Abacus of bacteria

How can we determine antibacterial effect?

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Within the frame of this project, our first aim was to identify bacterial strains by basic microbiological methods. We prepared antibiograms to inspect antibacterial effect of widely used antibiotics by conventionally applied diffusion tests. Our second aim was to examine the antibacterial efficiency by following the kinetics of their growth based on optical density changes.

We found among the tested antibiotics the streptomycin and tetracycline the most effective on the identified bacteria by diffusion based tests. We tested further the efficiency of these antibiotics on an *E. coli* strain with broth dilution test. Based on these results, we performed the growth kinetics based method as well.

INTRODUCTION

Bacteria are prokaryotic cells which can be found everywhere around us — sea, land, air and even in us: can divide themselves repeatedly, forming several colonies, creating the human flora with large numbers of bacteria on the skin and in gut, several bacteria have an important protective role in our immune system. In the industrial field, bacteria play the main role during the production of fermentation based products, meanwhile they have increasing role in biotechnology as well. However, there are several pathogenic bacteria which can cause serious, severe infections.

In the medical practice the time is a critical factor. We need to identify the different bacteria, and find out the appropriate and effective therapy based on antibiograms. We have gold standard methods, but these methods still takes days. Faster methods would be elementary and provide huge progress both in medicine both in research. In research we use many different ways to estimate antibacterial effect. Our aim was to test different possibilities.

MATERIALS AND METHODS

I. Strains

We carried out our experiments with the following bacteria: *Bacillus pumilus*, *Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli* DH5alpha.

II. Identification

We identified the strains based on macroscopic observation, observation of microscopic morphology, metabolism test, and biochemical reactions. For final conclusions regarding the "unknown" bacteria, we used literature data.

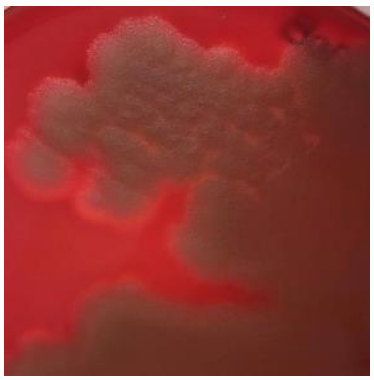
1. Macroscopy

Even the macroscopic examination of the unidentified strains after incubation on agar plates some conclusions can be drawn. As the incubation and the agar give the ideal circumstances for the bacteria to grow bacteria colonies appear over time. The different properties of these colonies: their shape, size and pigmentation can be strain specific. We

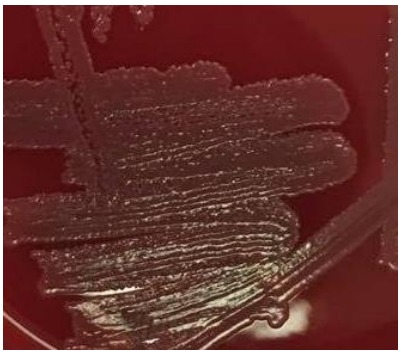
plated the bacteria on normal LB agar plates, and on blood agar plates.

2. Hemolysis on blood agar

This examination is based on the transformation of hemoglobin molecules caused by the hemolysin production of bacteria. An incomplete hemolysis, α-hemolysis, shows a greenish colour under the colony since the bacteria reduces the hemoglobin molecules to verdohemoglobin molecules only. A complete hemolysis called β-hemolysis, shows the original colour of agar under the colony, the area surrounding the colony is lighter (Figure 1.). This is caused by the complete hemolysis of the red blood cells. The γ-hemolysis, when the bacteria do not produce hemolysin, therefore there are not any visible changes on the blood agar plate (Figure 2.).



1. figure Example for β-hemolysis on *B. subtilis*



2. figure Example for γ-hemolysis on *B. pumilus*

3. Metabolism - respiration

The bacteria suspension was inoculated in a solid tube agar with a needle from agar plates. We incubated the samples for 24 hours at 33 °C. If bacteria are obligate aerobe they only grow on the top of the media. If they are obligate anaerobe, they will only grow on the middle or at the bottom of the agar. If they are facultative anaerobe it can grow in any direction of the tube test.

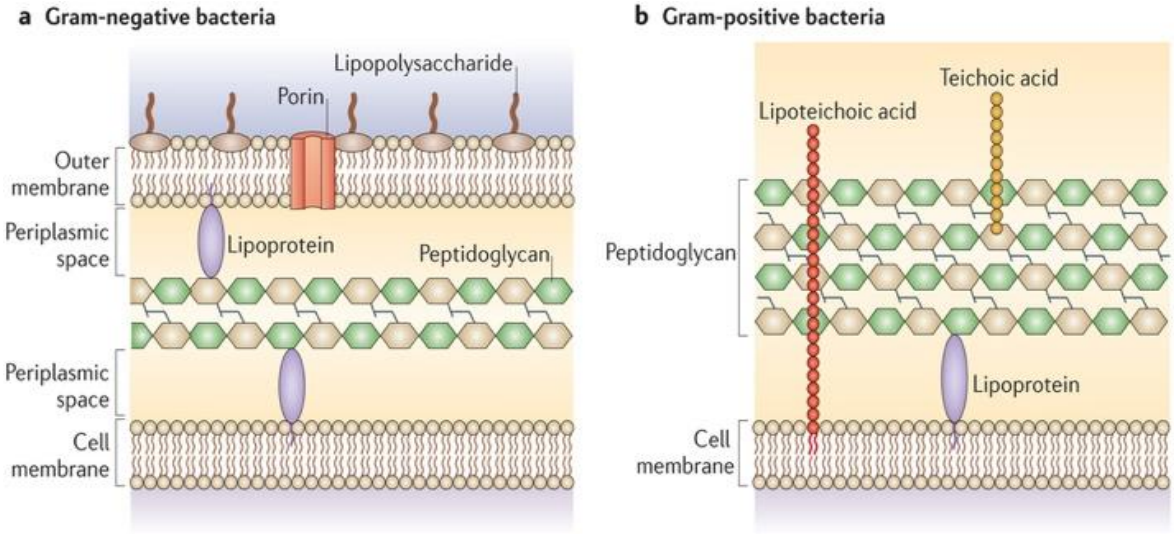
2. Figure Example for aerobe strain of *B. subtilis*

4. Gram-staining

This widely used method distinguishes bacteria based on the structure differences in their cell wall.

First a suspension solution is created by adding a drop of distilled water and bacteria by a loop onto the glass slide. Then, we performed heat fixation followed by the addition of Crystal Violet (CV+ ions got into the cells). The time in this process is crucial therefore, after applying and waiting two minutes we followed the next step: the addition of the Lugol solution (the CV+ ions form an insoluble complex with I- ions and it gets trapped in the cell wall), therefore all cells turn into a darker purple colour. After a minute, we poured 96% alcohol to the glass slide. Alcohol dissolves the lipid outer membrane of Gram-negative bacteria, thus leaving the peptidoglycan layer exposed and increases the porosity of the cell wall. The CV-I complex is then washed away from the thin peptidoglycan layer, leaving Gram negative bacteria

colourless. Alcohol has a dehydrating effect on the cell walls of Gram-positive bacteria which causes the pores of the cell wall to shrink. The CV-I complex gets tightly bound into the multi-layered, highly cross-linked Gram-positive cell wall thus staining the cells purple. Finally, carbol-fuchsin was applied to stain the colourless Gram-negative bacteria as well. Finally, we observed the results under microscope, and examine the microscopic morphology of the bacteria.



3. Figure Cell wall structure of Gram-negative and Gram-positive bacteria[1]

5. Urease test

Some bacteria have the enzyme urease, which in the presence of water converts urea into NH_3 and CO_2 and, forming ammonium carbonate. By the growing of the bacteria in the media containing the urea and pH indicator, it can be determined if the bacteria have the enzyme. If they have urease, the pH of the media will turn alkaline, therefore, the colour will change to purple (figure 6.). We performed this tests in glass tubes with 24 hours incubation at 33 °C.

4. Figure Example for urease positive on *M. luteus*

6. Catalase test

This test is based on the fact that some bacteria produce the catalase enzyme. We added five drops of 3% hydrogen-peroxide to the bacteria suspension on a glass slide. The catalase enzyme induces the following reaction: $2H_2O_2 \rightarrow H_2O + O_2$. The gas formation in the suspension indicates the presence of the enzyme. A positive result is observable on the figure 7.

5. Figure Example for catalase strain of *B. pumilus*

II. Antibacterial efficiency tests

A good therapy always based on an antibiogram: a collection of data usually in the form of a table summarizing the percent of individual bacterial pathogens susceptible to different antimicrobial agents. In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a chemical which prevents visible growth of a bacteria and give us comparable and quantitative information regarding the efficiency an antibiotic agent. We measured these values and compared the different means of antibacterial effect determination.

1. Diffusion based methods

During **disc diffusion test**, discs containing antibiotics are placed on an agar plate where bacteria have been placed, and the plate is left to incubate. If an antibiotic inhibits the bacteria from growing or kills the bacteria, there will be an area around the disc, where the bacteria have not grown enough to be visible. We call this a zone of inhibition. The size of this zone depends on how effective the antibiotic is at stopping the growth of the bacteria. We measured the diameters of the inhibition zones and evaluated the results with general method differentiating sensitive, moderately sensitive and resistant strains.

The **E-test** method is also able to determine MIC values, but this test is more precise than the disc diffusion test. E-test or epsilometer is a non-porous, plastic reagent strip with a predefined gradient of antibiotic, covering a continuous concentration range. When it is applied to the surface of an agar plate inoculated with the test strain, there is an instantaneous release of the antimicrobial gradient from the plastic carrier to the agar matrix to form a stable and continuous gradient. The bacterial growth becomes visible after incubation and a symmetrical inhibition ellipse centered along the strip is seen. The MIC value is read from the scale in terms of μg/mL where the ellipse edge starts next to the strip.

We performed disc diffusion test with self-prepared antibiotic discs containing 10 μg of the following antibiotics: ampicillin, chloramphenicol, streptomycin, tetracycline and commercial bacitracin discs containing 10 μg of the agent. We performed the E-test containing vancomycin, and tested it on our four strains. In each cases, we evaluated the results after 24 hours of incubation at 33 °C.

2. Broth dilution method

We performed this test on *E. coli* DH5alpha strain with chosen concentrations of streptomycin tetracycline. Based on the data provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), we chose 0.25 mg/l, 2 mg/l and 4 mg/l concentration of streptomycin and 4 mg/l and 16 mg/l concentrations of tetracycline to test. We coincubated the bacteria in HBSS buffer containing 2% LB as a control and the samples with the chosen concentration of the antibiotics for 40 minutes. Then, we spread-plated the samples on agar plates. We counted the colony forming units (CFU) after 24 hours of incubation at 33 °C. Finally, we performed the bacterial survival in percentages: sample coincubated with antibiotics / control sample *100.

3. Optical density changes based method

This test is based on the fact that we can determine concentrations of bacteria suspension by the measurement of the optical density (OD or absorbance). We coincubated the samples with the antibiotics and prepared a control sample in the same way as we did during the broth dilution method. After the coincubation we compared the growth

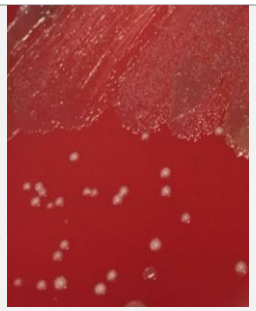
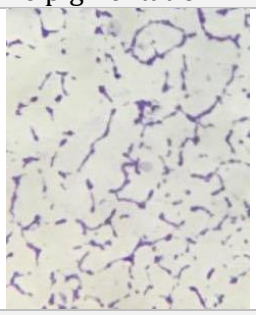
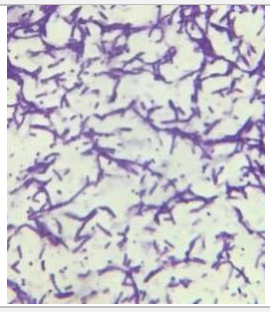
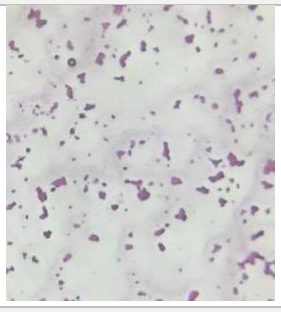
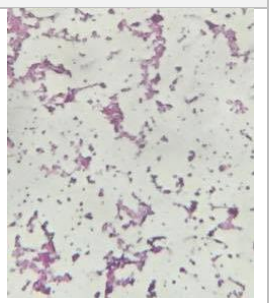
kinetics of our examined bacteria by measuring the OD at λ= 600nm wavelength every 10 minutes. We carried out calibration with known concentration of *E. coli* DH5alpha suspension. With the help of this calibration growth curve, we were able to compare the time point when the samples reached a chosen OD and calculate form the time point the number of the bacteria after the coincubation.

We evaluated the results with the following exponential equation based on the calibration:[bacteria] = $f*e^{g*t_{inc}}$ where t_{inc}: needed incubation time until the chosen OD value (0.1 in our case), g: steepness of the calibration curve, which is proportional to the division rate of the bacteria, f: multiplier factor. We compared time points when the samples' optical density reached the 0.1 value. Based on the calibration prepared with *E. coli* DH5alpha suspension with known concentrations, we were able to calculate the number of bacteria. Finally, we performed the bacterial survival in percentages in the same way as we did during the broth dilution method.

RESULTS

I. Identification of the bacteria

We could successfully identify our "unknown" bacteria. The results of the process are presented in Table 1 based on own observations, containing own captures.

	Bacillus subtilis	***Bacillus pumilus***	***Micrococcus luteus***	***Escherichia coli***
Macroscopy	 round, irregular, wrinkled, 2-4 mm diameter, white, creamy-brownish, no pigmentation	 round, irregular, can be wrinkled, 2-4 mm diameter, slightly yellowish, no pigmentation	 round, 1-2 mm diameter, yellow pigmentation	 Round, 3-6 mm diameter, opaque white, no pigmentation
Microscopy				
Gram-staining	Gram-positive	Gram-positive	Gram-positive	Gram-negative
Hemolysis	β-hemolysis	γ-hemolysis	β-hemolysis	γ-hemolysis
Metabolism	aerobe	aerobe	aerobe	aerobe
Urease	Negative	Negative	Positive	Negative
Catalase	Positive	Positive	Positive	Positive

1. table Result summary of the identification process, magnification of the microscopic images: 1000x

II. Antibacterial efficiency tests

1. Disc diffusion test

We summarized the results in the Table 2. We color-coded the different groups during the evaluation of the results. We found the two 30S subunit inhibitor (protein synthesis inhibitor) tetracycline and streptomycin as the most effective antibiotics in the examined concentration (10 µg). The bacitracin did not affect the two *Bacillus* strains as we expected since the bacitracin itself is related to a mixture of related cyclic peptides produced by a *Bacillus subtilis* strain.

6. Figure Example for disc diffusion test of *M. luteus*

Strain\ Antibiotic	Ampicilin	Chloramphenicol	Tetracycline	Streptomycin	Bacitracin
Micrococcus luteus	-	11	20	18	5
Bacillus subtilis	-	22	24	20	-
Bacillus pamilus	10	-	20	14	-
E. coli	12	18	20	10	7

≥ 18mm	Sensitive
18-10mm	Moderately sensitive
≤ 10mm	Resistant

2. table Result summary of the antibiotic efficacy

2. E-test

We also planned to test quantitatively the efficiency of the widely used vancomycin. Table 3. contains our findings. We got the same MIC value as we found data for this in the literature[2]. Since we know that the *Bacillus pumilus* shows high tolerance against several environmental stress as well (UV light, H_2O_2, desiccation, salt), it is not surprising that the MIC value was three times higher as we found in the case of *B. subtilis*. *E. coli* and other enteric Gram-negative bacilli are normally resistant to vancomycin and other glycopeptide antibiotics. We could not find literature data for the susceptibility of *M. luteus*.

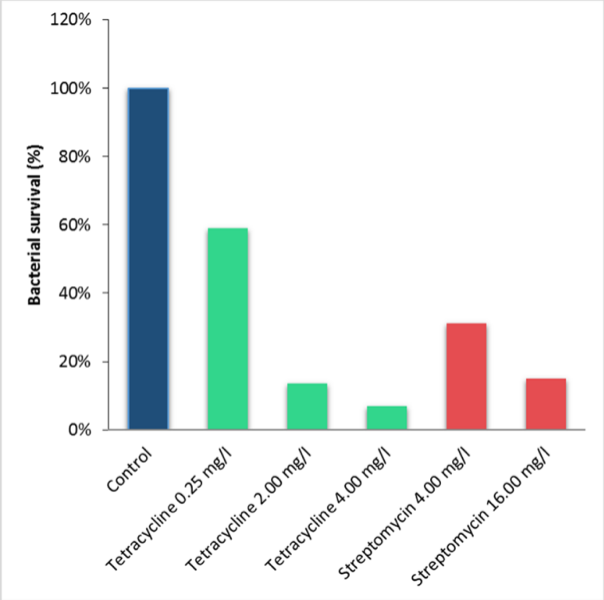
8. Figure Example for E-test of *B. subtilis*

Strain	MIC (mg/l)
Bacillus subtilis	4.00
Bacillus pumilus	12.00
Micrococcusluteus	0.25
Escherichia coli	2.00

3. table Result of the MIC for vancomycin for each strain of bacteria

3. Broth dilution test

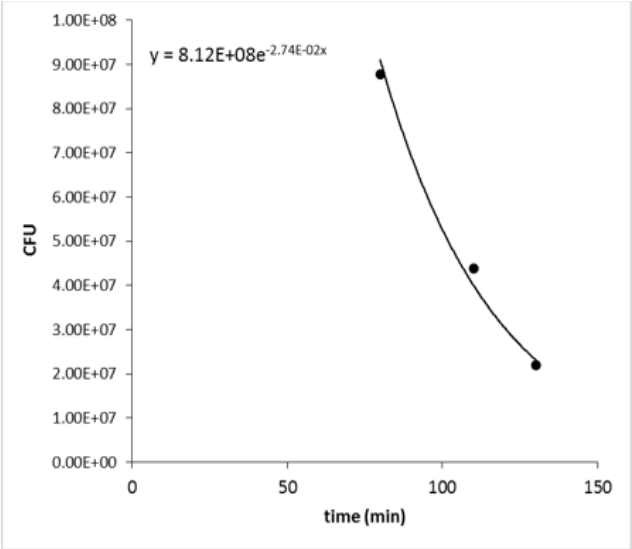
We got decreasing *E. coli* survival percentages by the increase of the concentration of the applied antibiotics (figure 9.). We observed more pronounced decrease of the bacterial survival with the same concentrations (4 mg/l of tetracycline and streptomycin). Therefore, we planned to examine further the effect of tetracycline with the OD changes based method.



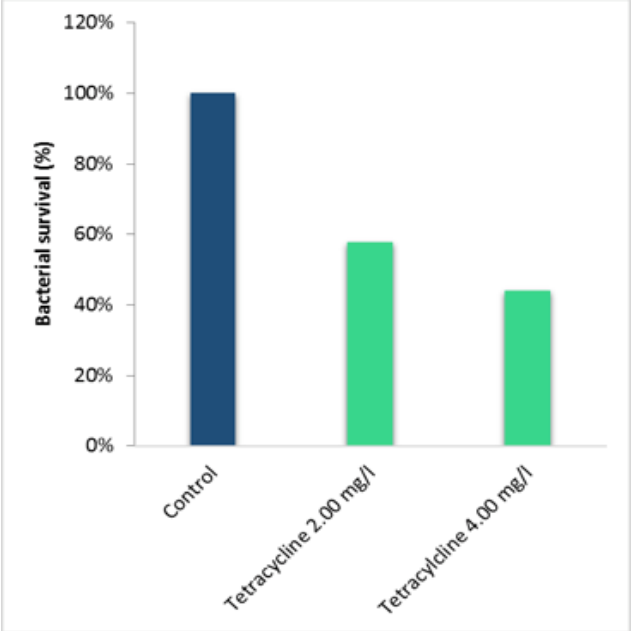
9. figure Results of bacterial survival (%) with different antibiotics in different concentrations

4. Optical density changes based method

We fitted exponential curve on our calibration datapoints (three different concentration of *E. coli* DH5 alpha). We calculated later with that equation of the calibration curve (figure 10.). The bacterial survival (%) results are shown on the figure 11. As we found previously in the case of the broth dilution method, by the increase of the concentration of the tetracycline the *E. coli* survival decreased, but the degree of the decrease consistent with the previous finding, however the values were higher in this case.



10. figure Growth curve for *E. coli* DH5alpha



11. figure Results of bacterial survival (%) of *E. coli* DH5alpha with different concentrations of tetracycline

Conclusion

Our project had two main aims: learn identification methods of bacteria, identify our "unknown" strains (knowing possibilities) and the examination of the effectiveness and the differences in effectiveness of various antibiotics on these strains. Both the identification of the unknown strains and the testing of the antibiotics were mostly successful, which helped us in the comparison of the

bacteria strains and the effectiveness of the antibiotics as it is presented in the results. However, this project also helped us in the comparison of the methods we used, as we could experience differences both in fastness and accurateness. By the examination of the growth curves of the bacteria we are able to get more detailed information regarding the viability of the bacteria, and how the antibiotics affect their growing behaviour. In the frame of this project we were able to test it only once, as we did only one experiment with the broth dilution method. Therefore these results are not comparable and reliable enough to declare a conclusion. In the meantime, we could clearly see the OD based growth kinetics following method's advantage: the time. In research this is a known and used method, but mostly measured on microplate readers which can collect data more frequently, with more parallels and for longer time than we could manually: more accurately. It is important to highlight the potential possibilities of the OD based method: while during the gold standard methods, we should wait until the bacteria grow up and form visible colonies – taking valuable time from therapy, with the help of this method we can get assessable results within hours (2-6 hours based on the generation time of the bacteria).

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