screening procedure. It needs to be stressed that even the composition or quality of standard media or selected ingredients may somewhat differ between batches, which will influence the sensitivity and reproducibility of the assay. This problem was highlighted by a report released in Germany [53], demonstrating that the use of a different peptone can influence the performance of the official three-plate-test procedure.

This study indicated that *Bacillus pumilus* might be a useful addition to the agar diffusion procedure by improving its sensitivity for certain target antibiotics as was previously shown for other strains like *Bacillus cereus* [40], *Escherichia coli* [51] or *Photobacterium leiognathi* [46].

4. Concluding remarks

The utilization of agar diffusion tests as screening method for the detection of antibiotics is still very useful as they are cheap, easy to perform, fast, flexible and enable a large sample throughput. Especially the flexibility is an advantage, as these tests can be easily adapted to different demands (e.g. different food matrices) via for example optimizing the pH range of the test media and implementing new test strains. However, a differentiation between simultaneously present antibiotics and/or other inhibitory substances (false positive results) as well as a reliable quantification of the substances is not possible with this method. Additionally, as agar diffusion tests are somewhat susceptible to variations in test medium composition and test strains, both the quality of the medium and the test strain has to be carefully monitored to insure reproducibility. As for all (microbiological) analytical procedures method validations have to be done before modified methods can be used routinely.

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