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DEVELOPMENT OF MICROBIOLOGICAL DIFFUSION INHIBITION TEST FOR THE DETERMINATION OF ANTIBIOTIC RESIDUES IN THE MILK

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Abstract

Antimicrobial agents are used in animal husbandry for the prevention and treatment of diseases in farm animals and have become an indispensable aspect of commercial livestock production. Antimicrobial therapy is well-established as a component of comprehensive preventive measures aimed at minimizing diseases in farm animals and has been incorporated into procedures directed at promoting livestock growth and productivity. *Bacillus licheniformis* strain T7 is susceptible to the antibiotics clidamycin, rifampicin, erythromycin, ciprofloxacin, tobramycin, tetracycline, penicillin, streptomycin, and chloramphenicol, has good sporogenic properties, grows rapidly on nutrient agar at 30-55 °C, pH 5.5–8.0, and can serve as a test culture in a microbial inhibitor test. *Bacillus licheniformis* T7 spores were obtained by growing the culture in Difco sporulation medium and then inactivating the vegetative cells at 90 °C for 20 minutes. Endospores of *Bacillus licheniformis* T7 co-polymerized with nutrient agar and germinated at 55 °C for 3–3.5 h, resulting in a pH shift from 5.5 to >6.5, which can be measured with an acid-base indicator. Bromocresol purple was suggested as a pH indicator for use in a microbiological inhibition test, wherein the presence of antibiotics or other compounds impeding the development of a microbiological culture could be determined by a change in the agar's color. A microtube- and plate-based microbiological inhibitor test prototype has been developed.

Key words: *Bacillus licheniformis*, spores, antibiotics, milk, inhibition.

INTRODUCTION

Antibiotics are medications used to treat infectious diseases that inhibit bacteria by suspending their critical processes and reproduction [1]. Antibiotic treatment is a fast and effective treatment option for many disorders in veterinary medicine. In addition, the use of antibiotics has long been established as an integral element of multifaceted disease prevention programs for agricultural animals [2]. In addition to treatment, the use of antibiotics in veterinary medicine is a component of productivity-boosting measures. Antibiotic usage has raised livestock productivity to the level of industrial meat and milk production, decreased mortality in yuong animals, boosted weight gain, and enhanced animal immunity [3].

As broad-spectrum antibacterial treatment for farm animals, antibiotics are used for the treatment of urogenital diseases, mastitis and endometritis, gastroenteritis and other GI diseases, respiratory diseases, and specific infections caused by gram-negative and gram-positive bacteria. Antimicrobials do not cause deleterious reactions when used properly, and milk and meat can be consumed for industrial and nutritional purposes after 14–30 days. Antibiotics are sometimes used as a preventive measure when there is a possibility of mass infection of the herd in epidemics. Antibiotics are commonly used in animal feed to enhance the health of the animals, boost their appetite, decrease the mortality rate of young animals, hasten the fattening process, and maximize the nutritional value of the feed. However, there has to be regulation of the use of therapeutic and feed antibiotics [4].

The widespread use of antibiotics, especially those with a broad spectrum, can have negative effects on human and animal microflora, which is a major drawback of these drugs [5]. The reservoirs of resistance exist both in animals and humans, and antimicrobial usage in animals can have serious consequences for human health [6]. Antibiotic residues have been found in a wide variety of foods, including milk, eggs, and meat. Transmission of antibiotic-resistant bacteria to humans; immunopathological effects; allergy; mutagenicity; nephropathy; hepatotoxicity; reproductive problems; bone marrow toxicity; and carcinogenicity [4] are just some of the adverse effects that can be brought on by these antibiotic residues. Antibacterial agents do not increase the quality of milk in any way, and instead reduce its sanitary quality and technological features, making their presence in excess of the authorized concentration particularly undesirable for dairy products. Antibiotics in milk reduce the viability of bacteria employed in the dairy industry. The extensive use of antibiotics in cattle and other farm animals has prompted public concerns due to the fact that their negative effects are at incompatible with their efficacy. Antibiotic residues are a public health concern [7], which has led to different regulations on antibiotic use in the dairy industry [5]. Because of this, it's important to test milk and other dairy products for antibiotic residues. The detection of antibiotic residues can be done using a number of various techniques based on chromatography and immunology [8]. Quantitative approaches such high-performance liquid chromatography [9], gas-liquid chromatography [10], thin-layer chromatography [11], mass spectrometry [12], and electrochemical methods [13, 14] are actively utilized to detect antibiotic residues in food. Meanwhile, microbiological approaches based on bacteria's susceptibility to antibiotics show promise [15].

It should be noted that drug treatment of dairy cattle for various diseases causes medication residues to remain in milk over time, which is highly undesirable from both a public health and dairy industry-specific technological standpoint. And there is an objective need for a simple and dependable system for assessing milk, both for dairy producers, who must

endeavor to prevent the introduction of drug residues into milk, and for dairy processors. These requirements are satisfied by a test for a broad-spectrum microbial inhibitor based on the co-polymerization of spores from a test bacterial culture with an agar medium.

The creation of a microbial inhibitory test required a study to identify a strain that could be utilized as a test culture [16]. *Bacillus licheniformis* strain T7 was selected as a test culture due to its sensitivity to the majority of antibiotics used in veterinary medicine, its rapid growth on solid and liquid nutrient media over a broad temperature and pH range, and its spore formation.

The purpose of this study is to develop a *Bacillus licheniformis* strain T7 spore-based microbiological diffusion inhibition assay for the determination of antibiotic residues in milk.

MATERIALS AND METHODS

A culture of *Bacillus licheniformis* strain T7 from the Microbial Genetics and Biochemistry Laboratory Collection and deposited at the National Centre for Biotechnology, B-NCB 0704, was used. The following nutrient media were used: nutrient broth (Himedia, India), nutrient agar (Himedia, India), Difco sporulation media, Arret-Kirshbaum agar, Modified nutrient agar, and Mueller-Hinton agar. Table 1 shows the composition of the nutrient media. Bromocresol purple (5,5′-dibromo-cresolsulfophthalein from Titan Biotech, Rajasthan, India) was used as an indicator dye.

Spore production

Bacillus licheniformis T7 night culture was inoculated into Difco sporulation medium, Arret Kirshbaum sporulation agar, and Modified nutrient agar. The dishes containing agarized media were incubated at 37 °C in a dry-air thermostat, while the tubes containing Difco sporulation media were incubated at 37 °C and 150 rpm in a shaker. Microscopic analysis of Schaeffer-Fulton-stained smears determined the incubation period. The culture was extracted after five days when the spore/cell ratio reached 1/1 or more in favor of spores. The agarized medium was scraped with a silicone scraper, and the liquid medium was centrifuged at 6000 × g for 7 minutes at 4 °C to capture the cells. The precipitate was dissolved in 0.9% NaCl. To eliminate vegetative cells, cell suspensions were heated for 20 minutes at 90 °C. The spore concentration was measured. The resultant spore suspension was utilized in subsequent research.

Milk sample preparation

For the determination of antibiotic residues, reconstituted skimmed cow's milk with the addition of standard solutions of the antibiotics tested was used as a sample. A solution of 0.12 g/mL of skim milk powder in distilled water with a pH of 6.8 was prepared.

Test for antibiotic detection in milk

Nutrient agar (pH 5.5) and Mueller-Hinton agar (pH 5.5) were used for the antibiotic sensitivity test. Spores at a concentration of 2×10^6 CFU/mL were copolymerized with agar and 10 mg/L of bromocresol purple was added. Reconstituted milk was used as a sample. Milk with the antibiotic gentamicin added at a concentration of 50 mg/mL was used as a negative control. It was incubated at 55 °C on a dry-air thermostat. The medium's transformation from yellow to purple reflected the growth of the culture Because gentamicin stopped *Bacillus licheniformis* T7 cells from growing; no color change occurred in the negative control.

Determination of the sensitivity of spores and lyophilized cells of *Bacillus licheniformis* strain T7 to antibiotics on Petri dishes

Spores and cells at a concentration of 1×10^6 CFU/mL were copolymerized with Nutrient agar and Mueller-Hinton agar at pH 5.5, and the mixtures were then poured into Petri plates with the addition of bromocresol purple. Wells of 10 mm diameter were cut using a puncher: six wells around the perimeter of the plate and one well in the center of the plate. Antibiotics such ampicillin, kanamycin, gentamicin, tetracycline, vancomycin, and chloramphenicol were added to reconstituted milk and poured into the wells. The central well was filled with a control sample (milk without antibiotics). Plates were incubated at 55 °C in a dry-air thermostat. The color change from yellow to purple was recorded.

Prototype microtube test

Sterile polypropylene microtubes without caps were used as test microtubes. Bromocresol purple was added to 1.5% nutrient agar at a concentration of 10 mg/L, and the mixture was then autoclaved at 1 atm (121 °C) for 20 minutes to sterilize it. *Bacillus licheniformis* strain T7 spore suspension with a concentration of 2×10^6 CFU/mL was added to the agar at 50–55 °C, swirled to ensure that the spores were evenly distributed, and 200 µL were then added to each microtube. Microtubes without milk were labeled and used as a positive control. Negative control: 500 µg/mL gentamicin was added to the test medium. The microtubes were incubated at ambient temperature for 1 hour before being refrigerated at 4 °C. In addition to the test samples, 1 negative control and 1 positive control were used for each series of experiments. A 100 µL sample of milk was added to a microtube. Test samples, positive and negative controls were incubated in a Biosan solid-state thermostat at 55 °C for 3–3.5 hours until the agar became purple in the positive control. The color of the negative control should remain yellow. Depending on the color of the

Table 1. Composition of the nutrient mediums

Medium	Composition	pH
Difco sporulation medium	8 g/L Nutrient broth, 1 g/L KCl, 0.12 g/L $MgSO_4$, 0.01 g/L $MnCl_2$, 0.05549 g/L $CaCl_2$, 0.15191 mg/L $FeSO_4$	7.2
Arret-Kirshbaum sporulation agar	6 g/L Pancreatic digest of gelatin, 4 g/л Casein enzyme, 3 g/L Yeast extract, 1.5 g/L Beef extract, 1 g/L Dextrose, 0.3 g/L $MnSO_4$, 15 g/L Agar	7.0
Modified nutrient agar	28 g/L nutrient agar, 0.1 g/L $CaCl_2 \times 2H_2O$, 0.05 g/L $MnSO_4 \times H_2O$	6.9
Mueller-Hinton agar	2 g/L beef extract, 17.5 g/L casein hydrolysate,1.5 g/L starch, 15 g/L agar	7.3

test samples, the results are interpreted: purple indicates the absence of the antibiotic, and yellow indicates its presence.

Prototype test in 96-well plate design

Plates made of sterile polystyrene were used for the 96-well format. The 1.5% nutritional agar was sterilized by autoclaving at 1 atm (121 °C) for 20 minutes. The medium contained 10 mg/L of bromocresol purple. A suspension of spores of *B. licheniformis* strain T7 at a concentration of 2×10^6 CFU/mL was added to the agar at 50–55 °C, stirred to ensure uniform distribution of spores throughout the agar, and 200 µL was immediately introduced into each well of the plate. Well A1 was the positive control - no milk samples were added to this well. The negative control was well A2, in which gentamicin at a concentration of 500 µg/mL was added in addition to the spores. The plate was incubated at room temperature for 1 h and stored at 4 °C in the refrigerator. 100 µL milk sample was added to the well of the plate and incubated in a dry-air thermostat at 55 °C for 3–3.5 hours until the agar in the positive control turned purple. In the negative control the color should remain yellow. Depending on the color of the test samples, the results are interpreted: purple indicates the absence of the antibiotic, and yellow indicates its presence.

RESULTS AND DISCUSSION

Bacillus bacteria are known to be capable of growth on a variety of nutrient media, in some of which the only source of carbon may be β-keratin and to have a high tolerance to adverse factors [17]. However, these bacteria are known to become spore-like when exposed to adverse factors or prolonged starvation, forming endospores. *Bacillus* endospores are characterized by resistance to high temperatures [18, 19], high salt content [20], ultraviolet radiation [20], high frequency radiation in the microwave range [21], and high pressure [22–24]. Therefore, it is preferable to use endospores that can withstand prolonged persistence and have the ability to germinate when exposed to favorable conditions when designing a test system.

Bacillus licheniformis T7 isolated from soil is sensitive to a number of antibiotics, including clidamycin, rifampicin, erythromycin, ciprofloxacin, tobramycin, tetracycline, penicillin, streptomycin, and chloramphenicol, according to previous research [16]. Different nutrient media, including broth and agar, are utilized for *Bacillus* spore formation [25].The effect of calcium and manganese ions in nutrient media on the efficiency of spore formation has been established [26]. Difco sporulation medium, Arret-Kirshbaum sporulation agar, and modified nutrient agar were tested to obtain endospores of the *Bacillus licheniformis* T7 strain. Table 2 shows the results of the spore counts in colony-forming units using these media.

In the course of the research, it was found that the DSM medium was optimal. The spore yield was 1.025×10^{12} CFU/mL. The spore yield on AKS and MNA agarized media was 1 and 5 orders of magnitude lower, respectively, indicating the low efficiency of these media for producing *Bacillus licheniformis* T7 endospores. To remove vegetative cells, the culture was heated at 80 °C and 90 °C for 20–60 min. A 20-minute heating at 90 °C was found to be the optimum condition for the complete removal of vegetative cells. Experimentally, the endospores of *Bacillus licheniformis* T7 themselves were found to survive autoclaving at 1 atm at 121 °C for 20 min.

Figure 1 shows the microscopy results of *Bacillus licheniformis* T7 endospores obtained under these conditions.

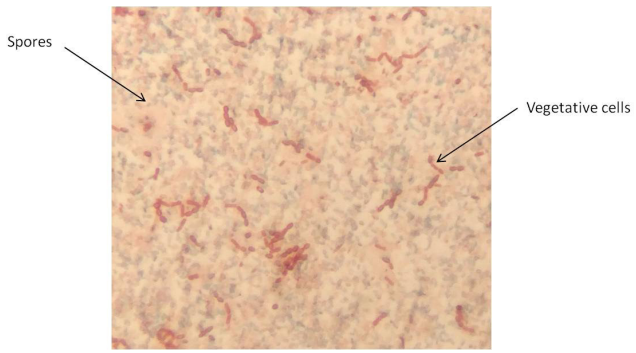
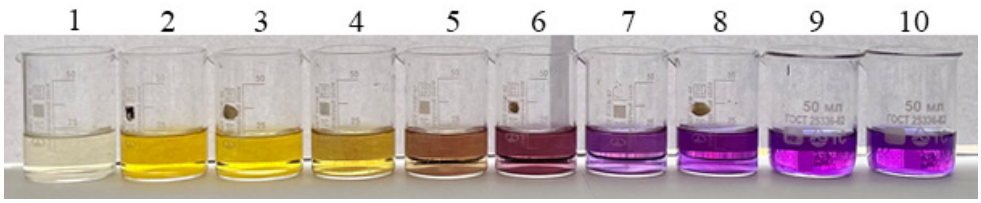


Figure 1 - Scheffer-Fulton staining of *Bacillus licheniformis* T7 spores and cells

Nutrient broth and Nutrient agar are the most suitable media for spore germination. To determine whether Bromocre-



1-NutBroth; 2- NutBroth+BrPurp (pH 4.0); 3- NutBroth+BrPurp (pH 4.5);
4- NutBroth+BrPurp (pH 5.0); 5- NutBroth+BrPurp (pH 5.5);
6- NutBroth+BrPurp (pH 6.0); 7- NutBroth+BrPurp (pH 6.5);
8- NutBroth+BrPurp (pH 7.0); 9- NutBroth+BrPurp (pH 7.5);
10- NutBroth+BrPurp (pH 8.0)

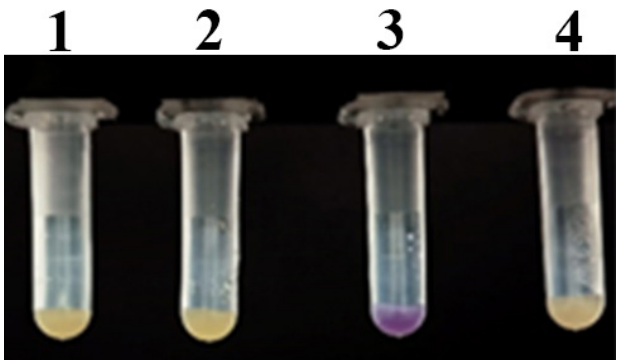
Figure 2 - Effect of pH on the color of nutrient broth (NutBroth) with bromcrezol purple (BrPurp)

Table 2. Yield of spores of the *Bacillus licheniformis* T7 strain depending on the medium

Medium	Abbreviation	CFU/mL
Difco sporulation medium	DSM	1.025×10^{12}
Arret Kirshbaum sporulation agar	AKS	2.072×10^{11}
Modified nutrient agar	MNA	5.382×10^{7}

sol purple can be used as an indicator, the effect of pH on the colour of the nutrient broth was determined. Figure 2 shows the change in color of the nutrient broth depending on the pH of the medium.

As can be seen in Figure 2, the color of the nutrient medium changes significantly when the hydrogen index changes from 4.5 to 8.0. For our studies, the values of 5.5 and 6.5 are of interest since strain *Bacillus licheniformis* T7 is alkaline and the optimum value for growth is pH 7.4, but the strain is also capable of growth at low pH values up to and including 5.5. Nutrient agar and Mueller-Hinton agar were tested for spore germination. When both nutrient media were used, spore germination was only observed in nutrient agar (Figure 3), and no spore germination was observed in Mueller-Hinton agar.



1-spores with NHA, 2- spores with NHA and gentamicin,
3-spores with NA, 4- spores with NA and gentamicin

Figure 3 - Results of testing Mueller-Hinton agar (MHA) and Nutrient agar (NA) as a medium for the germination of *Bacillus licheniformis* T7 spores

Titration was used to establish the optimal spore concentration for copolymerization, as well as the minimum spore concentration necessary for germination and the detection of a color change in an indicator. The following concentrations (spores/ml) were investigated: 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7}, 1×10^{6}, 1×10^{5}. As a result, a concentration of 1×10^{6} spores/mL was found to be sufficient.

Germination tests with spores copolymerized with nutritional agar in plates following the addition of milk and the appropriate antibiotic were carried out to ascertain the sensitivity of spores to penicillins, aminoglycosides, tetracyclines, glycopeptides, and chloramphenicols. For comparison, a similar experiment was carried out in parallel but using lyophilically dried cells of strain *Bacillus licheniformis* T7. Incubation of both plates was carried out at 55 °C. Figure 4 shows the results. Milk without added antibiotic was used as a control.

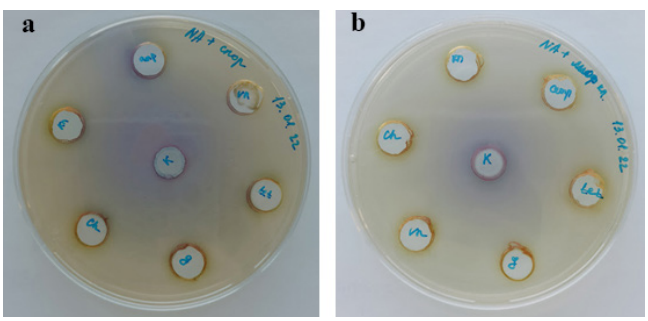


Figure 4 - Determination of the sensitivity of spores (**a**) and lyophilized cells (**b**) of strain *B. licheniformis* T7 to antibiotics: ampicillin (Amp), kanamycin (Kn), gentamicin (Gn), tetracycline (Tet), vancomycin (Vn), and chloramphenicol (Chl).

No color change was detected in the samples without antibiotics, whereas in the control sample the color of the nutrient agar around the well changed to purple, indicating germination of both lyophilic-dried cells and spores in the part of the agar where antibiotics were absent. A decrease in the sensitivity of spores to ampicillin compared with lyophilically dried cells was noted. A comparative analysis of the color change time in the two dishes showed that the color change occurred earlier with spores than with lyophilically dried cells. This is due to the faster germination of the endospores compared to the lyophilisate. Further studies were carried out comparing the rate of change in the pH of the nutrient medium between the spores and the lyophyllisates. Figure 5 shows the results for the incubation of spores and lyophilically dried cells of strain *Bacillus licheniformis* T7 in nutrient agar for 3 h.

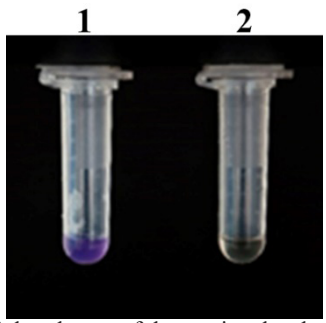


Figure 5 – Color change of the nutrient broth as a function of incubation time when adding spores (**1**) and lyophilically dried cells (**2**) of strain *Bacillus licheniformis* T7

As can be seen in Figure 5, the purple color of the spore culture is indicative of intensive germination, while the lyophilic-dried cells show stunted growth. The results obtained indicate that the endospores of *Bacillus licheniformis* strain T7 are promising for use as a test culture in the development of a microbiological inhibitor test.

Susceptibility to a broad class of antibiotics and high antibiotic sensitivity are two advantages of microbiological diffusion tests. Comparatively, immunological assays [27] can only detect a specific class of antibiotics. Physical-chemical techniques based on high-performance liquid chromatography, gas chromatography, and mass spectrometry require expensive equipment [28–31]. Microbiological inhibitor assays have a distinct advantage in this regard. It is known to use the plate method based on the ability of antibiotics in milk to diffuse into agar containing *Bacillus stearothermophilus* spores and inhibit their growth, resulting in the formation of distinct zones of inhibition [32]. The presence of antibiotics in milk creates inhibition zones, which requires operator skill. Use and interpretation of microbiological inhibition assays are less complicated. For practical use in milk and dairy products, the following commercial antibiotic and sulphonamide residue tests are available: Delvotest SP-NT, DSM Food Specialties, Denmark; Copan Milk Test, Copan Innovation, Italy; Milchtest CMT, Packhaus Rockmann GmbH, Germany; and Eclipse Farm 3G, Zeulab, Spain. The test is based on spores copolymerized with agar from a thermophilic strain of *Bacillus stearothermophilus* var. *Calidolactis*, which can thrive at 64–65 °C [33]. When milk is added to a test tube containing

spores, the milk diffuses into the agar, and 3–4 hours of incubation leads to spore germination [8]. Due to the metabolic activity of the *Bacillus stearothermophilus* strain induced by culture growth, the pH of the medium changes from a neutral pH of 7.0 to an acidic pH of 5.5, and the acid-base dye changes from blue to yellow with the Brilliant Black Indicator [34] or from purple to yellow with Bromocresol purple [8]. Therefore, the pigment change indicates that there are no inhibitory substances present in the milk. Milk samples are analyzed using microbiological diffusion tests both on a small scale, at the milk collection point and farm level, and on a large scale, in laboratories of milk processing facilities for quality control [33]. As a unique characteristic of the *Bacillus stearothermophilus* strain used as a microbiological test subject, this strain is resistant to a number of antibiotics, including quinolones, spiramycin, lincomycin, erythromycin, and streptomycin [35]. In the case of tetracyclines, the degradation rate is much higher under weakly alkaline or neutral conditions than under weakly acidic conditions [36], making it difficult to detect tetracycline class antibiotics in samples. Temperature also affects the stability of tetracycline antibiotics; a 10 °C elevate in temperature increases the rate of degradation a factor of 2.5 [36]. For the majority of quinolones, maximal activity is also observed at pH values between 5.0 and 6.0 [37, 38]. Due to these characteristics of existing microbiological assays, the search for a more appropriate microorganism is pertinent.

In the present work, a prototype microbiological inhibitor test system based on *B. licheniformis* T7 strain spores is proposed, which includes the following components: polypropylene microtubes containing *B. licheniformis* T7 strain spores copolymerized with nutrient agar and bromocresol purple. Figure 6 shows the general scheme of the proposed test.

Milk samples in a volume of 100 µL are added to experimental microtubes (Exp). For the positive control, a microtube without added milk is used (K+). For a negative control, a milk sample is added to a control microtube (K-), which additionally contains gentamicin. The four microtubes are heated at 55 °C for 3–3.5 h. Heating can be accomplished using a solid-state thermostat with a 2.0 mL microtube rack, a dry air thermostat, or a water immersion. A temperature variation of 1 °C is tolerable. At the end of incubation, the results for the antibiotic content of the milk can be observed visually. In the microtube with the milk tested, in the absence of antibiotics (beta-lactams, tetracyclines, sulphonamides, cephalosporins, and aminoglycosides), a color change from yellow to violet is observed (Figure 6, Exp1). No color change is observed in the presence of the antibiotics listed above; the color remains yellow (Figure 6, Exp2). In the negative control sample, the color will remain yellow, whereas it will turn purple in the positive control sample (Figure 6). For a small number of milk samples, a microtube vatiant (Figure 7a) can be used, for a larger number of samples, a 96-well plate is preferable (Figure 7b).

The interpretation of the results in the microtube and plate versions are identical. Instructions for the developed test system were prepared, as well as recommendations for the implementation of this prototype microbiological inhibitor test.

Figure 7 - Prototype microbial inhibitor test based on *Bacillus licheniformis* T7 spores in microtube (**a**) and 96-well plate (**b**) variant

Conclusion

Bacillus licheniformis strain T7's endospores can be prepared under the following optimal conditions: 120 hours on the Difco sporulation medium at 37 °C and 150 rpm. It has been determined that spores are preferable to lyophilized cells for use in test systems due to their greater germination rate. Endospores of *Bacillus licheniformis* T7 copolymerized with nutrient agar and the acid-base indicator Bromocresol purple germinated quickly at 55 °C for 3-3.5 h, shifting the pH of the nutrient medium from pH 5.5 to pH >6.5 and causing a change in color from yellow to purple. This result demonstrates the absence of any inhibitory elements in the growth medium, including antibiotics that *Bacillus licheniformis* T7 is susceptible to, such as ampicillin, kanamycin, gentamicin, tet-

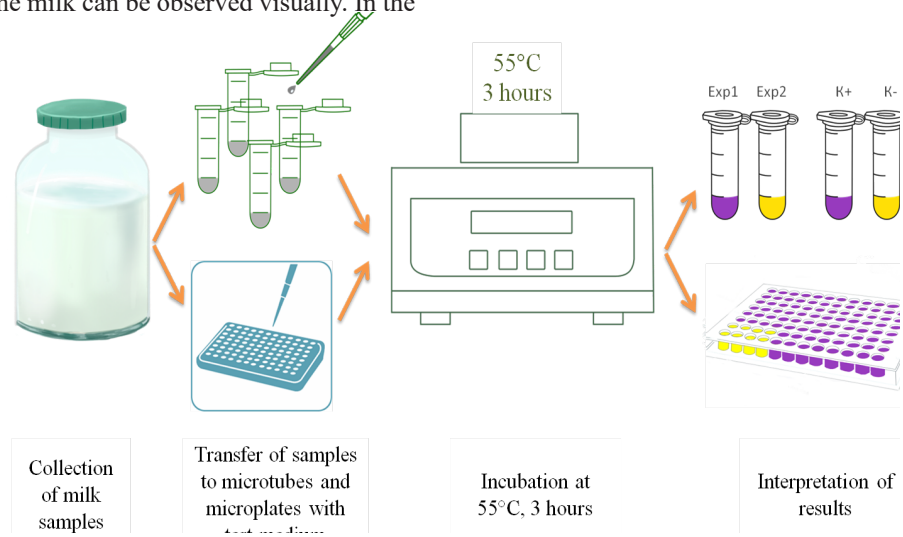


Figure 6 - Schematic diagram of the use of a microbial inhibitor test to determine the residual milk content based on spores of strain *Bacillus licheniformis* T7

racycline, vancomycin, and chloramphenicol. Based on this, a prototype microbiological inhibitor test for the detection of antibiotics in milk was developed. Milk samples from farms and dairy processors will be used to test the microtube and plate prototype.

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